PHOSPHOENOLPYRUVATE PROTEIN PHOSPHOTRANSFERASE OF PERIODONTOPATHOGEN
Fusobacterium nucleatum: STRUCTURAL INVESTIGATION AND INHIBITOR EXPLORATION USING COMPUTATIONAL APPROACHES

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ABSTRACT

Chronic periodontitis can cause the overgrowth of harmful bacteria in the mouth, which leads to the progressive destruction of the tissues that support the teeth. Fusobacterium nucleatum plays a role in the inflammatory response during periodontal disease. Phosphoenolpyruvate protein phosphotransferase (PEP) is found in bacteria and contributes to the virulence of F. nucleatum. Inhibition of PEP is a key role in finding more efficacious and safer drugs for treating chronic periodontitis. The research aimed to reveal the potential compounds able to inhibit PEP through molecular docking simulation by in silico analysis. The compounds were drawn from the DrugBank database, then the test ligands conducted virtual screening using the AutoDock Vina program on Google Colab Pro and assessed based on the affinity value against PEP by molecular dynamics analysis. Of 8364 compounds screened, pteroic acid showed the highest binding affinity of -6.8 kcal/mol. Root mean square deviations ranging from 0.3 to 5.5 nm were found in the molecular dynamics of pteroic acid and PEP. In vitro, investigation for pteroic acid against F. nucleatum is further needed.

Keywords: Drug Repurposing, Drugbank, In Silico, Phosphoenolpyruvate Protein Phosphotransferase, Oral Hygiene

INTRODUCTION

Periodontal disease is part of the global chronic disease problem and shares the same risk factors as other chronic diseases¹, such as alcohol², overweight and obesity³, smoking⁴, diabetes, and several other systemic diseases.⁵ Chronic periodontitis is underlined by the inflammatory cascade induced during the infestation of pathogenic in the oral cavity, which leads to the progressive destruction of the teeth-supporting tissues.⁶ The progression of this disease greatly affects the quality of life, especially when it worsens and spreads, causing more extensive damage.⁷ Based on the Global Burden of Disease Study from 2016, periodontal disease is one of the most widespread health conditions globally⁸, affecting anywhere from 20% to 50% of people.⁹ Approximately 19% of adults worldwide suffer from severe periodontal disease, which translates to over 1 billion cases globally.¹⁰ Fusobacterium nucleatum is involved in the inflammation cascade during periodontal disease. The main goal of periodontal treatment is to eliminate the bacteria, this can be done by

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pharmacological treatment and surgery. Behavioural correction is also needed to reduce the risk factors that contribute to periodontal disease, such as quitting smoking. Structure-based drug design, utilizing docking techniques, is a commonly employed strategy for developing new drugs and repurposing existing drugs to treat diseases, such as the discovery of hydroxy-3-arylcoumarins as a new antibacterial candidate against both gram-positive as well as gram-negative bacteria was found by molecular docking study. Phosphoenolpyruvate protein phosphotransferase (hereafter, PEP) is found in bacteria that can perform both catalytic and regulatory functions by transporting and converting various sugars and sugar derivatives, but also plays a role in metabolic regulation of phosphate, carbon, and nitrogen, chemotaxis, potassium transport, and the virulence of pathogens such as F. nucleatum. Because of PEP’s essential role in the pathogenesis of F. nucleatum, we conducted screening of the protein as a target. Herein, we have deciphered the structure of PEP isolated from Fusobacterium nucleatum subsp. nucleatum ATCC 25586 which was further used as a drug target via molecular docking and molecular dynamic (MD) simulations.

**EXPERIMENTAL**

**Study Design**
DELL Inspiron 15 7000 laptop with 16GB RAM specifications, Intel Core i7 7th Gen, NVIDIA GeForce GTX 1050 Ti 4GB, and the Microsoft Windows 10 Home operating system was used in the determination of protein structure, docking simulation, and molecular dynamics. The protocol of molecular docking in general followed the previous report. A potential PEP inhibitor was chosen based on the binding affinity and properties of the phytochemical molecules and their interaction with the receptor's pocket region.

**Determination of Bacterial Protein Structure using AlphaFold 2 Method**
The amino acid sequence of the PEP from F. nucleatum subsp. nucleatum ATCC 25586 was retrieved from the UniProt website with access codes Q8RI43. The AlphaFold 2 program was used to predict the three-dimensional structure of the three proteins. This program was carried out using Goggle Colab Pro where the protein sequence was entered in the sequence entry section. AlphaFold 2 first performed multiple sequence alignment (MSA) with protein sequences in the database.

**Determination of Protein Active Sites**
Determination of the active site or catalytic residue of each protein was carried out through a combination of 3 types of analysis, namely conservative residue analysis using ConSurf, druggability analysis using DoGSiteScorer, and comparison with the structure stored in the protein data bank using BLASTp. The determination of the active site was then validated against the existing structure by performing BLASTp analysis and selecting a protein whose active site was known.

