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Homology modeling and molecular docking studies for the identification of novel potential therapeutics against human PHD3 as a drug target for type 2 diabetes mellitus

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ABSTRACT

PHD3 (Prolyl Hydroxylating Domain) 3 protein contains the HRE (Hypoxia Response Element) and plays an important role in regulating HIF subunits. The hydroxylating ability of PHD3 of HIF subunits makes PHD3 is a prominent therapeutic target to control type 2 diabetes mellitus. The structure based approach is used to design novel molecular entities against PHD3 protein. In this present work, a 3D homology model of PHD3 was generated by MODELLER9.9 as the experimental structure of PHD3 is not reported in the protein database. The 3d structural model of PHD3 refined through energy minimization in VMD-NAMD interface. Active site of the target protein is identified by SiteMap module (Schrodinger suite), manual correlation technique using ClustalW software and literature studies. The asinex library of chemical structures subjected to the molecular docking at the PHD3 active site for the identification of potent inhibitors. The molecules resulted from molecular docking prioritized based on their docking score, glide energy. The ligand molecules are further prioritized with a rescoring parameter Prime-MM/GBSA by calculating binding free energies of final Ligand-Protein complexes. The identified novel leads are further evaluated with ADME properties for their druglikeness activity. The overall insights can further expedite for the development of novel molecular entities as potential inhibitors against PHD3 in type 2 diabetes mellitus.

Keywords: PHD3, homology model, VMD-NAMD, Prime-MM/GBSA, ADME, type 2 diabetes mellitus.

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INTRODUCTION

Type 2 diabetes mellitus deals with insulin resistance and low levels of insulin release by pancreatic beta cells. A state of chronic inflammation and beta cell dysfunction induces insulin resistance and causes type 2 diabetes mellitus **[1, 2].** The current major anti-diabetic medications have disadvantages which includes efficacy, cost, potential side effects, weight gain, comorbidities, hypoglycemia risk and patient preference. Therefore chronic diseases like diabetes mellitus requires the design of new drugs with high specificity and efficacy in the treatment **[3].** In the present study, PHD (Prolyl Hydroxyl Domain) Proteins hydroxylate the proline residues of HIF and regulates HIF and in an oxygen-dependent manner **[4].** But under Hypoxic conditions hydroxylation of HIF results in dysregulation of HIF inducing inflammation of pancreatic beta cells leading to insulin resistance which is a major hallmark of type 2 diabetes mellitus **[5].** Inhibition of PHD3 activity by small molecule inhibitors leads to stabilization of HIF subunits providing a potential therapeutic strategy in the treatment of T2DM.

PHD3 is the liver specific protein residing in the pancreas containing hypoxia response element (HRE) which is upregulated by HIF. HIF is a transcriptional controller that plays an important role in maintaining oxygen levels in pancreatic tissues. HIF subunits are regulated by PHD3 protein by hydroxylating their proline residues **[6, 7].** A recent study supported that HIF is required for normal β-cell function and reserve and that its dysregulation may cause to the pathogenesis of type 2 diabetes. Figure1 representing the biological pathway of PHD3 in type 2 diabetes mellitus. Inhibition of PHD3 regulates HIF levels under hypoxia which increases the insulin secretion suggesting the important role of HIF in regulating the β-cell amount and implicating PHD3 as potential therapeutic target for the β-cell dysfunction of type 2 diabetes mellitus **[5, 8]**.

The present study was carried out for the identification of novel potential inhibitors against PHD3 as a therapeutic target for diabetes mellitus with a lower risk of side effects using computational approaches. In this study structure based virtual screening, molecular docking, MM/GBSA approach and ADME studies were together considered for

the development of new potential therapeutics against PHD3 in type 2 diabetes mellitus treatment.

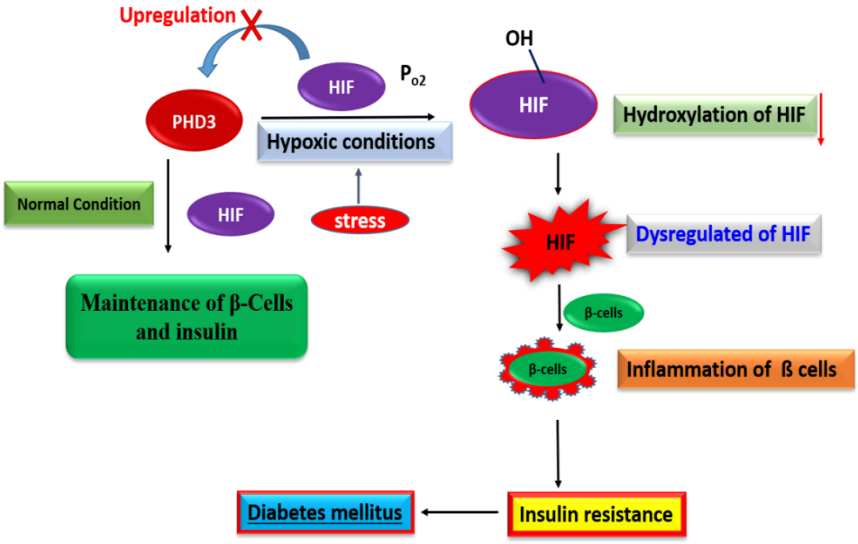

Figure 1. Biochemical pathway of PHD3 protein. Hypoxic conditions reduce the capacity of PHD3 to hydroxylate HIF, resulting in the dysregulation of HIF causing pancreatic inflammation leading to a state of insulin resistance results in type 2 diabetes mellitus.

