INVESTIGATION OF N2, N4-DIBENZYLQUINAZOLINE-2,4-DIAMINE (DBeQ) AS AN INHIBITOR AGAINST CASEINOLYTIC PEPTIDASE B (ClpB) PROTEIN - *IN SILICO* APPROACH

H. M. S. Shamodhi[1], R. S. J. Udari[1], N. R. M. Nelumdeniya[2], R. J. K. U. Ranatunga[3], S. P. N. N. Senadeera[4] and C. B. Ranaweera[1*]

[1]Department of Medical Laboratory Sciences, [2]Department of Pharmacy, General Sir John Kotelawala Defence University, Sri Lanka
[3]Department of Chemistry, University of Peradeniya, Sri Lanka
[4]Department of Zoology and Environment Sciences, University of Colombo, Sri Lanka

Antibiotic resistance is a global threat. Caseinolytic Peptidase B (ClpB), a molecular chaperone absent in metazoans, is crucial in reactivating protein aggregates and, therefore, is a compelling target for developing new antimicrobial agents. Loss of ClpB activity hinders the viability of several therapeutically critical pathogenic microorganisms. However, there are no known high-affinity inhibitors that are ClpB-selective. N2, N4-dibenzylquinazoline-2,4-diamine (DBeQ) was previously described as a human AAA+ ATPase p97 complex-targeting antitumor agent. To create ClpB-selective inhibitors, we assume that inhibitors of AAA+ ATPase distantly related to Hsp100 may function as model scaffolds. This study employed computational methods to evaluate DBeQ as a potential inhibitor of ClpB. We used the 3-dimensional structure of our target protein ClpB (PDB ID: 1QVR), and a docking study was performed using AutoDock Vina to assess their binding affinities. The drug-likeness and pharmacokinetics were predicted using the SwissADME web server, while the ProTox-II web server assessed toxicity profiles. A molecular dynamic (MD) study was conducted following the docking calculations to determine the stability of the ligand inside the binding pocket and the stability of the complexes formed over a 100 ns period using GROMACS software with CHARMM36 forcefield and TIP3P water model. Docking results reveal that DBeQ shows -8.0 kcal/mol binding affinity via interacting with residues in the middle domain. According to the SwissADME and ProTox-II results, DBeQ is predicted to be inactive with a lower probability of hepatotoxicity and cytotoxicity. Further, DBeQ was categorized as toxicity Class IV, slightly toxic with an LD_{50} value of 1187 mg/kg with no Lipinski's rule violation suggesting a favorable safety profile. Molecular dynamic trajectory analysis, including Radius of gyration (Rg), Solvent-Accessible Surface Area (SASA), and Root-Mean Square Fluctuation (RMSF), revealed that DBeQ causes conformational changes in the Nucleotide Binding Domain-2 (NBD-2) and the middle domain, corroborating docking results. As suggested by previous experimental data, conformational changes in the middle domain may disrupt the interaction with DnaK, a co-chaperone essential for ClpB's activation in protein disaggregation. The findings suggest that DBeQ might be a possible antibacterial agent, but further *in vitro* and *in vivo* studies are required to confirm the findings.

Keywords: Molecular Chaperone, ClpB, Anti-microbial compounds, DBeQ, Molecular docking, Molecular dynamics

[*]*Corresponding Authors: cbr2704@kdu.ac.lk*

INVESTIGATION OF N2, N4-DIBENZYLQUINAZOLINE-2,4-DIAMINE (DBeQ) AS AN INHIBITOR AGAINST CASEINOLYTIC PEPTIDASE B (ClpB) PROTEIN - *IN SILICO* APPROACH

H. M. S. Shamodhi[1], R. S. J. Udari[1], N. R. M. Nelumdeniya[2], R. J. K. U. Ranatunga[3], S. P. N. N. Senadeera[4] and C. B. Ranaweera[1*]

[1]Department of Medical Laboratory Sciences, [2]Department of Pharmacy, General Sir John Kotelawala Defence University, Sri Lanka
[3]Department of Chemistry, University of Peradeniya, Sri Lanka
[4]Department of Zoology and Environment Sciences, University of Colombo, Sri Lanka

INTRODUCTION

Antimicrobial resistance is an issue in which microbes bacteria, viruses, fungi, and parasites adapt gradually and cease responding to antimicrobials. As a result, it causes difficulty curing infections, a higher risk of disease transmission, life-threatening sickness, and even death (Antimicrobial Resistance, 2021; Capozzi et al., 2019).