**Molecular Docking**
Receptor preparation was carried out using the AutoDock Tools program which consisted of adding hydrogen atoms (polar only), adding Kollman Charges, and checking for residues with non-integral charges. The prepared receptors were then stored in PDBQT format. The test ligands in this study were taken from the DrugBank database of as many as 8364 compounds. Geometry optimization of these compounds was carried out using the OpenBabel program and converted into PDBQT format using the AutoDock Tools program. Next was to determine the size of the grid box where the ligand will bind to the receptor. A grid box was created around the receptor catalytic residues using the AutoDock Tools program. The grid box size parameters taken are size x, size y, size z, and center x, center y, and center z. Virtual screening was performed using the AutoDock Vina program on Google Colab Pro. Validation of the grid box was based on the docking of native ligand using RMSD as the parameter (RMSD<2), where the size of the grid box was adjusted within the range of 5—70 Å (elevation=1 Å). The input parameters for AutoDock Vina were receptor name, ligand name, grid box size, and exhaustiveness (set at 10). The output is a ligand file in PDBQT format and a log file.

**Molecular Dynamic Analysis**
The best compound resulting from the virtual screening was selected based on the affinity value and data on DrugBank, and then further validated by molecular dynamics. The first step was to prepare the topology of the receptor and ligand systems. The ligand topology was formed using the Avogadro program and the
The system topology was then created in the Gromacs program with the CHARMM36 force field. The next step was to perform solvation, the addition of ions, minimization, and balancing the system. Molecular dynamics was then carried out with a length of time of 100 nanoseconds (ns). The results of the molecular dynamics analysis are in the form of RMSD graphs showing changes in ligand poses during the simulation process.

**Visualization of Protein-Ligand Interactions**
The PyMOL program was used to visualize the interaction between the test ligands and amino acid residues in proteins in three dimensions. The output receptor and ligand files in PDBQT format are loaded into the PyMOL program. Receptors were displayed in the form of a surface with transparency set at 60%. Residues that interact with ligands were shown in cartoon form with contrasting colors, and are labeled. The residues interacting with the ligands were visualized in two dimensions and three dimensions using the Discovery Studio program. The output receptor and ligand files of AutoDock Vina docking results in PDBQT format are entered into the program. Interaction diagrams were displayed with different color visualizations for each type of interaction.

**RESULTS AND DISCUSSION**

**Structure of PEP and Its Active Sites**
The result of PEP structure modeling based on AlphaFold 2 has been presented in Fig.-1. The predicted local distance difference test (pLDDT) reveals the good quality of the structure, with almost all amino acid residues at very high and confident categories, especially those binding residues. Based on the BLASTp, we found protein 2XZ9 as the homolog of the PEP (99.69% percent identity and RMSD=0.729), where its active residues include Arg303, Arg339, Glu438, Asn461, Asp462, Arg472, and Cys509. A significant orientation difference between these proteins is observed for Arg472.

**Compounds Inhibiting Phosphoenolpyruvate Protein Phosphotransferase**
As many as 8364 compounds were taken from the DrugBank database, screened with molecular docking, and assessed based on the affinity value of the interaction with PEP. Thereafter, the ten compounds were revealed to have relatively higher binding affinity among others. Of which, pteroic acid; 7-alpha-D-ribofuranosyl-2-aminopurine-5'-phosphate; alpha-adenosine monophosphate has the highest binding affinity of -6.8 kcal/mol (Table-1). However, none of these compounds were found to form interactions with the predicted active sites (Arg303, Arg339, Glu438, Asn461, Asp462, Arg472, and Cys509). Pteroic acid is a synthetic substance that is similar to the natural amino acid phenylalanine. Pteroic acid can be activated with trifluoroacetic acid and binds with metal ions such as nickel, cobalt, copper, and zinc. Meanwhile, both alpha-adenosine monophosphate and 7-alpha-D-Ribofuranosyl-2-aminopurine-5'-phosphate belong to purine ribonucleoside monophosphates.
**Table-1. Molecular Docking and Molecular Properties of Some Potential Compounds Inhibiting PEP**