MATERIALS AND METHODS

Homology modeling of PHD3 protein

Homology modeling is used to make a 3D model of the target protein as the structure of a protein is unknown or it is not determined by experimental techniques like NMR and X-ray. **[9]**. A known template sequence with good similarity, identity and e-score is required to align with the sequence of the target protein to generate homology model. The 5LBF A was selected as potential template by providing the FASTA sequence of PHD3 to the BLAST server. The sequence with the highest similarity and e-score is considered as a reference sequence (5LBF A) to produce a 3d homology model of PHD3 **[10].** The alignment of both the sequences was operated in clustalX2 to find out the regions of similarity that represents the functional and structural evolution of sequences **[11].** The homology model of PHD3 was generated by using MODELLER9.9 software. Twenty (20) models were generated during the modeling, among these 3d structures of protein, the one with least modeler objective function (molpdf) was taken into the consideration for further refinement of 3d model and methodology.

Energy minimization and simulation studies

The homology modeling produces the 3d structure with high energy because of abnormal overlapping of nonbonding atoms of protein leading to severe steric clashes between residues of a protein**.** The refinement of protein is carried out using NAMD (Nanoscale Molecular Dynamics) and results visualized with VMD (Visual Molecular Dynamics). NAMD is a key molecular simulation software package to refine (energy minimization) the 3d structure of a protein with visualizing suite VMD **[12]**. The energy minimization of PHD3 in NAMD suite was carried out in a total of 100000 steps and monitored by RMSD values with time units(fs). The refined protein is used for further in-silico operations and docking studies**.**

Validation of PHD3 protein

Validation of the 3d model of PHD3 is required to know the quality of the 3d structure. Various online protocols are employed to check the reliability of the 3d model. The quality of the 3d model is evaluated with ProSA, PROCHECK **[13]** (Ramachandran plot). The ProSA server was recruited to estimate the energy profile and to validate protein in terms of z-score. PHD3 protein is validated with Procheck program in pdbsum server delivers Ramachandran plot which gives the favored regions of residues in accordance with stereochemistry.

Active site prediction

Predicting the potential active site residues of the target protein is an important outcome in drug discovery. Binding site residues of the protein bind to the ligand residues with its hydrogen bond donors and acceptors [**14**]. The Active site of PHD3 protein was analyzed with bioinformatics tools like SiteMap (Schrodinger), literature and manual correlation technique. The reported 3d structure of template 5LBF was retrieved from pdbsum server consisting of 224 amino acid chain which is reported with the ligand UN9 (N-[1-Chloro-4-hydroxy isoquinoline-3-Y1 carbonyl] glycine), with the molecular formula: ClH9ClN2O4 is taken into the consideration and the active site residues (pdbsum-ligplot) correlated to PHD3 protein sequence for active site prediction. The residues obtained by manual correlation are considered as active site residues responsible for functional evolution of PHD3. The nature of active site regions of protein is analyzed by SiteMap in Schrodinger suite which gives information about volumes of acceptor and donor regions of binding pockets in angstrom units [**15,16**].

Virtual screening and molecular docking

Virtual screening of chemical libraries is a quick and accurate technique to identify novel and potential leads in the drug discovery process. Asinex ligand data set of small molecules are selected to screen with PHD3 protein using Glide tool in maestro Schrodinger suite for identification of lead molecules. Ligands were prepared with the help of Ligprep module in Schrodinger software with an applied force field of OPLS_2005 in order to generate its 3D coordinates from 1D. Asinex databank with 10000 molecules was subjected to the Ligprep in maestro Schrodinger suite results optimized isomers. These molecules, in turn, are used as an input for virtual screening. Virtual screening of Asinex small molecules were carried out under HTVS (High

Throughput Virtual Screening), SP (Standard Precession) and XP (Xtra Precession) modes of filtering to results lead molecules. The final lead molecules were prioritized based on their glide score, glide energy and ADME properties **[17].**

Pharmacology

Elucidation of Pharmacology of new lead molecules obtained from molecular docking is an essential protocol to study pharmacokinetic properties (ADME) which are used to estimate druggability of new chemical entities. ADME properties of best fit molecules are predicted using Qikprop **[18]** module in Schrodinger suite.

Binding free energy calculations

The binding free energies of protein-ligand complex are calculated using Prime MM-GBSA approach (19). The Binding free energies of top lead molecules with protein receptor calculated using Prime- MM/GBSA method with an applied force field of OPLS_2005 in maestro Schrodinger suite.