Hsp100s are a member of the ATPases Associated with diverse cellular Activities (AAA+) superfamily proteins, including the bacterial chaperone Caseinolytic Peptidase B (ClpB). By reactivating aggregated proteins, ClpB is crucial in maintaining proteome homeostasis, otherwise termed proteostasis in bacteria. Therefore, ClpB is vital for bacterial survival in stressful situations. It's interesting that metazoan proteomes lack Hsp100s (Ranaweera et al., 2018).

Drug discovery and development is an intense, lengthy, and interdisciplinary venture. Recently, a trend toward using *in-silico* chemistry and molecular modelling for computer-aided drug design has gained significant momentum. Computer-aided drug designing (CADD) can be beneficial in ways such as generating new compounds from already existing molecules, sorting through vast libraries of ligand molecules to create smaller groups of active molecules with the desired structures and binding affinities, checking the drug-likeness and toxic nature of the drugs to be used, developing the best molecular interactions and stabilities with the target compound (Sliwoski et al., 2014). Computational studies are widely used to keep up with antimicrobial resistance.

N2, N4-dibenzylquinazoline-2,4-diamine (DBeQ) was an experimental inhibitor ligand against human AAA+ ATPase P97, an antitumor target. Since ClpB has a structure similar to p97, the same inhibitors were experimentally evaluated against ClpB to observe if any inhibitory activity was occurring. The ClpB ATPase was unaffected by the other ligands with a high affinity for p97. Still, it was observed that DBeQ inhibits the casein-activated ATPase activity by binding to ClpB, even though it was the least effective when experimented against p97 (Glaza et al., 2020; Ranaweera, 2021). Therefore, we studied binding interactions and interaction stability between ClpB protein and DBeQ, and confirmational changes of protein-DBeQ complex using a molecular docking and molecular dynamic (MD) simulation while performing an absorption, distribution, metabolism, excretion, and toxicity (ADMET) analysis computationally.

METHODOLOGY

The X-ray diffractive crystallographic structure of ClpB (PDB ID: 1QVR; resolution of 3.00 Å), derived from *Thermus thermophilus* (Lee et al., 2003), was used as the target protein structure for this study. The structure was cleaned and refined by removing trapped water molecules and co-crystalized ligands. PubChem (https://pubchem.ncbi.nlm.nih.gov/) database (Kim et al., 2023) was used to obtain the three-dimensional (3D) structures of DBeQ and Adenosine 5′-(β,γ-imido)triphosphate (AMP-PNP) in Structure Data File (.sdf) format. The experimental structure of the 1QVR structure is complex with AMP-PNP molecules, which is a nonhydrolyzable ATP analogue structure. Therefore, we use AMP-PNP as another ligand in

our study as a control to validate our docking and dynamic study results compared with the experimental PDB structure details.

SwissADME online webserver was used for the screening and predicting the physicochemical, pharmacokinetic, and drug-likeness (Daina et al., 2017) of DBeQ, while ProTox-II was used to analyze the toxicity (Banerjee et al., 2018). Docking was continued using the PyRx open-source platform using AutoDock Vina (Trott & Olson, 2010). The grid box was defined to cover the whole protein since it was blind docking. X× Y × Z sizes to 111.6939 Å × 124.4924 Å × 74.0728 Å. and center grid coordinates X, Y, Z were set to 45.8962, 23.4164, 33.1118, respectively. The binding poses produced by the docking process were further analyzed using BIOVIA Discovery Studio v21.1.0.20298 package (Systèmes, 2020).

