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Linagliptin and Empagliflozin Inhibit Microtubule Affinity Regulatory Kinase 4: Repurposing Anti-Diabetic Drugs in Neurodegenerative Disorders Using In Silico and In Vitro Approaches

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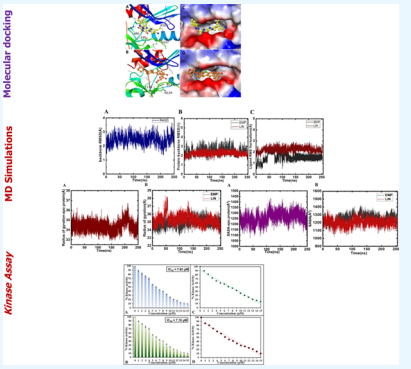
ABSTRACT: Type 2 diabetes mellitus (T2DM) and Alzheimer's disease (AD) are significant public health burdens. Many studies have revealed the possibility of common pathophysiology between T2DM and AD. Thus, in recent years, studies deciphering the action mechanism of anti-diabetic drugs with their future use in AD and related pathologies are on high demand. Drug repurposing is a safe and effective approach owing to its low cost and time-saving attributes. Microtubule affinity regulating kinase 4 (MARK4) is a druggable target for various diseases and is found to be linked with AD and diabetes mellitus. MARK4 plays a vital role in energy metabolism and regulation and thus serves as an irrefutable target to treat T2DM. The present study was intended to identify the potent MARK4 inhibitors among FDA-approved anti-diabetic drugs. We performed structure-based virtual screening of FDA-approved drugs to identify the top hits against MARK4. We identified five FDA-approved drugs having an appreciable affinity and specificity toward the binding pocket of MARK4. Among these identified hits, two drugs, linagliptin, and empagliflozin, favorably bind to the MARK4 binding pocket, interacting with its critical residues and thus subjected to detailed analysis. All-atom detailed molecular dynamics (MD) simulations revealed the dynamics of binding of linagliptin and empagliflozin with MARK4. Kinase assay showed significant inhibition of MARK4 kinase activity in the presence of these drugs, implying them as potent MARK4 inhibitors. In conclusion, linagliptin and empagliflozin may be promising MARK4 inhibitors, which can further be exploited as potential lead molecules against MARK4-directed neurodegenerative diseases.



1. INTRODUCTION

In the present era, much progress has been made in technology that has played a vital role in understanding the complexity of human diseases. This improved understanding aids in the better treatment of these diseases. Still, many drugs targeting these diseases fail at different stages of clinical trials creating chaos for pharmaceutical industries in terms of monetary and social aspects. Drug repositioning or repurposing, an effective strategy targeting new indications for existing drugs in other diseases, is an answer to tackle these issues.[1,2] Drug repurposing has various benefits with minimal monetary investment[3] and maximum paybacks. The risk of failure is lower than the new drug candidate since it has already passed different trials. The significant advantage of drug repurposing is to exploit different pathways and targets availed by a drug for its action.[4] Safety is another asset of drug repurposing because the drug toxicity data already exist, resulting in a drastic reduction in the processing time.[5]

Alzheimer's disease (AD), a chronic neurodegenerative disease, is characterized by decreased cognitive ability, memory impairment, and personality changes. A major obstacle in anti-AD drug discovery is the slowness of the onset and AD progression.[6] Diabetes mellitus (DM), the most prevalent chronic metabolic condition (463 million were affected by DM in 2019), has shattering complications with an increased risk of premature death.[7] Type 2 diabetes mellitus (T2DM) is mainly characterized by hyperglycemia and insulin resistance.[8] Studies have shown the association of T2DM with high dementia chances,[9] especially AD, by 45−90%.[10] This association can be attributed to the detrimental effect of insulin resistance and hyperglycemia on cognitive abilities because somatomedin C (IGF-1) plays a crucial role in cognitive ability, neural function, and development.[11] According to a study, individuals with cardiovascular diseases, hypertension, and diabetes have higher chances of suffering from AD later in life.[12]

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Table 1. List of Selected Drugs Based on the Binding Affinity with MARK4

s. no.	drug	binding affinity (kcal/mol)	pKi	ligand efficiency (kcal/mol/non-H atom)	torsional energy
1	linagliptin	−9.6	7.04	0.25	1.87
2	empagliflozin	−9.3	6.82	0.27	3.11
3	glimepiride	−9.2	6.75	0.25	2.18
4	glipizide	−8.8	6.45	0.26	2.18
5	canagliflozin	−8.8	6.45	0.26	2.80
6	5RC (PDB ID)	−9.1	6.67	0.28	1.87

