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Research article

In-silico studies on structural function of ampk through docking approach toward design drug for atherosclerosis

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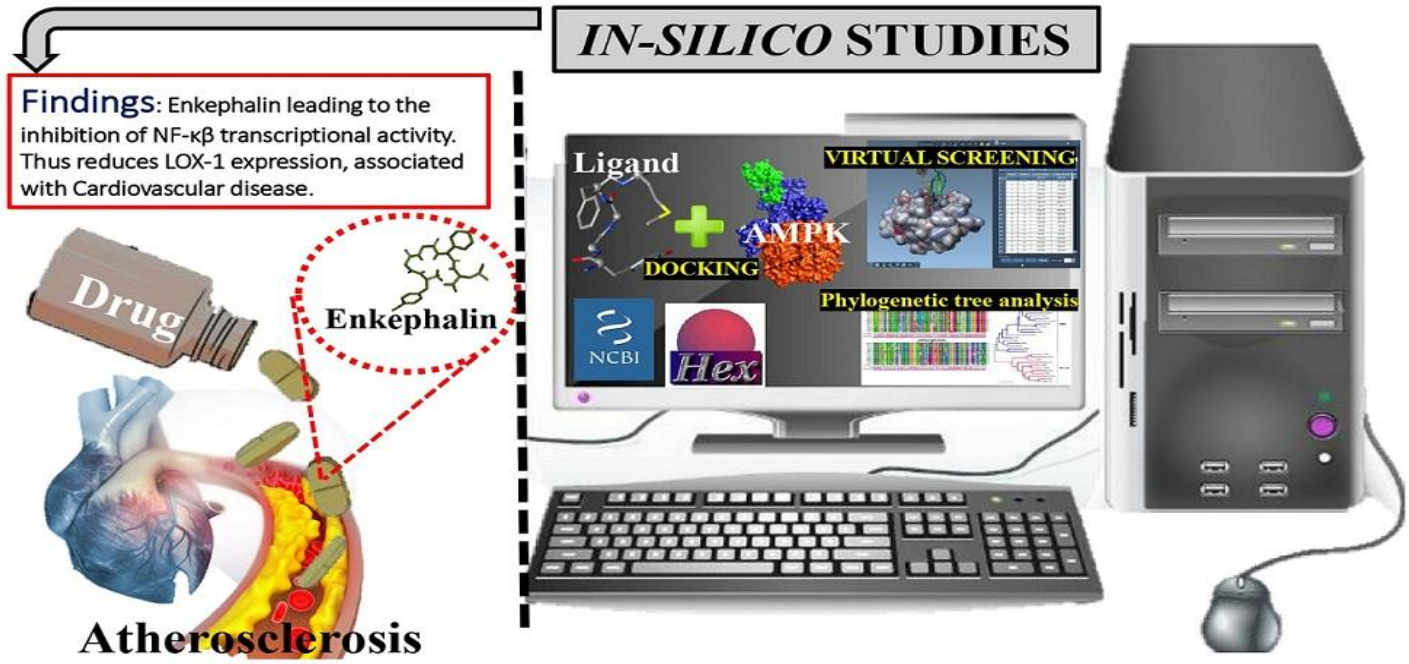
ABSTRACT

Atherosclerosis poses significant health risks, driving cardiovascular issues like heart attacks and strokes. Shortage of appropriate pathophysiological models and a suitable algorithm for the discovery of drugs and para-pharmaceuticals with direct anti-atherosclerotic action are major barriers to the effective pathogenetic medication for atherosclerosis. This study focused on using bioinformatics tools and molecular docking techniques to identify novel molecular agonists for the Activated Monophosphate Kinase (AMPK) enzyme via the AMPK/PP2A/NF-κB/LOX-1 pathway. Virtual screening and molecular docking were performed for activation and phosphorylation of AMPK and stimulation of Protein Phosphostase-2A (PP2A) which results in down-regulation of lectin-like oxLDL (LOX-1) receptor to reduce the internalization of oxidized low-density lipoprotein (oxLDL). Enkephalin (ZINC000014952116) emerged as a potent AMPK agonist, triggering a cascade of interactions leading to reduced LOX-1 expression and which inhibits the Nuclear Factor kappa betta (NF-κβ) transcriptional activity for inflammatory atherosclerosis.