Binding free energy of complex (receptor to ligand) calculated using the following equation

ΔG_{bind} = G (ligand-receptor) - (Gprotein + Gligand)

RESULTS AND DISCUSSION

Homology modeling and model refinement

Target protein PHD3 with accession id Q9H6Z9 with 239 amino acid residues was selected from UniProt from Server Expert Protein Analysis System (ExPASy SWISS-PROT/TrEMBL) which is a bioinformatics data source of proteins **[20]**. Homologous template 5LBF A is selected from BLAST and J-Pred online servers by submitting target protein sequence in Fasta format as an input. Both the servers provided similar templates for target PHD3 protein based on parameters sequence position and secondary structure prediction respectively. Template 5LBF A obtained from BLAST has an e-score of 6e-110, 93% query coverage, and 65% of identity. J-Pred also results 5LBF A as template with an e-score of 4e-86. The conserved domain of the PHD3 protein retrieved from BLAST server shown in Figure2. The 3d homology model of PHD3 protein was generated using MODELLER9.9 which it uses alignment of query sequence with its homologous template 5LBF A sequence. The sequence alignment of PHD3 to that of template 5LBF A is visualized with discovery studio4.1 shown in Figure3. Twenty (20) 3d models are generated, among this group of models, one of the model consists of least modeler objective function value 1213.63(molpdf) which is taken into consideration for further computational evaluation **[21].** The secondary structure of PHD3 shown using pymol tool (Figure4).

Energy minimization of PHD3 protein was carried out in NAMD-VMD interface. Simulation studies were applied for the energy minimization of PHD3 protein. Energy minimization was carried out in NAMD software with a CHARMM-AMBER force field **[22].** A total of 100000 cycles of the energy minimization was carried out using NAMD module. The protein coordinates are analyzed with RMSD values. The RMSD values observed during simulation studies of PHD3 protein represented with a graph shown in Figure5. The RMSD values observed between 1.0 to 1.2Å where it reaches energy minima at averaged RMSD value of 1.10 Å which become remain constant with time units (1001-2003 fs) where RMSD is a measure of equilibration and protein flexibility.

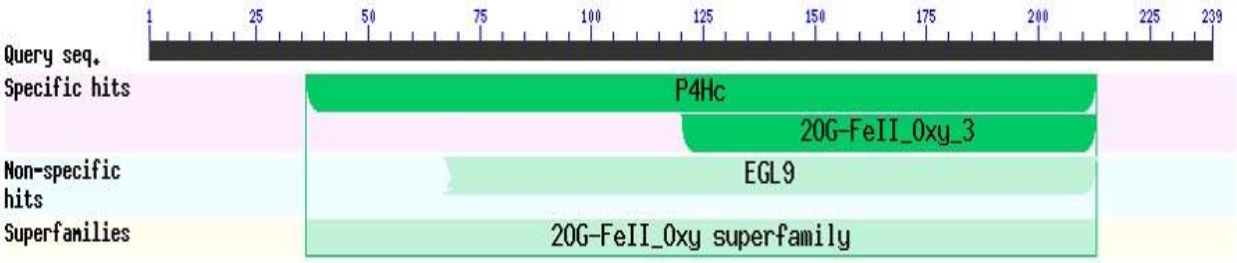

Figure 2. The conserved domain of PHD3 protein was saved from the BLAST server by submitting fasta sequence as an input.

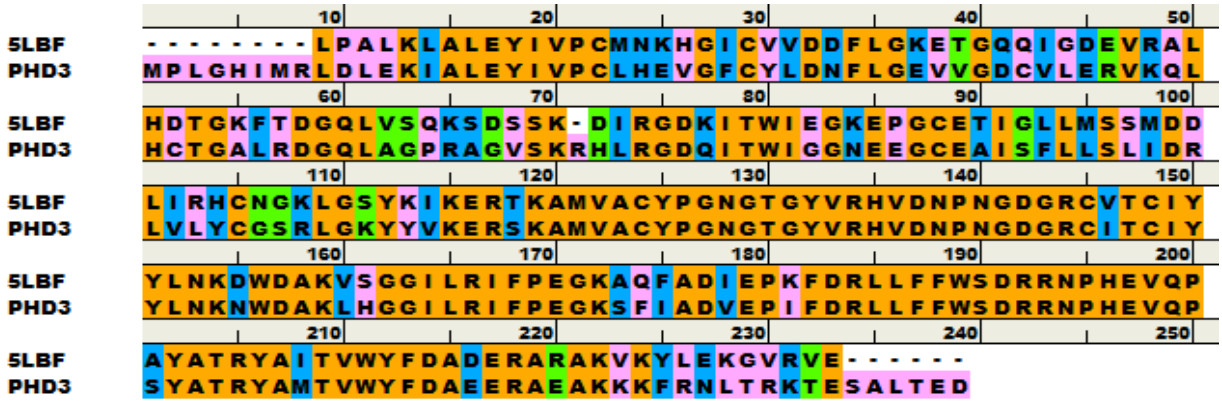

Figure 3. Sequence alignment of Residues of human PHD3 protein with the residues of its homologous template 5LBF is executed in discovery studio 3.5. Identical residues are highlighted with orange color, the residues fall in the strong zone are colored with blue, weak zone residues are indicated with green color, whereas the residues showed in pink color are not matched residues.