Cognitive impairment is another shared abnormality between T2DM and neurodegenerative and neuropsychiatric disorders, such as AD and schizophrenia.[13] Recent findings demonstrate that the brain is another important site of insulin resistance and its association with cognitive dysfunction.[14] MARK4 inhibition improves glucose homeostasis by upregulating AMPK kinase in tissues.[15] Altogether, these studies highlight the crucial role of MARK4 in energy metabolism and regulation, making the drug an irrefutable target for treating T2DM.

Repurposing approved drugs provides a newer avenue for developing safe and effective therapeutics against diseases.[16] To date, traditional therapeutics targeting AD-related subpathologies are ineffective; hence, the need arises to look beyond these.[17] Drug repositioning and repurposing might answer this as it enhances efforts in traditional drug development, aiding in identifying novel approaches for the treatment of AD dementia and mild cognitive impairment.[18] This study serves as a connecting link between diabetes and neurodegenerative disorders. Linagliptin, a dipeptidyl peptidase-4 inhibitor, was approved by the United States Food and Drug Administration (USFDA) on May 2, 2011, for the treatment of T2DM. Many recent studies show the neuroprotective potential[19] of it, thus attracting researchers' attention. The other drug, empagliflozin, is used as an adjunct to diet, exercise, and other drug therapies and plays a vital role in the reabsorption of glucose in the kidney.[20] According to a study, empagliflozin enhances the modulation of neurotransmission, establishing the growth, survival, and plasticity of neurons.[21]

Here, we employed structure-based virtual screening to find potential inhibitors of MARK4. We searched the literature database to find FDA-approved anti-diabetic drugs and performed virtual screening of these drugs against MARK4, a potent druggable target for cancer, neurodegenerative diseases, and obesity. We obtained the top five hits and selected the top two for further detailed analysis. The top two drugs, linagliptin and empagliflozin, were subjected to detailed analysis using an extensive molecular dynamics (MD) simulation study to decipher the binding and conformational dynamics of MARK4−drug complexes. Additionally, a kinase assay was deployed to understand the inhibitory effect of these drugs on MARK4 kinase activity, i.e., to understand the implication of these drugs on the functionality of MARK4.

2. MATERIALS AND METHODS

2.1. Virtual Screening. We retrieved the MARK4 crystal structure (high resolution and no mutation) from Protein Data Bank[22] (PDB ID 5ES1). We created a library of FDA-approved anti-diabetic drugs (23 drugs) and performed virtual screening using the protein−ligand docking method for the target MARK4. InstaDock software was used to perform molecular docking-based virtual screening in a blind search space.[23] InstaDock is a front-end graphical user interface written in Python language to perform molecular docking-based virtual screening that can be done in just one go. The docking analysis of MARK4 was done to observe the bond conformations and binding affinity of ligands with the MARK4. The screening results were analyzed from the out files and log files when virtual screening was completed using InstaDock. The most suitable docked conformations were then taken for further analysis. PyMOL and LigPlot were used to visualize and structure for the analysis of the docked complexes.

2.2. Re-Docking Analysis. The elucidated drugs from the virtual screening study were taken for re-docking analysis with the MARK4. Empagliflozin and linagliptin satisfy Lipinski's drug like tests and are water-soluble. Table S1 shows the critical molecular descriptors of both drugs.

2.3. System Preparation Prior to the MD Setup. We started MD simulation studies using the top-docked pose. Initially, geometry optimization was carried out of the fragments using the B3LYP/6-31G(d) method[24] in the Gaussian 16 program.[25] After that, we estimated the electrostatic potential charges.[26] The next step was to parametrize the ligand, and this was done using antechamber in Amber Tools,[27] and ligand frcmod and library files were obtained. All other parameters were followed as per earlier published studies.[28]

2.4. MD Simulation Details. For MD simulations, we performed minimizations using a host of combinations of steepest descent and conjugate gradient algorithms. The minimized complex was then subjected to temperature ramping steps at NVT, fine-tuned equilibration steps at NVT, followed by NPT equilibration to stabilize the complex. After we checked the equilibration steps, the complex was subjected to 250 ns of production runs. A detailed methodology of all the MD steps has been described in previous publications.[2,29] The same MD protocol was followed for both ligand complexes and for simulating apo MARK4 kinase without any ligands. This serves as our control simulation.