Keywords: Atherosclerosis, AMPK/PP2A/NF-KB/LOX-1 pathway, Enkephalin, Molecular docking studies.

INTRODUCTION

Atherosclerosis, marked by intimal layer inflammation leading to arterial blockages, faces limited treatment success with traditional drugs such as statins, niacin, and others target cholesterol, they overlook the inflammation factor [1,2]. Emerging strategies focus on AMPK activation, notably with metformin, a promising AMPK activator. However, metformin acts indirectly via mitochondrial Complex I suppression, impacting ATP production. Despite the complexity, expensiveness and unpredictability of drug development, many pharmaceutical industries are investing in research for advancements in treating atherosclerosis through direct activation of AMPK [3,4].

When there is a lack of energy AMP activated protein kinase (AMPK) gets activated. This activation happens when kinases like Liver Kinase-B1 (LKB1) phosphorylates AMPK at Thr172. The catalytic activity of AMPK is further enhanced by the addition of AMP, which also has benefits, in preventing atherosclerosis. It helps regulate metabolism and displays inflammatory properties [5].

In addition, Ser298 and Ser336 were reported as residues of PP2A phosphorylated under the AMPK stimulation. Stimulated PP2A dephosphorylates Ser536 in RelA/p65 (Subunit of nuclear factor) and inactivates NF-κβ (Nuclear Factor Kappa beta), a transcription factor that plays a key role in regulating inflammation [6]. Hence, lead to the downregulation of LOX-1 expression. This highlights their interaction and potential use in treating atherosclerosis [7].

MATERIALS AND METHODOLOGY

Retrieving of Sequences

Protein accession numbers and FASTA files of liver kinase B1 (LKB1) (accession number: Q15831) and Protein Phosphatase- 2A (PP2A) (accession number: AAB69751) retrieved from National Center for Biotechnology Information (NCBI) online database [8]. Protein Data Bank (PDB) database also used to retrieve the PDB file of AMPK (accession number: 7JHH). In fact, the PDB file consists of 3D atomic coordinates obtained from experimental methods [9].

Pairwise Sequence Alignment

We aimed to search for homologous proteins (i.e., which have been published with their 3D structure) of Protein Phosphatase- 2A (PP2A) and liver kinase B1 (LKB1) by submitting their FASTA file (generated by sequence retrieval step) to basic local alignment search tool (BLAST). Where we used Position-Specific Iterative (PSI)-BLAST algorithm that set up 500 maximum target sequences against the PDB database. Specifically, PSI-BLAST computes statistical significance of match for examining distant relationship between proteins [10].

Multiple sequence alignment

Related sequences are identified through the database similarity searching. Multiple Sequence Alignment of query sequence of PP2A and LKB1 hit ten and twenty subjects respectively, aligned through Clustal Omega. The input sequences for Clustal Omega were set to "PROTEIN SEQUENCES". The FASTA files of the LKB1 and PP2A files (obtained through pairwise alignment) uploaded independently in two different procedures. The Phylogeny Inference Package (PHYLIP) format output configured at each procedure.

Phylogenetic tree construction

Through Phylogenetic Tree analysis of evolutionary relatedness of our query sequences of LKB1 and PP2A. PHYLIP software has been used to construct a consensus tree. The construction of the trees for LKB1 and PP2A used the same construction parameters in different trials. SEQBOOT, PROML, and CONSENSUS, among other programs from the package utilized in constructing phylogenetic trees [11].

Three-Dimensional (3D) Structure Prediction

Via free an online server called" SWISS-MODEL'' accessed at SWISS model webpage a 3D structure of LKB1 and PP2A predicted, by homology modeling through SWISS-MODEL [12]. While performing comparative modeling, a 3D protein model of a target sequence created by extrapolating experimental data from a template protein structure that is evolutionarily linked to the target sequence of LKB1 and PP2A. The sequences from FASTA files pasted into SWISS-MODEL, and searching for templates, where LKB1 and PP2A's FASTA sequences used as a query to look for protein structures that connected to evolution using the SMTL SWISS-MODEL template library [13]. Next step was model building, 3D protein model automatically created for each chosen template by first transferring conserved atom coordinates as determined by the target-template alignment.

