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Original Article

MOLECULAR DYNAMICS SIMULATIONS OF THE CAFFEIC ACID INTERACTIONS TO DIPEPTIDYL PEPTIDASE IV

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ABSTRACT

Objective: The research presented in this article aimed to examine the applicability of a recently published software PyPLIF HIPPOS to identify the interactions hotspots between dipeptidyl peptidase IV (DPP4) and its inhibitor caffeic acid during molecular dynamics (MD) simulations.

Methods: Caffeic acid was docked to the binding pocket of DPP4 followed by 50 ns MD simulations, during which snapshots were taken every 10 ps. The molecular docking and the MD simulations were performed in YASARA-Structure 21.12.19. The snapshots were analyzed using the MM/PBSA analysis in YASARA-Structure and PyPLIF HIPPOS to calculate the binding energy (BE) and the caffeic acid-DPP4 interactions hotspots, respectively.

Results: The 50 ns MD simulations of DPP4-caffeic acid had converged since the early stage of the simulations. The BE and the RMSD values of the ligand movement indicated a probable DPP4 allosteric site. PyPLIF HIPPOS identified 15 interacting DPP4 residues to caffeic acid. The residues interacting with caffeic acid in more than 10% snapshots of the MD simulations were Ser59, Arg61, Glu206, and Phe357. The binding residues Ser59 and Arg61 were suggested to be part of the plausible DPP4 allosteric site.

Conclusion: PyPLIF HIPPOS serves as a valuable complement to the MM/PBSA method in the examination of enzyme-inhibitor interactions.

Keywords: PyPLIF HIPPOS, YASARA-Structure, Caffeic acid, Dipeptidyl peptidase IV, Molecular dynamics simulations

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INTRODUCTION

Dipeptidyl peptidase IV (DPP4) has served as a prominent target in the drug discovery for the treatment of type 2 diabetes mellitus (T2DM) [1]. Inhibition of DPP4 regulates glucose control by enhancement of the glucagon-like peptide-1 (GLP-1) action [1, 2]. Several DPP4 inhibitors are available in the market for T2DM management, i.e., sitagliptin, vildagliptin, saxagliptin, alogliptin, linagliptin, anagliptin, gemigliptin, teneligliptin, evogliptin, omarigliptin, trelagliptin, and gosogliptin [1]. On the other hand, some natural products comprising several flavonoids, resveratrol, and caffeic acid were reported as potent DPP4 inhibitors, which could implicate in T2DM management through the daily intake as food [2]. Moreover, in 2010, our research group predicted a natural product, curcumin, as a potential DPP4 inhibitor *in silico* [3], which was recently verified *in vitro* and *in vivo* by Cao *et al.* [4].

The DPP4 inhibitory activity belonging to caffeic acid (IC_{50} = 3.37±0.14 μM) [2] has attracted our attention since it is one major phenolic compound identified in coffee beans [5]. This could explain the inverse association of coffee consumption with the risk of T2DM [6]. The structure-activity relationships (SAR) analysis of the DPP4 inhibitors [1, 2, 4] indicated that they share the aromatic moiety and some hydrogen bond donors and acceptors. Caffeic acid is the 2nd smallest compound after gallic acid among them. With a molecular weight of 180 Da, caffeic acid could serve as a potent fragment to be developed further [7], as well as a lead compound to discover more natural products in the structure-based virtual screening (SBVS) campaigns targeting DPP4. On the other hand, our effort to quantify caffeic acid in spent ground coffee (SGC) identified that SCG contains 0.17%±0.006 (w/w) of caffeic acid [8]. Recycling SCG from waste to glucose-controlling products benefiting from the caffeic acid content could be part of the near-future T2DM management.