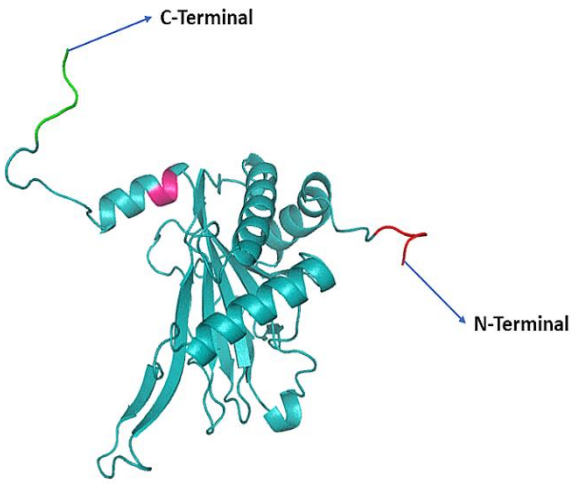

Figure 4. The secondary structure of PHD3 consists of 6α-helices represented in spirals and 3 beta sheets. The conserved part of the protein exposed with the pink color. C-terminal and N-terminal end of the 3d structure indicated with green and red color respectively.

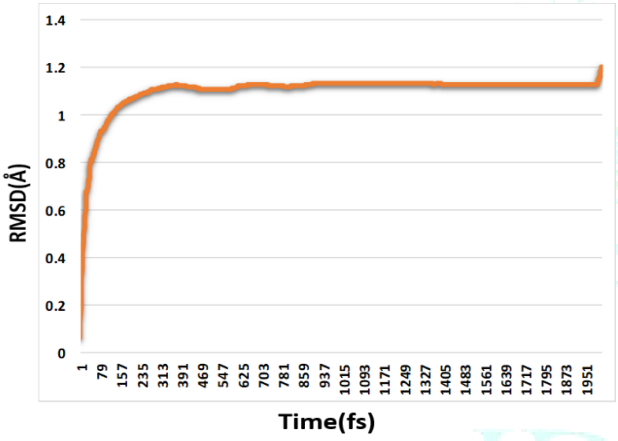

Figure 5. The graph representing the RMSD values of various energy states at different intervals of time as a plot of RMSD values against time frames. The averaged RMSD value of 1.10Å is observed between 1001-2003 time frames.

Validation of PHD3 protein

The reliability of PHD3 model evaluated with standard validation protocols. Ramachandran plot of PHD3 protein is retrieved from PROCHECK in pdbsum server. The steric clashes between psi-phi angles of amino acid residues of protein were explained with a contour plot. Ramachandran plot of PHD3 protein consists a total of 99.6% of residues falls in the core region of the plot which is a sterically free zone indicating that PHD3 is a reliable model to continue for further computational studies. The detailed contour plot of allowed and disallowed regions represented with Ramachandran plot shown in Figure6 **[23]**. The overall model quality of PHD3 is evaluated by using ProSA **[24]** in terms of z-score. The ProSA analysis of PHD3 provides a structural similarity with a z-score of -5.8 signifying the good quality of protein which falls in the region of similar groups of structures of protein determined from X-ray and NMR showed in the ProSA with light blue and blue color respectively shown in Figure7.

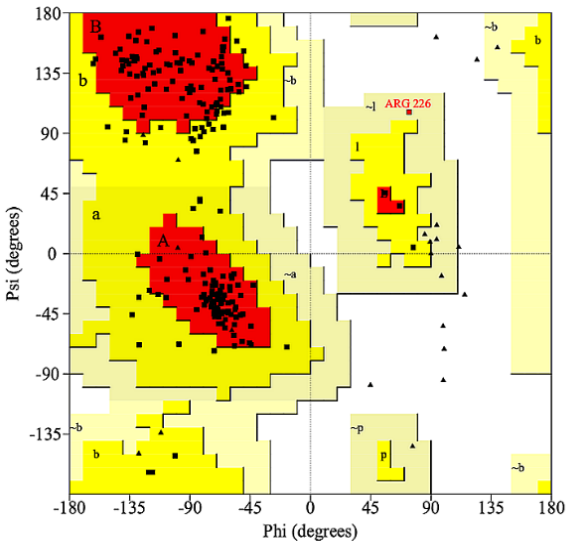

Figure 6. Ramachandran plot of PHD3 is saved from Procheck server. The overall plot of PHD3 Protein consists of 99.6% of residues lies in the sterically free zone, that is favored region, which reporting good quality of the 3D model.