Then, MD simulations were run for both free unbound and bound ligand-protein complexes using the GROMACS-2024 software package (Berendsen et al., 1995) with CHARMM36 force field (Huang & MacKerell, 2013) to assess the stability of binding poses and to find out their impact on the stability/ structural changes of the ClpB protein. The topology file of the clean protein was generated using inbuilt GROMACS commands using CHARMM36 all-atom forcefield, while the ligand topology files were created by SwissParam (http://www.swissparam.ch/) online server (Zoete et al., 2011). The complex was solvated in a truncated octahedron periodic box (111.01 x 124.01 x 74.91 Å), using a distance of 12 Å between the protein and the boundary of each side using TIP3P water model (Mark and Nilsson, 2001), keeping the complex in the middle. Then, the system was neutralized by adding Na^+ ions, and an energy minimization step was carried out. Finally, the system was equilibrated using NVT and NPT conditions, at 300 K and 1 bar pressure, respectively, for 100 ps. The production run was done for 100 ns, keeping the time step at 2 fs (Bhattarai & Emerson, 2021). In the end, the trajectory files of the free protein and protein-ligand complexes were analyzed by calculating RMSD and Root Mean Square Fluctuation (RMSF). Radius of Gyration (RG), Solvent-accessible surface area (SASA), and cluster analysis were also carried out for the protein-DBeQ complex to observe conformational changes throughout the simulation period (Jephthah et al., 2021). Visualization Molecular Dynamics (VMD) (Humphrey et al., 1996) was used to display the molecular system. Graphical representations were produced using GraphPad Prism 8.4.2 software.

RESULTS AND DISCUSSION

The study was carried out in three significant steps: molecular docking, ADMET analysis, and MD simulation, and the results generated were analyzed accordingly.

Docking results

Binding affinities between the ligand and the protein of the most favourable docked pose for AMP-PNP and DBeQ were assessed, giving results of -7.8 (kcal/mol) and -8.0 (kcal/mol), respectively. The interacting amino acids of DBeQ with conventional hydrogen bonds are GLU 481 and ARG 474 in the middle domain (Figure 1), while ARG 370, GLU 523, GLU 545, LEU 541, LEU 542, LYS 548 in the NBD-2 make conventional hydrogen bonds with AMP-PNP (Figure 2). AMP-PNP binds to NBD-2 with high affinity, which is one of the

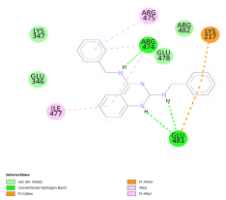

Figure 1. DBeQ Binding Interactions

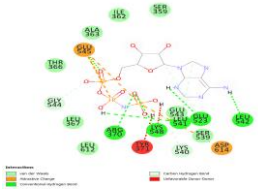

Figure 2. AMP-PNP Binding Interactions

locations in which AMP-PNP is complex in the 1QVR crystal structure. DBeQ molecule binds to the middle domain, as shown in Figure 1, which was also suggested by Przemyslaw Glaza and colleagues in their study conducted on the ClpB chaperone system (Glaza et al., 2020).

ADMET Analysis by SwissADME and Protox-II

The SwissADME server provides results of analyzed chemicals in three main categories: physicochemical, pharmacokinetic, and drug-likeness. Lipinski's rule of five (RO5) was considered when analyzing the drug-likeness of a ligand for oral bioavailability. For the compound to be orally bioavailable, the ligand should not have more than one violation of the RO5 (Lipinski et al., 2001). As for the DBeQ results, 2 H-bond donors, 2 H-bond acceptors, a molecular weight of 340.42 Da, and a computed Log P (ClogP) of 4.15 predicted zero violations, thereby considered orally bioavailable. When assessing pharmacokinetic properties, BBB, GI Absorption, Log Kp, and CYP isoforms were considered. DBeQ is predicted to be a BBB permeate and P-gp substrate with high GI absorption.