Structural assessment and validation

After 3D prediction we had verified their 3D-structure quality via The PROCHECK and ERRAT programs that are beneficial for evaluating the quality of protein structures. Both programs accessed via SAVES V6.0 server (https://saves.mbi.ucla.edu/) for protein 3D-structural verification. PDB files of LKB1 and PP2A uploaded into SAVES V6.0 workplace and analyzed for structure validation. PROCHECK assesses the stereo chemical quality of protein structures, while ERRAT evaluates the statistical reliability of non-bonded interactions, providing comprehensive validation for protein structure models [14].

Molecular docking

In this study, molecular docking involved two main stages: Firstly, screening of ligands that have high affinity to the AMPK by mimicking the interacting mode of Adenosine Monophosphate (AMP). To find the compounds with the optimum binding affinity, several compounds screened from a small-molecule library to a target macromolecule (often a protein). Secondly, docking approaches for activating and phosphorylating AMPK with screened ligand and LK-B1 respectively. As well as docking between stimulated AMPK and

PP2A for increasing activity of PP2A to regulate expression of LOX-1 [15].

To enhance the accuracy of docking we carefully prepared AMPK (PDB ID; 7JHH) by identifying residues removing water molecules adding hydrogen atoms and eliminating chains i.e.in accordance with the literature review, the AMPK chains A, B, and G which involve in catalytic activity of AMPK were the only retained chains in this study, while the other chains had been eliminated [16]. This improved protein structure was then used for Virtual Screening, a technique in drug discovery that allows for accurate predictions of ligands during docking studies [17]. The goal was to obtain an estimation of the binding strength, within the protein cavity [18,19].

Therefore, MTiOpenScreen webserver utilized for ligand screening and the various steps were established including web search of MTiOpen; MTiOpenScreen-RPSB; Run MTiOpenScreen and different parameters such as PDB file as receptor format, lead like molecule selected to act as drug were set to perform a virtual screening of AMPK against ligand library [20]. By molecular docking, PyRx Software, an open source, free, and released under the Simplified BSD license was employed in our experiment to explore AMPK activator (ligand) whereby AMPK docked with screened ligand. After that, we had run second docking of AMPK-ligand complex (AMPK-ZINC000252441408, an output file of first docking by PyRx) with LKB1 via Hex Software, by uploading PDB output file of AMPK-ZINC000252441408 as receptor and LKB1 as ligand then dock procedure performed [5,21].

Finally, regarding to the literature review, activated and phosphorylated AMPKα catalytic subunit (A chain) stimulates PP2A to increase its activity by phosphorylating B56γ at Ser-298 and Ser-336 [22,23]. Thus, we preferred to modify an output file generated by the previous docking between Ligand bound AMPK and LK-B1 by retaining only residues at chain A of AMPK interacted with LK-B1. This was for ensuring the minimization of docking errors from other non-interested chains or residues [24]. Therefore, AMPK INC000014952116-LK-B1 complex docked with PP2A via HEX Software. HEX 8.0.0 Software superimposes pairings of molecules using only information of their 3D forms and it can compute protein-ligand docking under the assumption that the ligand is stiff [25].

RESULTSAND DISCUSSION

Phylogenetic tree analysis for PP2A and LK-B1

A consensus tree was constructed by utilizing the PHYLIP package to identify the level of relationship among the compared homologous proteins; it indicated PP2A and LK-B1 with their homologous proteins [11].

From phylogenetic analysis, “PP2A and 6NTS” were sister clade since they belong to the same tree branch, while 2NYL is considered as a precursor of PP2A. So, as this sister clade occurs, maximum times in 30 times, 6NTS has been used as a template for homology modeling intention [26].

While “1MRV and 1GZN” and “2JDO and 16OL” were sister clades and LK-B1 is an ancestor of these two sister clades. Since “1MRV and 1GZN” have occurred maximum times in 100 times we preferred to use 1MRV as homology model of liver kinase B1 (LK-B1).

Three-dimensional structural model prediction Structure Assessment and Validation of predicted Models (PP2A and LK-B1)

Template proteins 6NTS and 1MRV were selected for 3D structure prediction of Protein Phosphatase 2A and Liver Kinase B1, respectively. SWISS Model was used, meeting requirements of sequence identity, resolution, and clade proximity for accurate predictions [13,27].