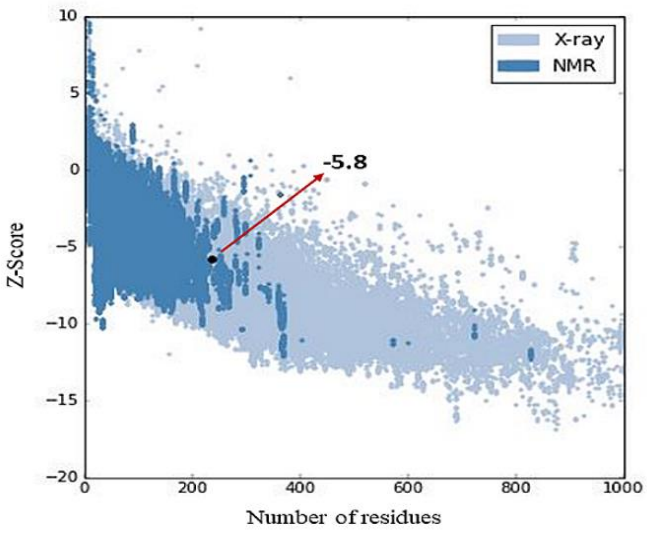

Figure 7. The ProSA plot of PHD3 is showing a z-score of -5.8 representing the good quality of protein structure. The regions with blue and light blue colors are about the groups of similar structures of protein, determined from NMR and X-Ray methods respectively. The black spot resembling the PHD3 pdb.

Identification of the binding site of PHD3 protein

The binding site of PHD3 is identified by the manual correlation technique, SiteMap and other computational tools. The Ligplot of known template 5LBF taken from Pdbsum server and consists the ligand named UN9 (N- [1-Chloro-4-hydroxy isoquinoline-3-Y1 carbonyl] glycine) showing the interactions with the residues Gly215, Lys216, Glu217, Asp254, Ile256, Met299, Tyr303, Tyr310, His313, Asp315, Ile327, Tyr329, Leu349, His374, val376 and Arg383. These residues of 5LBF were manually correlated to the residues of the target sequence in ClustalW Omega software by means of multiple sequence alignment in order to identify the active site residues of PHD3 protein (Figure8). The

putative binding regions of the PHD3 protein are identified using SiteMap with an applied force field of OPLS_2005 in Schrodinger suite. The SiteMap of PHD3 provides information about the nature of binding of regions (Figure9). SiteMap identifies potential binding regions by correlating to active site cavities that are most likely participates in protein-ligand binding and the area of binding pockets measured in terms volume in angstrom units as listed in Table1**[25].**

Table 1. The putative active site, nature of binding regions that bind with ligand hydrophobic and hydrophilic regions are calculated from the SiteMap in Schrodinger software.