2.5. Kinase Assay. Next, we carried out an adenosine-triphosphatase (ATPase) assay to see the effect of empagliflozin and linagliptin on MARK4 kinase activity, keeping the concentration of MARK4 and adenosine 5′-triphosphate (ATP) constant and varying ATP concentration. This assay uses a malachite green reagent (Biomol, Enzo Life Sciences). The experiment was performed as has been reported in earlier published studies.[30] Briefly, we fixed the amount of protein, i.e., MARK4, varied the ligand concentrations, and incubated at 25 °C for 1 h. Freshly prepared ATP solution (200 μM) and $MgCl_2$ (10 mM) were added to the reaction mixture and incubated for 30 min at 25 °C. Finally, we added malachite green to this reaction mixture to terminate the reaction and incubated for 20−30 min until the development of green color, which was read spectrophotometrically at 620 nm.

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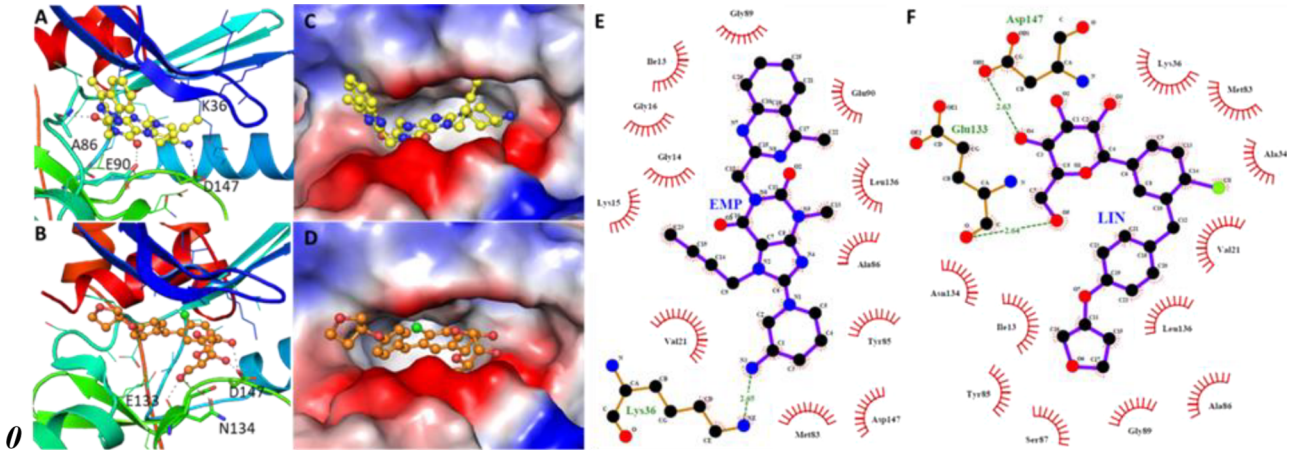

Figure 1. MARK4 interactions with empagliflozin and linagliptin. (A) Magnified cartoon view of MARK4 with empagliflozin and (B) linagliptin. (C) Potential surface view MARK4 with empagliflozin and (D) linagliptin, (E) MARK4-empagliflozin, and (F) MARK4-linagliptin 2D interaction plots generated through LigPlot.