Table 1: Structure assessment results for predicted models (PP2A and LKB1)

Tools / Model	SWISS MODEL	PROCHECK		ERRAT
	Ramach Fav	G-factor	Ramach fav	Quality factor
PP2A	95.26%	-0.09	99.4%	96.67
LK-B1	92.52%	-0.18	87.6%	85.27

SWISS-MODEL workspace aids users in identifying weak points in models through MolProbity plots and Ramachandran plots. Ideally, Ramachandran favored above 98% signifies optimal residue distribution as found on SWISS Model Webpage [28]. However, both PP2A and LK-B1 models achieved 95.26% and 92.52%, respectively. To ensure model validation, PROCHECK and ERRAT analysis were consulted [14].

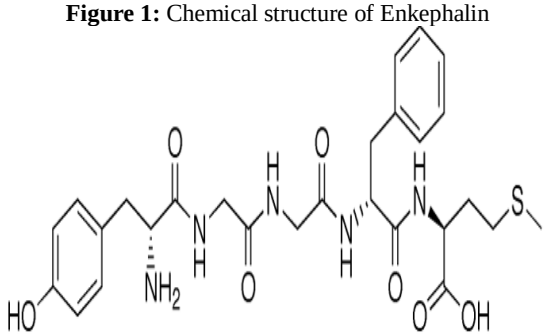
Error! Reference source not found. reveals a high-quality model of PP2A, evident by its G-factor of -0.09, within the acceptable range. The model's 99.4% Ramachandran favorability underscores its reliability, aligning with previous quality assessments. LK-B1's model, with a G-factor of -0.18 and 87.6% favored residues, suggests good quality [13], [27]. ERRAT's overall quality factors stand at 96.675 for PP2A and 85.2294 for LK-B1. Literally, for good high-resolution structures, the total quality factor value must be 95% or greater [29].

For that reason, LK-B1 has been remodeled through the refining process, while the PP2A model bypassed refinement. LK-B1 underwent refinement via GalaxyWEB server, improving to 91.2% favored residues and a positive G-factor, confirming its structural validity [30]. In addition to the increased overall quality factor, the positive G-factor usually indicates a good quality model aligning with Eshan Khan's emphasis on G-factor importance in model assessment since 2014 on research gate.

Virtual Screening of a highly potent ligand towards AMPK.

The study's first objective sought a suitable biomolecule binding to AMPK from a diverse compound library. MTiOpenScreen allows screening more than 10,000 molecules from its compound repository [20]. Thus, the virtual screening against AMPK by MTiOpenScreen was deemed highly reliable. Among the screened ligands, ZINC000014952116 (Enkephalin) emerged as the top contender with a binding energy of -11.2 Kcal/mol, aligning with Simon's findings since 2018 [31]. His work accepted ligands with less than -7.0 Kcal/mol binding energy. Low negative binding energy ensures more binding affinity [22,32,33]. This substantiates capability of Enkephalin (ZINC000014952116) as an effective AMPK modulator.

Figure 1: Chemical structure of Enkephalin



Activation of AMPK by ZINC000014952116 (Enkephalin)

It is crucial to have a basic understanding about the interaction between AMPK and ligand docked with it for validating the docked results in line with the wet laboratory experiments [18]. In this study we intended to find a Biomolecule that mimicked the activation activity of AMP towards AMPK. PyRx helped to generate many different docked conformations between ZINC000014952116 and AMPK during activation. Among the docked conformations, the precious one was recorded to have binding affinity (-9.4 kcal/mol) [34]. Regarding to the figure below, the different residues interacted with the ligand, Enkephalin, as displayed by using Pymol and Discovery Studio Visualizer. The interacting residues are: Ser[45,46,242], Arg[70,152,171,172,299], Ala[71,228,295], Ile[166,240], Tyr[165], Leu[48,229], Thr[168], Lys[47,170,174], Val[29,294], Phe[244], His[390], Pro[43,227], Gln[391], Gly[68], Asp[245] and Glu[296]. Arginine, Lysine and Histidine are the only basic amino acids which are positively charged while the Aspartate and Glutamate have the negatively charged R groups.