Sitemap cavity	Region name	Volume (Å)
1	Hydrogen acceptor	148.12
2	Hydrogen donor	226.99
3	Hydrophilic	387.06
4	Hydrophobic	56.57

```
5LBF        --------LPALKLALEYIVPCMNKHGICVVDDFLGKETGQQIGDEVRALHDTGKFTDGQ
PHD3        MPLGHIMRLDLEKIALEYIVPCLHEVGFCYLDNFLGEVVGDCVLERVKQLHCTGALRDGQ
                     *   *:*********::: *:* :*:***: .*: : :.*: ** ** : ***

5LBF        LVSQKSD-SSKDIRGDKITWIEGKEPGCETIGLLMSSMDDLIRHCNGKLGSYKIKERTKA
PHD3        LAGPRAGVSKRHLRGDQITWIGGNEEGCEAISFLLSLIDRLVLYCGSRLGKYYVKERSKA
            *.. ::. *.:.:***:**** *:* ***:*.:*:* :* *: :*..:**.* :***:**

5LBF        MVACYPGNGTGYVRHVDNPNGDGRCVTCIYYLNKDWDAKVSGGILRIFPEGKAQFADIEP
PHD3        MVACYPGNGTGYVRHVDNPNGDGRCITCIYYLNKNWDAKLHGGILRIFPEGKSFIADVEP
            ************************:********:****: ***********: :**:**

5LBF        KFDRLLFFWSDRRNPHEVQPAYATRYAITVWYFDADERARAKVKYLEKGVRVE------
PHD3        IFDRLLFFWSDRRNPHEVQPSYATRYAMTVWYFDAEERAEAKKKFRNLTRKTESALTED
            ******************:******:*******:***.** *: :    :.*
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Figure 8. Identification of active site residues of PHD3 protein by manual correlation of template 5LBF residues with that of PHD3 residues using ClustalW, indicated with green and yellow colors respectively.

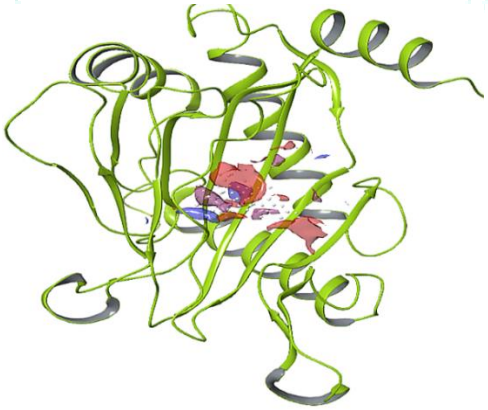
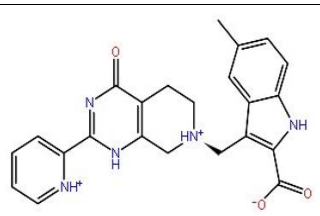
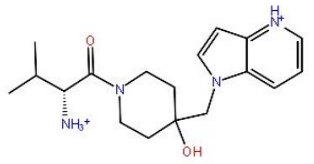
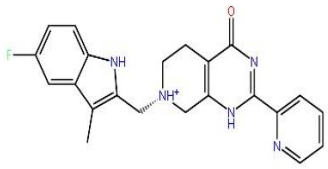
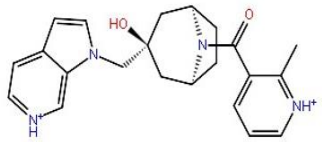


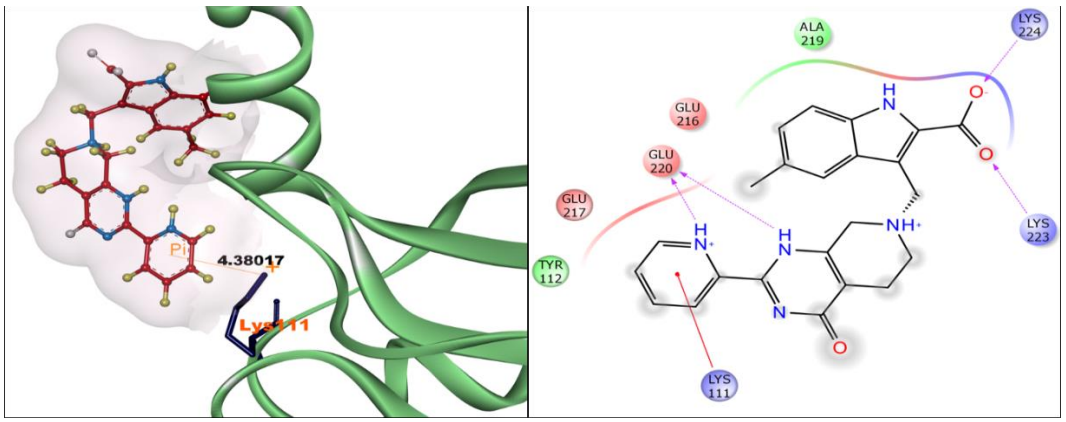
Figure 9. The putative active site of PHD3 protein identified using the SiteMap tool in Schrodinger suite. The blue color region of the protein is indicating that hydrogen acceptors area; the red color region is displaying that hydrogen donor area. The Hydrophobic region of the active site is shown in maroon color. Grey colored dots represent the cavity of the putative active site of the protein.

Virtual screening and docking analysis

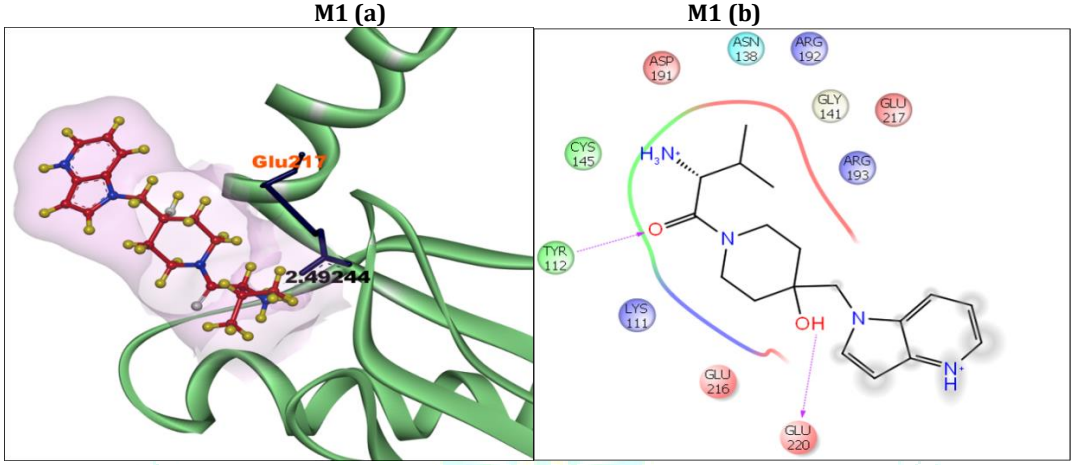
Structure based virtual screening is an important technique for obtaining lead molecules from data sets chemical structures to act against the biological target. PHD3 protein is screened with a data set of ligand library Asinex consisting of 10000 molecules. A three dimensional grid is generated by Glide module in Schrodinger suite using active site residues of PHD3 protein as input for virtual screening studies with the dimensions of 32 Å x32 Å x32Å and 80 Å x80 Å x80Å with a box size of 1.00Å. The Ligand preparation was carried out using Ligprep **[26]** application in Schrodinger suite for molecules of Asinex database in order to generate energetically favorable structures and its atomic coordinates tautomers and ionic isomers of ligand molecules to avoid poor interactions to PHD3 protein in docking. These generated ligands used as an input source for virtual screening **[27].** The Ligprep out file of Asinex database consisting of 31820 molecules is subjected to HTVS mode of screening which filters a large number of molecules in part of docking with 5 poses per ligand. 