The insights of the molecular determinants of the protein-ligand binding could increase the effectiveness and efficiency of the drug discovery and development projects [9, 10]. We developed and made PyPLIF HIPPOS publicly available [11]. PyPLIF HIPPOS is a tool to identify protein-ligand interactions bitstrings of poses resulted from docking simulations, either using PLANTS or AutoDock Vina [11]. Assisted by PyPLIF HIPPOS, we increased the prediction quality of SBVS by targeting some G-protein coupled receptors (GPCR), identified the molecular determinants of the protein-ligand binding in the retrospective SBVS campaigns [9]. We recently reported the usefulness of PyPLIF HIPPOS in identifying dominant DPP4-ABT341 interactions during 10 ns production run of molecular dynamics (MD) simulations [12]. The non-hydrophobic interactions identified in more than 50% of snapshots were aromatic interactions to Phe357, aromatic interactions to Tyr666, and hydrogen bond to Glu206 when the residue serves as the acceptor [12]. The identified dominant interactions reflect the binding of DPP4 to a phenolic moiety. The research presented in this article aimed to explore the applicability of PyPLIF HIPPOS in identifying interactions hotspots of caffeic acid to DPP4 during 50 ns MD simulations. The interaction hotspots could be identified by employing energy decomposition analysis in the MM/PBSA analysis of results from MD simulations [13]. However, we employ YASARA-Structure, which does not provide a tool to perform energy decomposition analysis in its MM/PBSA analysis module [14, 15]. Therefore, PyPLIF HIPPOS's applicability to assist in identifying the protein-ligand interactions hotspots serves as the complementary tool to MM/PBSA analysis module in YASARA-Structure.

MATERIALS AND METHODS

Materials

Two main materials of the research were the DPP4 structure obtained from the Protein Data Bank (PDB) with PDB ID of 2ONC (https://www.rcsb.org/structure/2onc, accessed on January 20th, 2022) [16] and the structure of caffeic acid in the Canonical-SMILES format "C1=CC(=C(C=C1C=CC(=O)O)O)O" obtained from PubChem (https://pubchem.ncbi.nlm.nih.gov/, accessed on March 1st, 2022) with the PubChem CID of 689043. There were two instruments used in the research, i.e., (i) an Ubuntu 20.04 LTS virtual private server (VPS) with 8 Intel(R) Xeon(R) CPU E5-2680 v3 @ 2.50GHz as the processors, and 16 GB of RAM; and (ii) a Windows 11 personal computer client (pc-client) with 11th Gen Intel(R) Core(TM) i5-1135G7 @ 2.40GHz as the processor, 8 GB of RAM, and NVIDIA(R) GeForce(R) MX350 as the graphic card. Both VPS and pc-client were

equipped with YASARA-Structure 21.12.19 [15]. PyPLIF HIPPOS and its dependencies [11], as well as PLANTS [17] and SPORES [18], were installed in the VPS.

Methods

Input file preparation

The file 2ONC.pdb was downloaded directly to YASARA-Structure in the pc-client, with the SeqRes was assigned as "Yes" in the downloading options. Except for atoms from chain A, the command *DelMol !A* was applied to remove atoms. Then, the command *DelRes Hoh Nag or Sy1 801* was applied to obtain only the DPP-4 and the co-crystal ligand alogliptin. The SeqRes module showed that some residues were missing, i.e., His36, His37, Ala38, S39 from the N-loop, and Gln72, Glu73, Asn74 from the loop. Therefore, the following command *BuildLoop None, Sequence=HHASA,5-7, Structures=1, Mutate=All, Bumpsum=1.0, SecStr=* and followed by *BuildLoop 311-313, Sequence=KQENN,323-325, Structures=1, Mutate=All, Bumpsum=1.0, SecStr=* were applied to build the missing residues. The commands *pH value=7.4, update=Yes* and *CleanAll* were subsequently performed to add hydrogens correctly at the physiological pH. The Force Field was then set to AMBER14, and the energy minimization experiment was performed. The simulation cell resulted from the energy minimization was removed, and a new simulation cell was constructed in the cubic shape with a distance of 5 Å around the co-crystal ligand alogliptin. The co-crystal ligand alogliptin was removed, and the scene was saved as 2ONC-cac_receptor.sce (Supplementary File S1).