The interacting residues align with both wet laboratory findings and published data [5,22,23,31]. AMP binding to AMPK involves phosphate groups binding to residues including Arg[69], His[150], Arg[151], Lys[169], His[297], and Arg[298], along with Ser/Thr hydroxyl groups [22,23]. Leu[70,81], His[139], Lys[62,47], and Phe[160] are implicated in AMPK activation. Many of these residues also play roles in lipid metabolism, such as Threonine's role in controlling lipid biosynthesis and Isoleucine's impact on AMPK responses. Antioxidant and metabolic functions are associated with Leucine and Arginine, while Serine's dysregulated phosphorylation can impact proliferation signaling [35,36]. These identified interactions between AMPK and ZINC000014952116, supported by negative binding affinity predictions, validate their feasibility in biological systems [37,38].

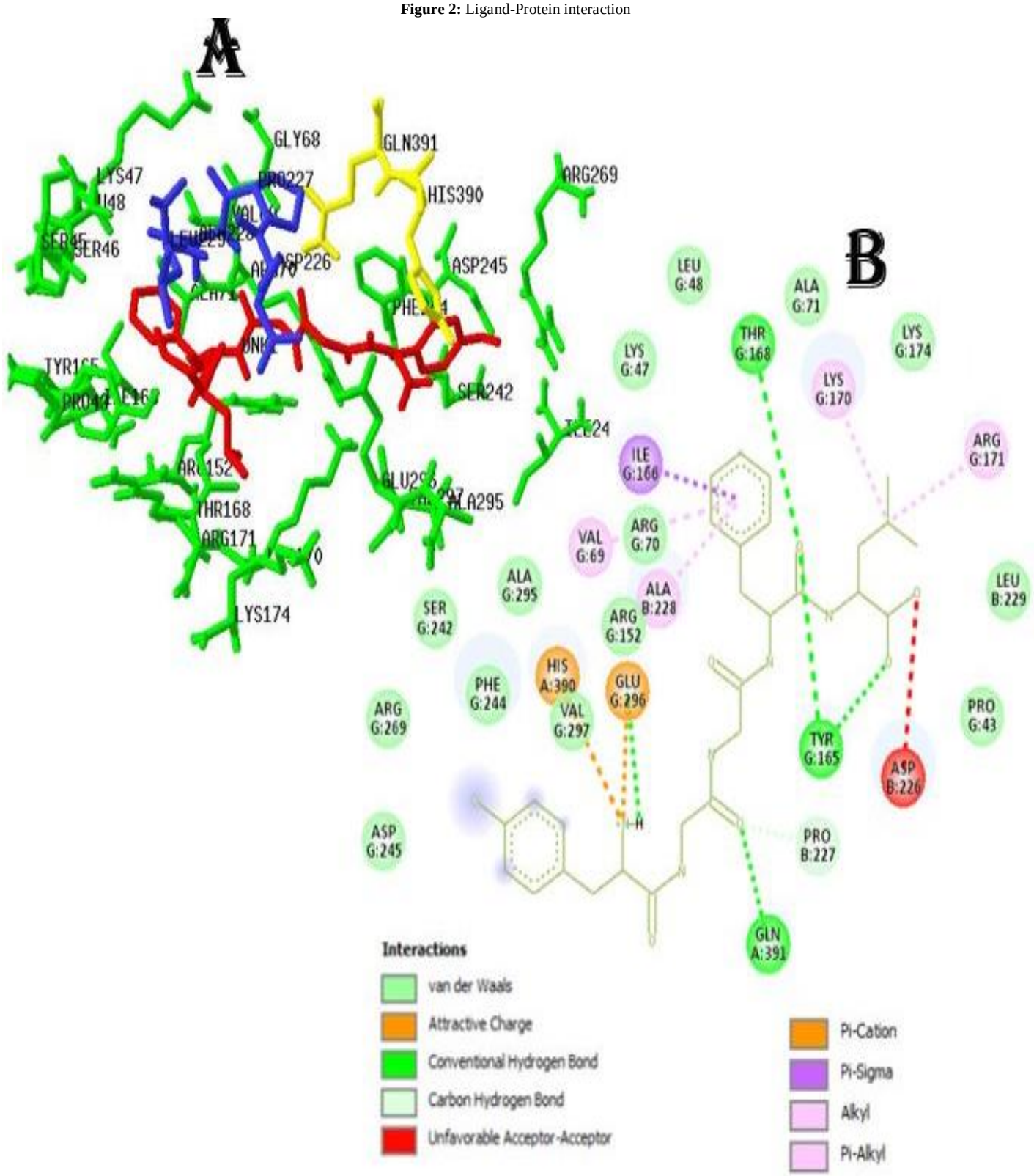
Phosphorylation of AMPK bound with ZINC000014952116.