10% (3182) of the molecules are comes out from the HTVS mode of screening based on glide screening functions. These 3182 molecules are redocked under SP (Standard Precession) mode in order to produce more effective ligands which results in 318 molecules which are in turn redocked in XP (Extra Precession) mode to filter ten percent of molecules from SP docking. The final out file of XP results in 32 hit molecules which are considered as best fit molecules for PHD3 protein. [**21, 28**]. The screened best fit molecules are ranked based on binding free energies (MM/GBSA), docking scores and glide energy as tabulated in Table2. The four lead molecules M1-M4 are identified as potential inhibitors based on binding free energies (MM/GBSA), docking scores, glide energies and hydrogen bond interactions. The binding poses of M1-M4 with PHD3 active site are visualized in three dimensional patterns using discovery studio4.1 as shown in Figure10. The identified new lead molecules M1-M4 are consistently binding to the residues Lys111, Glu216, Glu217, Glu220, and Tyr112 of PHD3 protein by making hydrogen bonding interactions indicating their binding specificity.

Table 2. Structures of lead molecules M1-M4 identified from virtual screening of Asinex dataset with PHD3 protein and their intermolecular interactions; bond distances prioritized with glide score, glide energy and binding free energies.

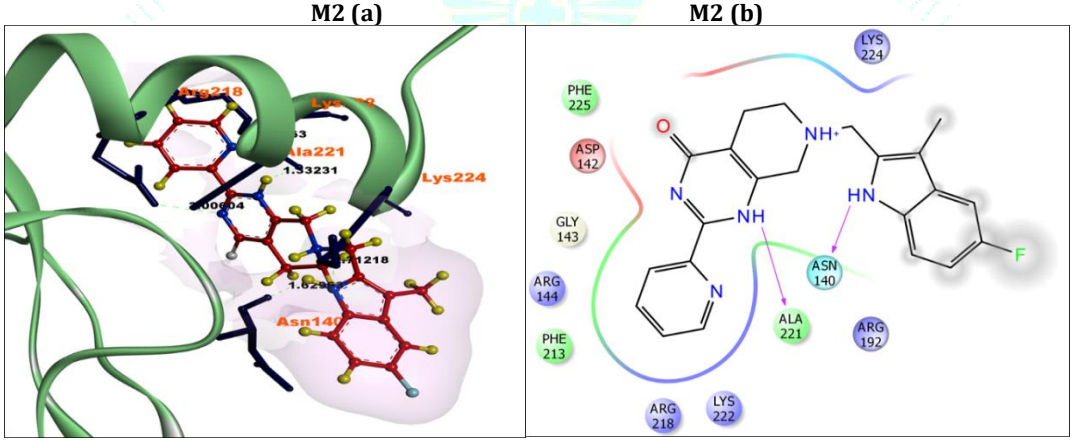
S.No	Structure of lead molecule	Glide score	Glide energy (kcal/mol)	Intermolecular interactions (H-Bonds)	Bond distance(Å)	Prime-MM/GBSA, ΔG_{Bind} (kJ/mol)
M1		-8.71	-34.38	M1::LYS-111 M1::LYS-223 M1::GLU-220 M1::GLU-220	4.38 2.43 3.47 1.30	-37.55
M2		-7.13	-23.59	M2::GLU-217 M2::GLU-217 M2::GLU-220 M2::TYR-112	2.49 1.51 2.98 3.47	-32.50
M3		-6.87	-33.10	M3::ARG-218 M3::LYS-222 M3::LYS-224 M3::ALA-221 M3::ASN-140	3.00 2.56 2.71 1.33 1.62	-31.28
M4		-6.18	-30.43	M4::TYR-112 M4::LYS-111 M4::LYS-111 M4::GLU-220	3.17 4.51 4.54 4.31	-46.61



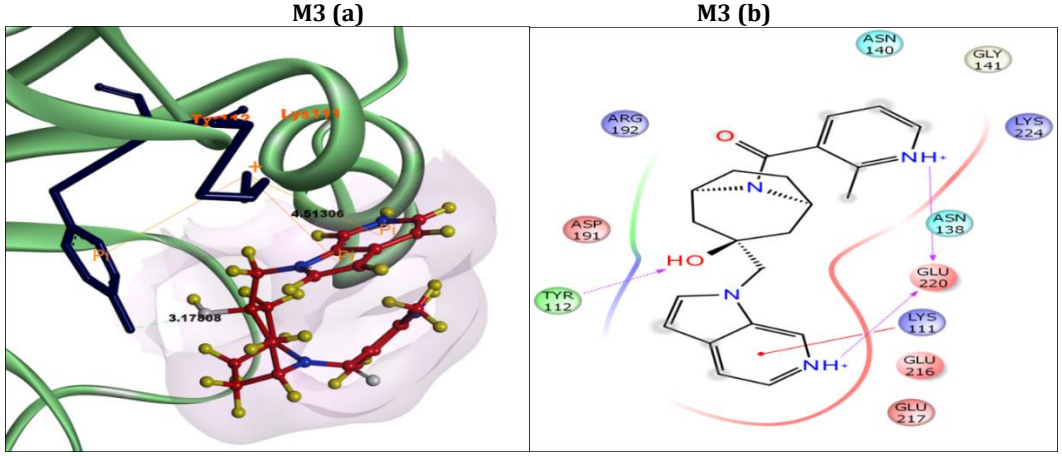
M1 (a) M1 (b)



M2 (a) M2 (b)



M3 (a) M3 (b)



M4 (a) M4 (b)

Figure 10. The binding orientations of best fit molecules M1-M4 identified from virtual screening. The protein showed in solid ribbon manner with green color and ligand displayed as ball-stick form. The amino acid residues are represented in the form of sticks with blue color. The intermolecular hydrogen bonds are denoted with light green dotted lines and pi-pi interactions are shown with orange color lines. The 2d ligand interaction figures retreived from schrodinger suite.

Binding free energies and ADME studies