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Affinity Energy (kcal/mol)</th>
<th>H bond</th>
<th>CH bond</th>
<th>Other bonds</th>
<th>Unfavorable interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pteroic acid</td>
<td>-6.8</td>
<td>Ser526, Asn454, Arg332</td>
<td>-</td>
<td>Arg296, Glu504</td>
<td>Mg1</td>
</tr>
<tr>
<td>7-alpha-D-Ribofuranosyl-2-aminopurine-5'-phosphate</td>
<td>-6.8</td>
<td>Asn454, Arg296, Gly452</td>
<td>Gly503</td>
<td>Glu431, Asp455, Glu504</td>
<td>-</td>
</tr>
<tr>
<td>Alpha-Adenosine Monophosphate</td>
<td>-6.8</td>
<td>Glu504, Asn454, Asn274, Arg296, Cys502</td>
<td>Gly503</td>
<td>Asp455, Glu431</td>
<td>Mg1</td>
</tr>
<tr>
<td>N-[2-(5-methyl-4H-1,2,4-triazol-3-yl)phenyl]-7H-pyrrolo[2,3-d]pyrimidin-4-amine</td>
<td>-6.6</td>
<td>Asn454, Arg296, Cys502</td>
<td>-</td>
<td>Arg322, Glu504, Met429</td>
<td>Mg1</td>
</tr>
<tr>
<td>(z)-3-benzyl-5-(2-hydroxy-3-nitrobenzylidene)-2-thioxothiazolidin-4-one</td>
<td>-6.6</td>
<td>Arg296, Ser526</td>
<td>-</td>
<td>Glu431, Mg1, Arg322, Glu504, Cys502</td>
<td>Asn454</td>
</tr>
<tr>
<td>2-(3-((4,5,7-trifluorobenzothiazol-2-yl)methyl)-1H-pyrrolo[2,3-b]pyridin-1-yl)acetic acid</td>
<td>-6.6</td>
<td>Ser526, Asn264, Arg296</td>
<td>-</td>
<td>Mg1, Asp455, Asn454, Glu504</td>
<td>-</td>
</tr>
<tr>
<td>7-Alpha-D-Ribofuranosyl-Purine-5'-Phosphate</td>
<td>-6.5</td>
<td>Asn454</td>
<td>Asn274, Gly503</td>
<td>Glu504, Arg296, Asp455, Glu431</td>
<td>Mg1</td>
</tr>
<tr>
<td>2',3'-Dehydro-2',3'-Deoxy-Thymidine 5'-Diphosphate</td>
<td>-6.5</td>
<td>Asn274, Arg296, Asn454, Cys502</td>
<td>Gly452</td>
<td>Glu504, Arg322</td>
<td>-</td>
</tr>
<tr>
<td>Neflamapimod</td>
<td>-6.5</td>
<td>Asn274, Asn454</td>
<td>Gly452</td>
<td>Asp455, Glu504, Arg296, Arg322, Cys502, Met429</td>
<td>-</td>
</tr>
<tr>
<td>5'-O-(L-Prolylsulfamoyl)adenosine</td>
<td>-6.5</td>
<td>Asn274, Ser526, Arg296, Glu431</td>
<td>Cys502, Gly452, Asn454</td>
<td>Arg322</td>
<td>Glu504, Mg1</td>
</tr>
</tbody>
</table>

**Visual Analysis of the Ligand—Protein Complex**

To focus on the study on finding alternative drugs that can target and likely form a stable interaction with PEP, three compounds were selected due to their binding affinity showing the highest value. Pteroic acid, alpha-adenosine monophosphate, and 7-alpha-D-ribofuranosyl-2-aminopurine-5'-phosphate 2D representations of the molecular docking simulation have been presented in Fig.-2, while the 3D representation of the ligand-protein complex has been presented in Fig.-3a. Pteroic acid was chosen among the three compounds due to its accessibility in the laboratory and was further conducted for validation on
molecular dynamics (MD). Moreover, the other two, as explained previously, are likely to cause serious adverse events.

**Validation based on Molecular Dynamics**

The Root Mean Square Deviation (RMSD) was examined on pteroic acid to validate its molecular dynamics, as presented in Fig.-3b. Results showed that pteroic acid had above 5 nm RMSD, which issues unstable interaction. An RSMD < 3 nm has been used in a previous study as a cut-off point to determine the stability of the protein—ligand complex. This implies the possibility of inaccurate docking results. However, the RMSD value obtained herein is close to that reported in a study screening antibacterial compounds, where the RMSD reached 6 nm and still maintained the stability of the docked complex.

This implies the possibility of inaccurate docking results.

Fig.-2: 2D Representation of (a) Pteroic acid, (b) 7-alpha-D-Ribofuranosyl-2-aminopurine-5'-phosphate, and (c), Alpha-Adenosine Monophosphate Interacting with the Receptor's Residues in the Pocket Region

MD simulates the effect of atomic movement in the molecules involved in the ligand—protein complex. Moreover, MD could inform the effects of the molecular environment including salt concentration and composition of membrane lipids. In a previously reported study, MD was found to be capable of discriminating the binding ligands with the decoy compound obtained from a Vina docking simulation. Hence, it increases the necessity to confirm the docking results via in vitro experiments.

**CONCLUSION**

Pteroic acid showed the highest binding affinity (-6.8 kcal/mol) against PEP. Molecular dynamics of pteroic acid and PEP showed RMSD values of 0.3—5.5 nm. The limitation of the study includes the inadequacy to present other inhibition mechanisms of the compounds against the targeted protein (such as non-competitive inhibition). In vitro, investigation for pteroic acid against F. nucleatum is warranted.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

All the authors contributed significantly to this manuscript, participated in reviewing/editing, and approved the final draft for publication. The research profile of the authors can be verified from their ORCID ids, given below:

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