Experimental evidence indicates that AMPKα's phosphorylation at Thr[172] involves LK-B1 and CaMKKβ kinases. The Adenosine derivative IMM-H007 strongly binds to AMPK-α, boosting Thr[172] phosphorylation [39]. AMP binding to AMPK's gamma subunit stabilizes a complex with LK-B1, enhancing Thr[172] phosphorylation [6]. However, the structural details of this interaction remain unknown [16].

In the following Figure 3:**A)** 3D structure for a complex of AMPK bound with ZINC000014952116 and LK-B1 Visualized by Discovery Studio Visualizer (DSV). **B)** 3D-Ribbon structure of the interacting residues between AMPK and LK-B1, generated by SPDBV package. **C)** Screenshot of the residues of A chain (catalytic subunit of AMPK) interacted with LK-B1), captured from SPDBV package in 2D manner.

This study surprisingly found that while Thr172, a known residue for AMPK phosphorylation was not involved in our interactions. The docking energy of -792.06 Kcal/mol indicated feasibility of LK-B1 and AMPK interaction. Residues such as Ser99, Lys36,43,60,109,156, Gly100, Tyr82,97,106, His77,152, Thr40, Val98, Arg120, Glu96,117, Asn134, Met153, Pro76, Asp150 and Leu39 made an interaction. The contradiction with prior lab experiments might be because we have retrieved AMPK PDB file which has been submitted based on Electron Microscopy Assay while the laboratory experiments that we referred to, have implied X-ray crystallography. Despite the 7JHH accession sequence not having Thr172, Thr40 emerged as a key interacting residue. The study employed the PDB file 7JHH based on Electron Microscopy (EM) Assay, offering high-resolution structural data. While EM provides detailed information, it may not capture finer details, possibly explaining the absence of Thr172 in the structural data [40].