Binding free energies of identified new lead molecules are calculated using the prime-MM/GBSA tool in Schrodinger suite. Binding free energies of newly identified lead molecules with protein receptor are calculated using OPLS-AA (2005) force field and GBSA continuum solvent model. A lead molecule M4 from final hits showing the good binding free energy of -46.61 kJ/mol with a 100% human oral absorption which consists 1H-pyrrolo[2,3-c]pyridine scaffold acts as novel pharmacophore moiety may be considered as more potent for the inhibition of PHD3 against T2DM. All the four identified novel lead molecules M1-M4 are showing the permissible binding free energy values in range of -46.61 to -31.28 kJ/mol and showing consistent interactions with the residues Lys111, Tyr112, Glu217 and Glu220 which are responsible for their specific binding affinity. The docking score, glide energy, hydrogen bond interactions and MM/GBSA binding free energies are important factors to design potential inhibitors against PHD3 protein**.** The pharmacokinetic properties (ADME) of identified lead molecules are calculated using Qikprop in Schrodinger suite to estimate druglike activity. All the four lead molecules are following the Lipinski rule of five, Jorgensen rule of three and existing within the range of acceptable CNS ranges and showing permissible ranges of human oral absorption (Table3). **[29]**.

Table 3. The ADME properties are calculated using QikProp module. All the identified leads M1-M4 are satisfying the pharmakokinetic properties in the permissible range of ADME values.

S. No	CNS	m.wt	Donor Hb	Acceptor Hb	Q plog Po/w	Q plog BB	% of Human oral Absorption	Rule of three	Rule of five
M1	-2	415.45	3.0	8.50	0.55	-1.43	46.06	1	0
M2	0	330.42	3.0	5.75	1.58	-0.47	79.10	0	0
M3	1	389.43	2.0	6.50	3.32	-0.27	88.19	0	0
M4	0	376.45	1.00	6.25	3.79	-0.473	100	0	0

CONCLUSION

In this study, PHD3 has been considered as a therapeutic drug target against type 2 diabetes mellitus. The homology model of the molecular target PHD3 protein was generated by MODELLER9.9 program for structural elucidation. The refinement of 3d model was done through energy minimization in NAMD software. Virtual screening was carried to find out new molecular entities to act against the binding site of PHD3. A total four potential leads M1, M2, M3 and M4 are identified as novel chemical entities against target protein which are prioritized based on glide score, glide energy, binding free energies (MM/GBSA), and % human oral absorption (Bioavailability). The lead molecule M4 is showing maximum bioavailability (100%), predictable binding free energy (-46.61kJ/mol) from Prime-MM/GBSA and contain 1H-pyrrolo[2,3-c] pyridine moiety as it acts as pharmacophore which is more probable to design as potent inhibitor. The results showing that the identified lead molecules M1 to M4 are showing specific binding with the residues Lys111, Tyr112, Glu217, and Glu220 and satisfying the druglikeness by showing permissible ranges of ADME properties. The whole procedure gave insights into the structure based approach to design potential therapeutics against PHD3 for type 2 diabetes mellitus.

CONFLICTS OF INTEREST

There is no potential conflict was reported by authors.

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