The toxicity was evaluated using the ProTox-II server. DBeQ was expected as slightly toxic (Class IV) with an LD_{50} of 1187 mg/kg. For hepatotoxicity and cytotoxicity, DBeQ was predicted to be "inactive" with lower probability, thereby giving intermediate results. It was also free from any predicted stress response pathways. Therefore, based on the overall ADMET results, we can suggest that DBeQ can be modified accordingly.

MD results

RMSD computes the average distance created by the displacements of the tested atoms in the system over a certain period relative to a reference structure. RMSD indicates the stability of a complex; the more fluctuations, the less stable. RMSF is used to determine the structural change in the protein by evaluating how much a particular residue varies as the simulation is run (Opo et al., 2021). According to Figure 3, the free protein displayed stable RMSD starting from around 45 ns to 100 ns with an averaging RMSD value of 0.5185 nm. Variations in the RMSF (Figure 4) were observed at a few positions of the free protein and they were significant among the residue numbers 270-290 (NBD-1), 415-500 (middle domain), and 620-650 (NBD-2). The protein-AMP-PNP complex displayed stable RMSD averaging 0.5987 nm and RMSF showed no prominent oscillations (Figures 3 and 4). When considering the protein-DBeQ complex, variations of the RMSD (Figure 3) were observed at a few positions of the protein-DBeQ complex with a significant fluctuation at around 30 ns and again starting from 40 ns till the end of the simulation. The average RMSD of the complex throughout the simulation is 0.617 nm. More deviations indicated instability or structural change of the protein-DBeQ complex throughout the production phase.

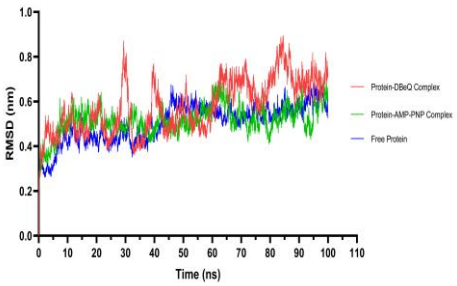

Figure 3. RMSD Plot

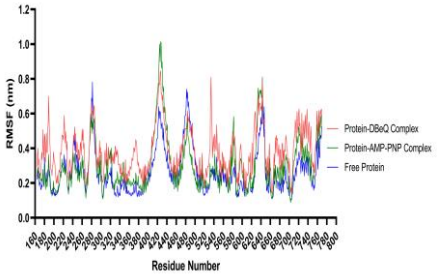

Figure 4. RMSF Plot

When considering the RMSF (Figure 4) plot of the complex, it illustrated overall RMSF fluctuations throughout all the residues. More prominent fluctuations can be observed towards the C-terminal starting from around residue number 520, at the middle domain and almost covering the NBD-2 of the protein sequence indicating significant flexibility change in the residues after DBeQ binding.

The region of a protein surface that comes into contact with its surrounding solvent molecules is known as SASA (Mazola et al., 2015). According to Figure 5, the computed SASA of the entire protein illustrated increasing trends for DBeQ. Overall, the trends point toward the possibility of conformational changes of the protein-DBeQ complex. Throughout the simulation, the protein structure's overall compactness was shown by the Rg. The fewer the

fluctuations the better stability of the folded protein structure (Choudhary et al., 2023). Figure 6 represents variations in Rg value over the simulation period and indicates that all the protein-DBeQ complex was more extended than the free protein.