Figure 2: Ligand-Protein interaction

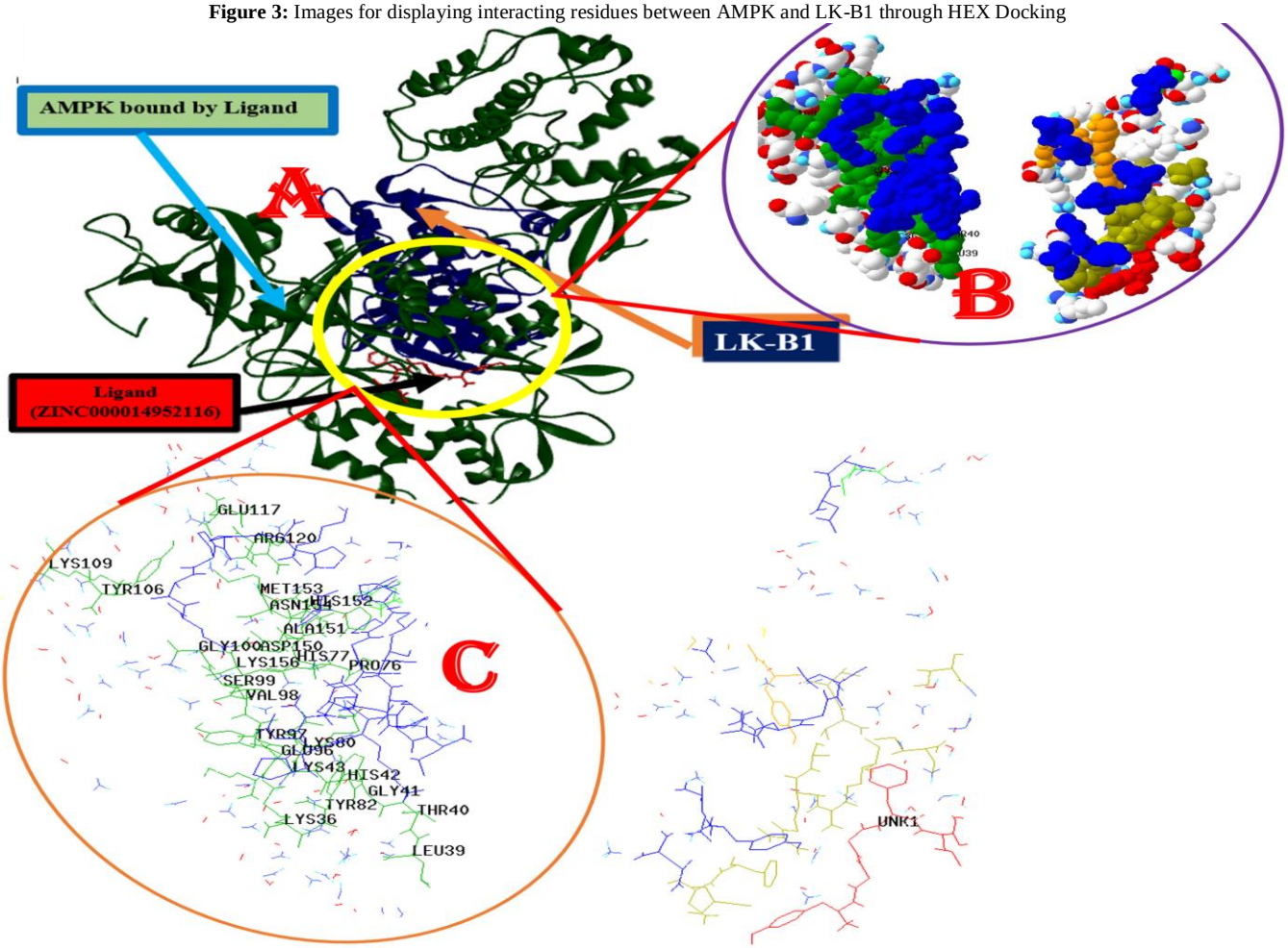


A) the image was generated by SPDBV Package: Red is a ligand (ZINC000014952116), green is α-catalytic subunit of AMPK, while yellow and blue are regulatory subunits of AMPK. **B)** Protein-Ligand interaction image from Discovery Studio Visualizer (DSV).

This limitation could stem from the resolution constraints of the EM technique. we consider the possibility of improving the mentioned lab data using a lower-resolution X-ray crystallography determined structure, which is currently unavailable for us.

Activation loop phosphorylation, enhancing AMPK activity, was suggested by Steinberg & Carling, while Leu, Ser, and Lys in interacting residues connect to kinase phosphorylation [41]. This insight suggests AMPK phosphorylation occurs at different residues than Thr-172. The phosphorylation could occur at any residue, as molecules are not static and have different active sites responsible for inducing expected behaviors [41].