By employing the following command, *BuildSMILES String="C1=CC(=C(C=C1C=CC(=O)O)O)O"*, the structure of caffeic acid was built in YASARA-Structure. The structure was set to have the correct hydrogens at physiological pH by commands *pH value=7.4, update=Yes* and *CleanAll*. Energy minimization was then performed using NOVA as the Force Field, followed by geometry optimization using the AM1 semiempirical method. The object was renamed to ligand, and the residue name was changed to CAC. The 1st object was saved as 2ONC-cac_ligand.yob (Supplementary File S2).

The caffeic acid (2ONC-cac_ligand.yob) was docked to the DPP4 (2ONC-cac_receptor.sce) using the default docking macro dock_run. mcr in YASARA-Structure. The docking was performed using AutoDock Vina [19] embedded in YASARA-Structure using the default parameter. The best docking pose was then loaded to YASARA-Structure, and the ligand CAC was split to the 2nd object. The 1st and the 2nd objects were renamed 2ONCR and 2ONCL, respectively. A cubic simulation cell with a distance of 10 Å around all atoms was defined, and then the system was saved as 2ONC-cac-r2md.sce (Supplementary File S3).

The MD simulations in YASARA-Structure require a macro file that defines the parameters of the simulations. A macro file md_run_50ns-ss10ps-8cpu-0gpu.mcr (Supplementary File S4) was prepared by modification of the default macro md_run.mcr. The modification was done to use all 8 CPUs available in the VPS to perform 50 ns MD simulations, during which snapshots were taken every 10 ps.

Molecular dynamics simulations

The input file 2ONC-cac_r2md.sce and the macro file md_run_50ns-ss10ps-8cpu-0gpu. mcr were uploaded to VPS. The MD simulations were performed using the text mode of the YASARA-Structure in the VPS. The following are the modified simulation parameters described in YASARA-Structure [15]: "The simulation was run with YASARA. The setup included optimizing the hydrogen bonding network to increase the solute stability and a pKa prediction to fine-tune the protonation states of protein residues at the chosen pH of 7.4. NaCl ions were added with a physiological concentration of 0.9%, with an excess of either Na or Cl to neutralize the cell. After steepest descent and simulated annealing minimizations to remove clashes, the simulation was run for 50 ns using the AMBER14 force field for the solute, GAFF2, and AM1BCC for ligands and TIP3P for water. The cut-off was 8 A for Van der Waals forces, no cut-off was applied to electrostatic forces (using the Particle Mesh Ewald algorithm). The equations of motions were integrated with a multiple timestep of 1.25 fs for bonded interactions and 2.5 fs for non-bonded interactions at a temperature of 310K and a pressure of 1 atm (NPT ensemble) using algorithms described in detail previously [15]. After inspection of the solute root-mean-squared deviation (RMSD) as a function of simulation time, the first 15 ns were considered equilibration time and excluded from further analysis."

Analysis

A custom macro to analyze the results md_analyze_2ONC-cac.mcr (Supplementary File S5) was made by modifying the md_analyze. mcr provided by default in YASARA-Structure [15]. The analysis focused on the solute RMSD, i.e., RMSD of the backbone atoms of the protein (RMSDBb) and RMSD of the ligand movement (RMSDLigMove). The MM/PBSA module in md_analyzebindenergy. mcr provided by default in YASARA-Structure was used to calculate the binding energy (BE). The pdb2plif.sh (Supplementary File S6) and the md2plif.sh (Supplementary File S7) were developed to employ PyPLIF HIPPOS to identify the interactions hotspots of caffeic acid to DPP4 during MD simulations.

RESULTS

The file 2ONC-cac-r2md_analysis.tab (Supplementary File S8) resulted from executing md_analyze_2ONC-cac.mcr was analyzed, resulting in the graphs RMSDBb vs. simulation time and RMSDLigMove vs. simulation time (fig. 1). The graph BE values vs. simulation time resulted from the MM/PBSA analysis is presented in fig. 2, while the percentage values of the non-hydrophobic interactions identified during the MD simulations assisted by PyPLIF HIPPOS are presented in table 1. The values in table 1 were calculated from the number of interactions in the file all. nobb.plif.xlsx (Supplementary File S9). The results show that the DPP4-caffeic acid complex was stable during the simulations, but the ligand caffeic acid was trying to fig. out the most stable pose in the DPP4 cavity. This has led to the discovery of a possible DPP4 allosteric site. Nevertheless, the results presented in table 1 show that PyPLIF HIPPOS was applicable to identifying interactions hotspots during MD simulations.