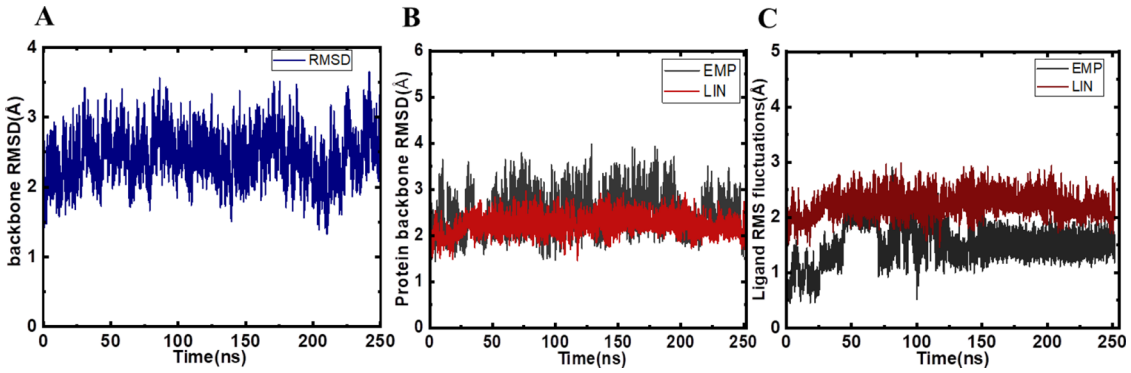

Figure 2. (A) Free MARK4 RMSD fluctuation during the 250 ns production runs. (B) RMSD fluctuations of the protein backbone (red: linagliptin bound; black: empagliflozin bound MARK4 complexes) during 250 ns production. (C) RMSD of ligands, empagliflozin and linagliptin, during the production runs.

3. RESULTS AND DISCUSSION

3.1. Molecular Docking-Based Virtual Screening. Computational approaches are very useful in the drug discovery process.[31] We performed a virtual screening of the 23 drugs to identify the high-affinity binding partners of MARK4 (Table S2). Log files and out files were generated containing affinity scores and docked poses for every drug in the library. Depending on the binding affinities, docking score, and binding poses, these log files and out files were further subjected to drug screening.[32] We found that some of the screened drugs show an appreciable binding affinity score toward the MARK4 binding pocket and thus can be selected further for detailed analysis. Screening of generated output led to identifying five hits out of 23 drugs having appreciable binding affinity scores with MARK4 (Table 1). Both drugs show higher affinity toward MARK4 than the co-crystalized known inhibitor 5RC, ~{N}1~{R},6~{R})-6-azanyl-2,2-bis-(fluoranyl)cyclohexyl]-5-ethyl-4-[6-(trifluoromethyl)pyrazolo-[1,5-*a*]pyrimidin-3-yl]thiophene-2-carboxamide (PDB ID: 5ES1). The selected five drugs' binding modes and interaction patterns were analyzed based on interacting residues. It was observed that critical residues of the kinase domain of MARK4 offer a significant number of interactions, such as Lys36, Ala86, Glu90, Glu133, Asn134, and Asp147 toward two drugs, empagliflozin and linagliptin (Figure 1). These observations suggest empagliflozin and linagliptin have specific interactions toward the bonding pocket of MARK4 with appreciable binding affinity and were analyzed in detail. Empagliflozin stabilized by four close polar binds with Lys36, Ala86, Glu90, and Asp147, along with several hydrophobic interactions (Figure 1A). Similarly, linagliptin forms three hydrogen bonds with Glu133, Asn134, and Asp147, along with several hydrophobic interactions (Figure 1B).

The elucidated drugs empagliflozin and linagliptin were explored with MARK4 for their detailed interactions. The LigPlot generated plots showed that empagliflozin forms a hydrogen bond to Lys36 and other interactions (Figure 1E). Similarly, linagliptin forms hydrogen bonds with Glu133 and Asp147 and several hydrophobic interactions (Figure 1F). Both drugs show several common interactions with MARK4 as the co-crystalized known inhibitor 5RC (PDB ID: 5ES1). Empagliflozin and linagliptin showed a structural resemblance with several known small molecules toward MARK4.[33] These observations suggest both drugs' specific and significant binding affinity to the MARK4. The selected pose for each drug was considered the starting point for our stability MD simulations.

3.2. MD Simulations. *3.2.1. Structural Changes and Analyses Post MD.* When a small molecule binds to the protein surfaces, it results in significant perturbations in the tertiary structures of proteins, and these changes are implicated in drug design and discovery.[32,34] MD simulations are

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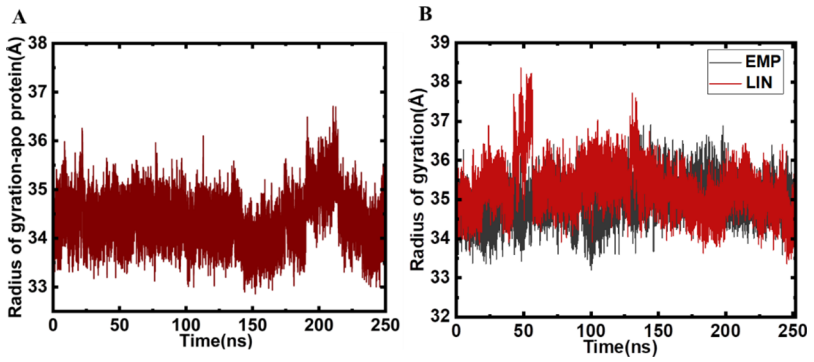

Figure 3. Radius of gyration for (A) free MARK4 and (B) empagliflozin and linagliptin bound protein plotted as a function of snapshots during 250 ns runs.