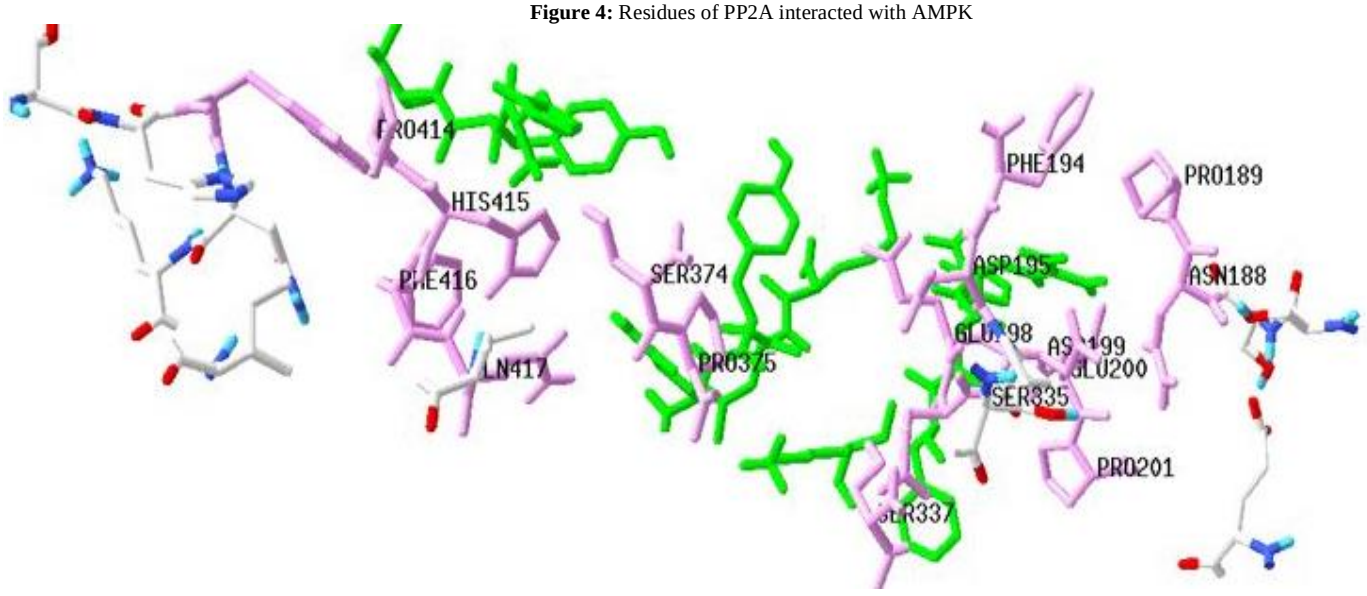
Figure 3: Images for displaying interacting residues between AMPK and LK-B1 through HEX Docking



Stimulation of PP2A activity by phosphorylated AMPK

Higher phosphorylation at Thr^{172} of AMPK leads to its influence on the kinase enzyme PP2A, resulting in increased PP2A activity through direct phosphorylation of B56γ subunits by adiponectin-activated AMPK [22]. Through Hex software, we predicted the docking energy between Protein Phosphatase-2A (PP2A) and AMPK, as well as uncovering interaction details. Hex software can predict the docking energy between two proteins, and it has been utilized by Gundampati who have been working on Aspergillus niger RNase and human actin docking [34]. The affected residues of PP2A such as, Pro, Phe, Ser, His, Asp, Asn, Gln, and Glu were all conserved motifs among the aligned sequences of homologous proteins of PP2A. while the predicted docking energy of -730.25 Kcal/mol suggested thermodynamic stability [33].

Figure 4: Residues of PP2A interacted with AMPK



In recent studies, phosphorylation at Ser-298 and Ser-336 of PP2A B56γ subunit correlates with our findings on Ser-337, as noted by Kim since 2009. AMPK activation potentially triggers NF-κB dephosphorylation, wherein PP2A plays a role in dephosphorylating NF-κB's subunit RelA/P65 at Ser536 [6,42,43]. This process reduces NF-KB transcriptional activity in macrophages, leading to decreased levels of LOX-1, responsible for oxLDL internalization [44]. Key interacting residues like Glutamine, Lysine, and Phenylalanine exhibit antioxidant and anti-inflammatory effects, potentially inhibiting MAPK-controlled signaling pathways and interfering with Lipoprotein binding to toll-like receptors [36,45].

CONCLUSION

In summary our research offers possibilities for comprehending and potentially tackling atherosclerosis a health concern. The potential of enkephalin as an AMPK activator could represent an advancement in combating the inflammation associated with atherosclerosis. However, it is important to recognize the constraints of our computer-based simulations and the requirement for confirmation, through laboratory studies. Future investigations, including molecular dynamics simulations and in vitro tests, will be crucial to advance our understanding and eventually translate these findings into therapeutic solutions. This study lays a foundation for the exploration of innovative atherosclerosis treatments, offering hope for improved cardiovascular health.

Authorship statements

Parfaite BIZIMANA

Literature review, software's' installation and visualization.

Leon Fabrice ISHIMWE Writing – Manuscripts draft, results analysis, visualization, interpretation, and validation.

Jean Fidele KATABARWA Methodology and hands on software.

Dr. Dieudonne MUTANGANA Supervision, writing - review and editing, project administration.

Declaration of interest

The authors declare that there are no conflicts of interest related to this research study or its publication.

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