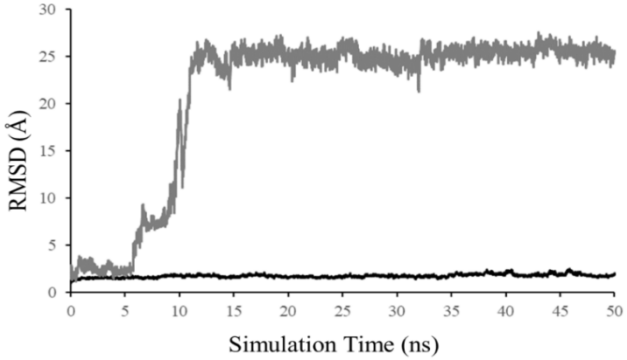

Fig. 1: The graphs between the RMSD values of the backbone atoms of the protein (black) and the RMSD values of the atoms of the ligand (grey) vs. simulation time

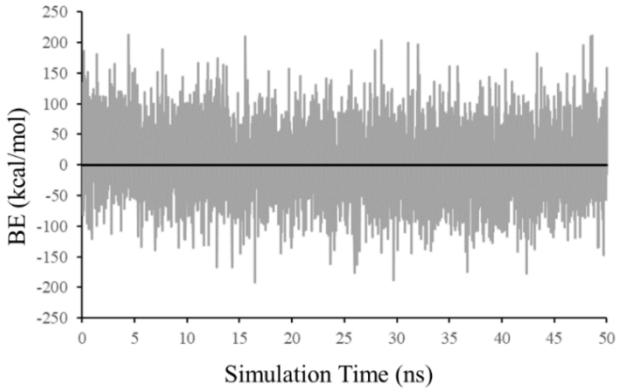

Fig. 2: The graphs between the BE values calculated using MM/PBSA vs. simulation time

Table 1: Interactions hotspots of DPP4-caffeic acid identified by PyPLIF HIPPOS from the MD simulations snapshots

Interacting residue	Interaction type [11]	Interaction percentage
Ser59	H-bond (residue as the donor)	46.03 %
Arg61	H-bond (residue as the donor)	5.74 %
Arg61	ionic (residue as the cation)	23.56 %
Lys71	H-bond (residue as the donor)	1.86 %
Lys71	ionic (residue as the cation)	0.28 %
Asp104	H-bond (residue as the acceptor)	6.42 %
Tyr105	H-bond (residue as the acceptor)	0.14 %
Tyr105	H-bond (residue as the donor)	0.08 %
Tyr105	aromatic (edge-to-face)	8.24 %
Tyr105	aromatic (face-to-face)	0.56 %
Ser106	H-bond (residue as the acceptor)	0.06 %
His126	H-bond (residue as the donor)	0.36 %
Glu206	H-bond (residue as the acceptor)	10.64 %
Ser209	H-bond (residue as the acceptor)	0.36 %
Ser209	H-bond (residue as the donor)	0.18 %
Phe357	aromatic (edge-to-face)	3.36 %
Phe357	aromatic (face-to-face)	12.34 %
Arg358	H-bond (residue as the donor)	3.36 %
Arg358	ionic (residue as the cation)	1.76 %
Arg471	H-bond (residue as the donor)	0.06 %
Tyr547	H-bond (residue as the donor)	7.46 %
Tyr666	H-bond (residue as the donor)	1.80 %
Tyr666	aromatic (edge-to-face)	0.24 %
Tyr666	aromatic (face-to-face)	0.44 %
Arg669	H-bond (residue as the donor)	9.64 %