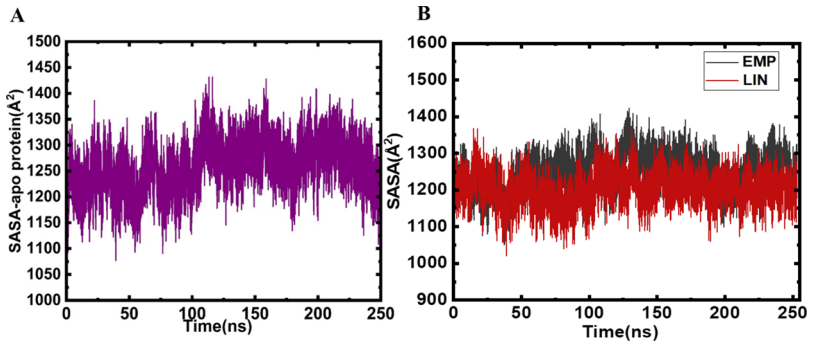

Figure 4. Solvent accessible surface area plotted as a function of snapshots for these (A) apoprotein and (B) protein−ligand complexes.

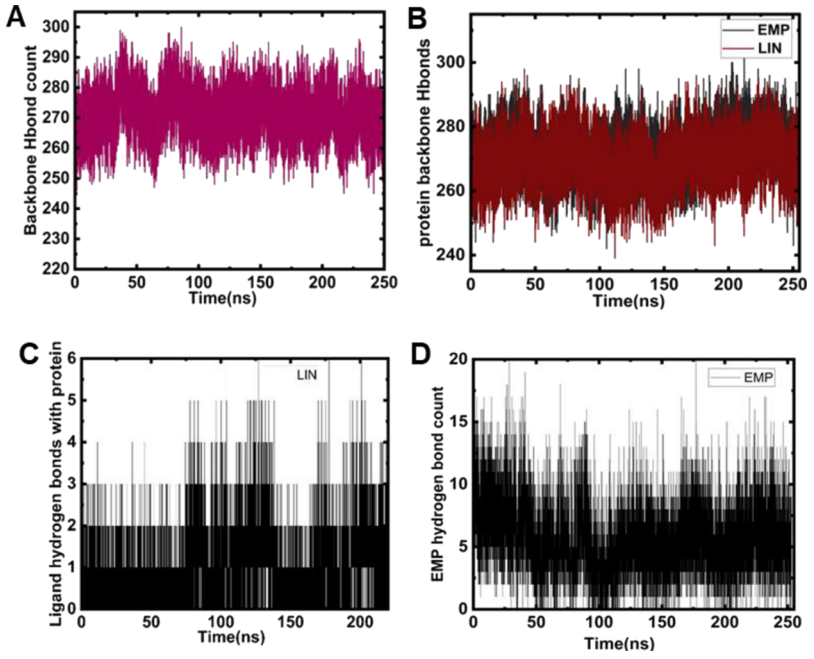

Figure 5. (A) Protein backbone hydrogen bonds monitored during apo MARK4 kinase production runs. (B) Protein backbone hydrogen bond analysis of MARK4-empagliflozin and linagliptin complexes during 250 ns runs. (C) Intermolecular protein−ligand hydrogen bonds (H bonds) of linagliptin and (D) empagliflozin with MARK4.

incredibly vital to probe these biophysical phenomena atomistically and demonstrate these changes in terms of various parameters.

We understand protein residue-based fluctuations with root-mean-squared deviations (RMSDs). Figure 2 depicts the RMSD values of protein before and after the binding of ligands. RMSD of free MARK4 is shown in Figure 2A. The RMSDs of empagliflozin and linagliptin bound protein were also computed to analyze the fluctuations and translations during the 250 ns production runs (Figure 2B). The RMSD plot reveals significant stability of the protein backbone during the simulation, and the average RMSD is ∼3 Å from the initial