Therefore, together with the docking and MD results of DBeQ, which showed high affinity to the middle domain, as well as analysis of the trajectory files, that further suggests that DBeQ affects the flexibility of the protein and causes conformational changes, which may affect the function of ClpB, and the changes are prominent in the middle domain and the NBD-2. However, the stability of the altered structure needs to be studied further. Moreover, according to the study by Przemyslaw Glaza and colleagues, DBeQ may bind to a site in the middle domain and hence potentially cause conformational changes. (Glaza et al., 2020). Therefore, this information can be used in our study to justify the RMSD, RMSF, SASA and Rg fluctuations in the protein-DBeQ complex and validate our docking results.

CONCLUSIONS/RECOMMENDATIONS

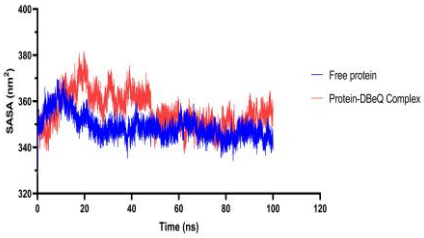

Figure 5. SASA Plot

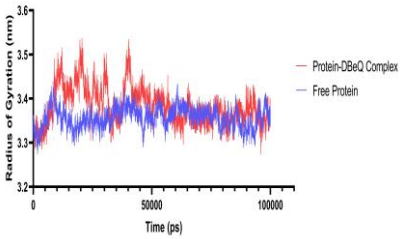

Figure 6. Rg Plot

In this study, we tested previously described known inhibitor DBeQ against ClpB protein computationally to identify binding affinities, drug-likeness, and physicochemical, pharmacokinetic, and toxicity properties. Docking results reveal that DBeQ shows higher binding affinity towards the middle domain, and the following MD simulation results indicated conformational changes in the protein structure. Further, DBeQ displayed favorable ADMET properties; hence, it could be considered a potential drug candidate in further drug development and discovery processes. Further *in vivo* and *in vitro* studies can be performed to proceed with the drug development with DBeQ.

REFERENCES – Name of the book title ought to be italicized.

Antimicrobial resistance. (2021, November 17). World Health Organization. https://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance

Banerjee, P., Eckert, A. O., Schrey, A. K., & Preissner, R. (2018). ProTox-II: A webserver for the prediction of toxicity of chemicals. *Nucleic Acids Research*, 46(W1), W257–W263. https://doi.org/10.1093/nar/gky318

Berendsen, H. J. C., van der Spoel, D., & van Drunen, R. (1995). GROMACS: A message-passing parallel molecular dynamics implementation. *Computer Physics Communications*, 91(1), 43–56. https://doi.org/10.1016/0010-4655(95)00042-E

Bhattarai, A., & Emerson, I. A. (2021). Exploring the conformational dynamics and flexibility of intrinsically disordered HIV-1 Nef protein using molecular dynamic network approaches. 3 *Biotech*, 11(4), 156. https://doi.org/10.1007/s13205-021-02698-8

Capozzi, C., Maurici, M., & Panà, A. (2019). [Antimicrobial resistance: It is a global crisis, 'a slow tsunami'*]. Igiene E Sanita Pubblica*, 75(6), 429–450.

Choudhary, S., Kesavan, A. K., Juneja, V., & Thakur, S. (2023). Molecular modelling, simulation and docking of Rv1250 protein from Mycobacterium tuberculosis. *Frontiers in Bioinformatics*, 3. https://doi.org/10.3389/fbinf.2023.1125479

Daina, A., Michielin, O., & Zoete, V. (2017). SwissADME: A free web tool to evaluate pharmacokinetics, drug-likeness and medicinal chemistry friendliness of small molecules. *Scientific Reports*, 7(1), 42717. https://doi.org/10.1038/srep42717

Glaza, P., Ranaweera, C. B., Shiva, S., Roy, A., Geisbrecht, B. V., Schoenen, F. J., & Zolkiewski, M. (2020). Repurposing p97 inhibitors for chemical modulation of the bacterial ClpB–DnaK bichaperone system. *The Journal of Biological Chemistry*, 296, 100079. https://doi.org/10.1074/jbc.RA120.015413