DISCUSSION

Based on the criterion suggested by Liu *et al.* [20], the MD simulations have reached convergence since the first 5 ns (fig. 1). The MD simulations were considered convergence if the deviation of the RMSDBb values during 5 ns runs was<2 Å [20]. However, the RMSDLigMove values, as can be seen from fig. 1, had never reached below 2 Å. This could be interpreted that the ligand being unstable inside the DPP4 binding pocket, as suggested by Liu *et al.* [20]. But, the movement of the ligand around the binding pocket made it possible to explore the DPP4 allosteric site [21]. PyPLIF HIPPOS served as a powerful tool to perform the task to discover the allosteric site since it could identify the binding residues for every snapshot resulted from MD simulations (table 1).

Visual inspection on the snapshots indicated that although the RMSDLigMove values were above the criterion [20], the ligand caffeic acid stayed in the DPP4 binding pocket. Indeed, the caffeic acid left the active site at the beginning of the simulations, but until the end of the simulation, it stayed in contact with DPP4 residues. The results from PyPLIF HIPPOS analysis (all.nobb.plif.xlsx; Supplementary File S9) confirmed the visual inspection. The previously identified DPP4 active site was composed of Glu206, Phe357, and Tyr666 [12, 16]. Caffeic acid interacted with at least one of them from the beginning until the simulation time reached 9.05 ns. At the simulation time of 10.48 ns, caffeic acid started to interact with the plausible allosteric site by performing H-bond to Arg61, which stayed until the end of the MD simulations with the H-bond to Ser59 as the anchor (table 1). The snapshots at the simulation time of 4.75 ns and 44.35 ns are presented in fig. 3 and 4 as the representatives of caffeic acid in the DPP4 active site (fig. 3) and in the DPP4 possible allosteric site (fig. 4), respectively. PyMOL[22] was used to produce fig. 3 and 4.

Most of the DPP4 inhibitors in the market are competitive inhibitors, e. g., alogliptin, linagliptin, saxagliptin, sitagliptin, and vildagliptin [1, 16, 23]. On the contrary, several inhibitors were reported as non-competitive inhibitors *in vitro* [21, 24], which indicated the availability of allosteric sites in the DPP4 binding pocket [24]. Unfortunately, there is no information on whether caffeic acid is a competitive or non-competitive inhibitor for DPP4 [2]. Therefore, it was assumed that caffeic acid was a competitive DPP4 inhibitor, and then it was docked to DPP4 using the binding residues defined by a competitive inhibitor alogliptin [16] as the starting point for the MD simulations (see Methods subsection). Notably, caffeic acid immediately left the predefined pocket and moved to the probable DPP4 allosteric site identified in this research. Hence, instead of being a competitive DPP4 inhibitor, caffeic acid might serve as a non-competitive one.

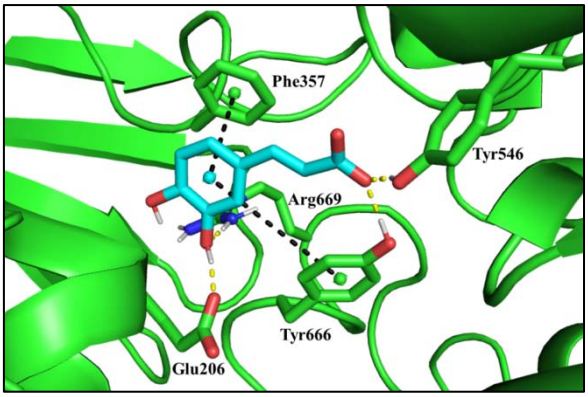

Fig. 3: The representative pose of caffeic acid (carbon atoms are cyan) in the DPP4 active site (carbon atoms are green). The H-bonds and the aromatic interactions are depicted with green and black dashed lines, respectively. Aromatic centers were added to assist the depiction of the aromatic interactions