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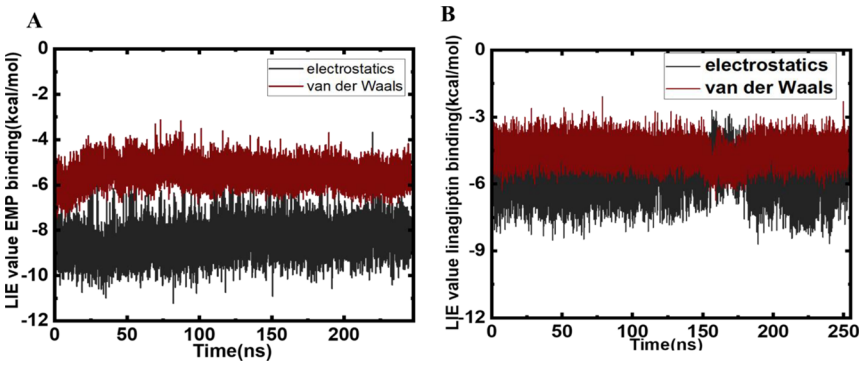

Figure 6. (A) Empagliflozin binding affinity estimated via LIE methodology (electrostatics plotted in black and net van der Waals plotted in red). (B) Linagliptin binding affinity was estimated via LIE methodology (electrostatics plotted in black and net van der Waals plotted in red).

solvated complex. The entire trajectory can be partitioned into two phases: in the first 100 ns, the RMSD slightly shows an increasing trend whereby the sidechains adapt to each other's movements, and there are global conformational changes expected as the protein−ligand complexes adapt to the binding event. In the latter half of 250 ns, the RMSD values slowly seem to taper down and maintain a constancy, which indicates the complexes have stabilized, and the readjustment of side chains has happened to accommodate the ligand binding. This proves that empagliflozin and linagliptin stabilize the protein slightly, as evident from the RMSD of the bound protein compared to the apo form. The ligand's RMS fluctuations are monitored to track the ligand's conformations during the 250 ns MD runs. Figure 2C clearly shows that empagliflozin and linagliptin RMSDs are ∼1−2 Å, and they reside in the docked pocket during the MD progression. The radius of gyration (R_g) demonstrates the compactness and folding pattern of the protein and is associated with the overall conformation of the protein.

Figure 3 shows the R_g pattern for free MARK4 ligand bound MARK4. It is apparent that no structural switching was observed for MARK4 in the presence of both ligands, suggestive of the stability of the protein−ligand complexes. As evident from Figure 3, compactness increases the last 100 ns of the 250 ns for protein−ligand complexes compared to free protein implying the overall stability of the protein−ligand complexes.

With the solvent-accessible surface area (SASA) analysis, we evaluate the solvent accessibility of a protein molecule under a solvent environment. It is an important parameter to understand conformational dynamics.[35] To understand the binding effect of both the ligands with MARK4, we estimated the SASA values of free protein and protein−ligand complexes (Figure 4). A minor increase in SASA was evident for ligand-bound MARK4 compared to free protein for both the ligands, suggesting that some of the internal residues of the protein might be uncovered on the surface after the ligand binding. The SASA values show a fair equilibration without significant switching throughout the simulation. It is apparent from Figure 4 that stable equilibrium is attained during the simulation, implying the overall stability of the protein−ligand complexes.

3.3. Hydrogen Bond Analysis. Protein structure formation relies on accurate hydrogen bonding interactions between residues. Exploration of intramolecular hydrogen bonds aids in analyzing the stability of structures during MD simulations. The backbone hydrogen bonds of MARK4 are also computed to analyze the differences in the apo and ligand-bound simulations. It appears (seen in Figure 5A) that the apoprotein hydrogen bonds average between 260 and 270, whereas in the ligand-bound conformations, the raw backbone hydrogen bond count stands at 270 (Figure 5B). These observations imply that consistency is observed in the protein even after binding both ligands, implying the system's overall stability. The empagliflozin and linagliptin complexes are plotted in Figure 5.

The protein−ligand hydrogen bonds are crucial to probing key interactions of some residues with ligand atoms. As seen in Figure 5C,D, we plotted the hydrogen bonds formed with ligands empagliflozin and linagliptin during the 250 ns production runs. The hydrogen bonds formed with MARK4 and linagliptin the ligands vary between 1−3 for linagliptin and 5−10 for empagliflozin. It is evident that empagliflozin forms higher H-bonds compared to linagliptin, indicative of the higher stability of the MARK4−empagliflozin complex. The analysis advocates that MARK4-empagliflozin and linagliptin complexes are quite stable (Figure 5C,D). Both complexes remain stabilized, and no huge fluctuations were observed.