Huang, J., & MacKerell, A. D. (2013). CHARMM36 all-atom additive protein force field: Validation based on comparison to NMR data. *Journal of Computational Chemistry*, 34(25), 2135–2145. https://doi.org/10.1002/jcc.23354

Humphrey, W., Dalke, A., & Schulten, K. (1996). VMD: Visual molecular dynamics. *Journal of Molecular Graphics*, 14(1), 33–38. https://doi.org/10.1016/0263-7855(96)00018-5

Jephthah, S., Pesce, F., Lindorff-Larsen, K., & Skepö, M. (2021). Force Field Effects in Simulations of Flexible Peptides with Varying Polyproline II Propensity. *Journal of Chemical Theory and Computation*, 17(10), 6634–6646. https://doi.org/10.1021/acs.jctc.1c00408

Lee, S., Sowa, M. E., Watanabe, Y., Sigler, P. B., Chiu, W., Yoshida, M., & Tsai, F. T. F. (2003). *The Structure of ClpB. Cell*, 115(2), 229–240. https://doi.org/10.1016/S0092-8674(03)00807-9

Lipinski, C. A., Lombardo, F., Dominy, B. W., & Feeney, P. J. (2001). Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Advanced Drug Delivery Reviews*, 46(1–3), 3–26. https://doi.org/10.1016/s0169-409x(00)00129-0

Mark, P., & Nilsson, L. (2001). Structure and Dynamics of the TIP3P, SPC, and SPC/E Water Models at 298 K. *The Journal of Physical Chemistry A*, 105(43), 9954–9960. https://doi.org/10.1021/jp003020w

Mazola, Y., Guirola, O., Palomares, S., Chinea, G., Menéndez, C., Hernández, L., & Musacchio, A. (2015). A comparative molecular dynamics study of thermophilic and mesophilic β-fructosidase enzymes. *Journal of Molecular Modeling*, 21(9), 228. https://doi.org/10.1007/s00894-015-2772-4

Opo, F. A. D. M., Rahman, M. M., Ahammad, F., Ahmed, I., Bhuiyan, M. A., & Asiri, A. M. (2021). Structure based pharmacophore modeling, virtual screening, molecular docking and ADMET approaches for identification of natural anti-cancer agents targeting XIAP protein. *Scientific Reports*, 11(1), 4049. https://doi.org/10.1038/s41598-021-83626-x

Ranaweera, C. B., Glaza, P., Yang, T., & Zolkiewski, M. (2018). Interaction of substrate-mimicking peptides with the AAA+ ATPase ClpB from Escherichia coli. *Archives of Biochemistry and Biophysics*, 655, 12–17. https://doi.org/10.1016/j.abb.2018.08.002

Ranaweera, C.B. (2021). Bacterial AAA+ Disaggregase ClpB: *mechanism and inhibition*. Kansas State University, ProQuest Publishing. 28417660. [online] pp.1–40. Available at: https://hdl.handle.net/2097/41435

Sliwoski, G., Kothiwale, S., Meiler, J., & Lowe, E. W. (2014). Computational methods in drug discovery. *Pharmacological Reviews*, 66(1), 334–395. https://doi.org/10.1124/pr.112.007336

Systèmes, D. (2020, March 20). Free Download: *BIOVIA Discovery Studio Visualizer*. Dassault Systèmes. https://discover.3ds.com/discovery-studio-visualizer-download

Trott, O., & Olson, A. J. (2010). AutoDock Vina: Improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *Journal of Computational Chemistry*, 31(2), 455–461. https://doi.org/10.1002/jcc.21334

Zoete, V., Cuendet, M. A., Grosdidier, A., & Michielin, O. (2011). SwissParam: A fast force field generation tool for small organic molecules. *Journal of Computational Chemistry*, 32(11), 2359–2368. https://doi.org/10.1002/jcc.21816

ACKNOWLEDGMENTS

We acknowledge the General Sir John Kotelawala Defence University for providing facilities for this research.