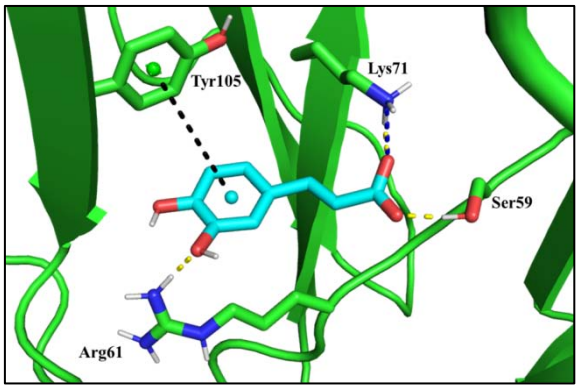

Fig. 4: The representative pose of caffeic acid in the DPP4 possible allosteric site. The rendering is similar to fig. 3, with an additional blue dashed line to present the ionic interaction

The phenomenon of caffeic acid immediately leaving the best docking pose highlighted the limitation of molecular docking simulations [25]. Most molecular docking approaches define the simulation box or sphere based on the co-crystalized ligands of the reference crystal structures [9, 26]. Fan *et al.* [2] used the same technique in the docking of caffeic acid to DPP4. In 2015, Chen [25] has warned us of this limitation and suggested performing MD simulations to validate docking results. Liu *et al.* [20] suggested performing MD simulations production runs of 10 ns for this purpose. In the field of natural products research, molecular docking followed by molecular dynamics works of Febrina *et al.* [27] could serve as a good-practice reference. Taken together, caffeic acid might never reach the DPP4 active site. This could be explored further by MD simulations using the lowest BE snapshot resulted from this research as the starting point.

The BE values resulted from the MM/PBSA method in this research were erratic (fig. 2), which were in line with the findings by Genheden and Ryde [28]. Moreover, the RMSDLigMove had never reached below 2 Å (fig. 1). Therefore, it is not reliable to derive the free energy of binding value based on the MD simulations [20]. The MD simulations of the DPP4-caffeic acid complex could reach its stability if it starts from the lowest BE snapshot resulted from this research. The MM/PBSA method was useful to identify the snapshot at the simulation time of 16.47 ns as the lowest BE snapshot (fig. 2). Results from PyPLIF HIPPOS (table 1 and Supplementary File S9) of the snapshot showed that caffeic acid interacted with the allosteric interactions hotspots Ser59 and Arg61. The lowest BE snapshot 2ONC-cac-r2md01647. pdb is provided as Supplementary File S10 for further exploration.

The allosteric site suggested by this research could be explored further by employing the lowest BE snapshot to study DPP4 non-competitive inhibitors from natural products, e. g., luteolin and apigenin [2]. Recent *in silico* studies comprising molecular docking simulations of Indonesian medicinal plants identified some potential DPP4 inhibitors [29]. Similar approaches to the discovery of DPP4 inhibitors by linking traditional medicine [30] and molecular docking studies [31] could benefit from the approach presented in this manuscript. Further MD simulations employing the allosteric site identified by MD simulations of DPP4-caffeic acid here could serve as further validation, especially for the similar compound to caffeic acid, i.e., mimosine and L-histidine [29]. These compounds resemble the size and the carboxylic acid group of caffeic acid.

CONCLUSION

PyPLIF HIPPOS served as a powerful complementary tool for MM/PBSA method in the identification of the DPP4-caffeic acid interactions hotspots during MD simulations. The H-bonds of caffeic acid to Ser59 and Arg61 were the interactions hotspots. Those interactions hotspots were located in the possible DPP4 allosteric site, which could be explored further to obtain more insights into the discovery and development of non-competitive DPP4 inhibitors.

DATA AVAILABILITY

All supplementary files (Supplementary Files S1 to S10) are publicly available and can be accessed on https://github.com/paamc-camm/md-dpp4-cac.

FUNDING

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AUTHORS CONTRIBUTIONS

E. P. I. and F. D. O. R conceptualized the research project. E. P. I. conducted all the computational simulations and initiated the

original draft of the manuscript. F. D. O. R reviewed and edited the manuscript. All authors have given approval to the final version of the manuscript.

CONFLICTS OF INTERESTS

All authors have none to declare

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