3.4. Free Energy Calculations of MARK4 Ligand Complexes. Protein-ligand binding free energies are crucial as these aid in filtering out key ligands from a large pool. In this work, we have employed molecular mechanics Poisson−Boltzmann surface area and linear interaction energy (LIE) methodologies to estimate the binding affinities of empagliflozin and linagliptin as per earlier published studies.[36]

The binding affinity value calculated for empagliflozin and linagliptin is −7.92 kcal/mol. The energies are plotted in Figure 6 for both MARK4 complexes.

The importance of MMGBSA and LIE calculations arises from quick and less demanding approaches. Additionally, many studies have estimated these values to decipher the structural stabilities. We computed the binding affinities of empagliflozin and linagliptin using MM/PBSA as it is more computationally exhaustive and hence yields a more accurate free energy estimate than its GBSA model. The generalized born computed value for empagliflozin and linagliptin binding stands at −26.2 kcal/mol and −12.1 kcal/mol. LIE estimates for empagliflozin and linagliptin binding are −16.5 kcal/mol and −9.8 kcal/mol, respectively. The LIE energy contributions are plotted in Figure 6. Figure S1 depicts the MMGBSA energy affinity for MARK4-empagliflozin and MARK4-linagliptin.

3.5. Kinase Assay. After ensuring through in silico approaches that linagliptin and empagliflozin bind to MARK4 with a significant affinity leading to the formation of stable complexes with both the ligands, the next aim was to

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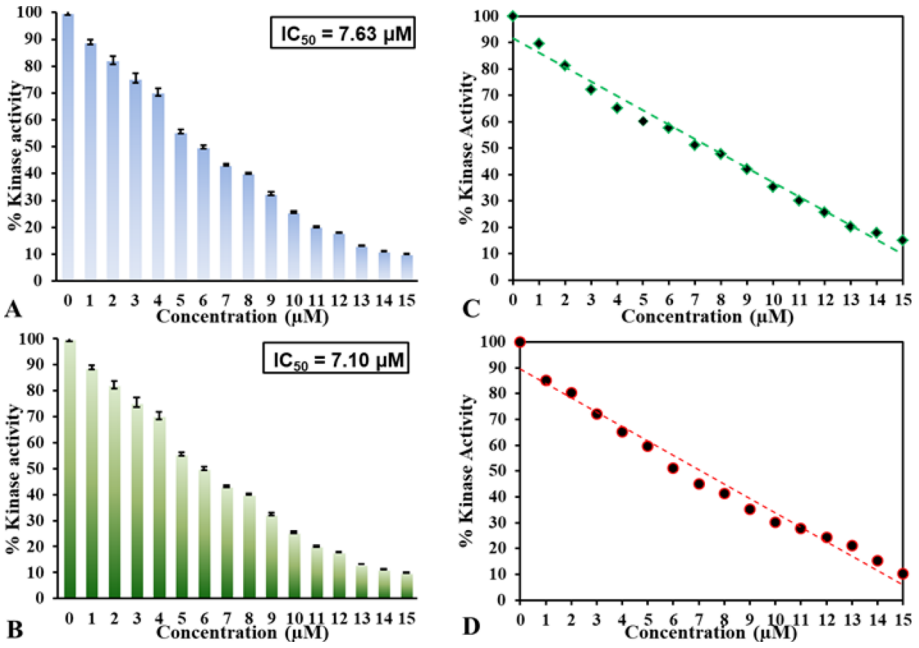

Figure 7. ATPase assay of MARK4 in the presence of varying concentrations of (A) linagliptin and (B) empagliflozin. IC_{50} calculation of (C) linagliptin and (D) empagliflozin.

ascertain the effect of binding on the functional aspect of the protein, i.e., to have an insight into the impact of these drugs on the kinase activity of MARK4. MARK4 was successfully cloned, expressed, and purified as in the earlier published literature.[37] In the presence of varying concentrations of both drugs, there is a corresponding decrease in the kinase activity of MARK4 (Figure 7A,B). Thus, it is evident that both the drugs inhibiting the activity of MARK4 and IC_{50} were 7.63 μM and 7.10 μM for linagliptin and empagliflozin, respectively (Figure 7C,D). Hence, this assay deciphered the inhibitory effect of both drugs on MARK4, i.e., both drugs compromise the functionality of MARK4. They consequently can be implicated in the treatment of MARK4-directed diseases wherein overexpression of MARK4 directly or indirectly contributes to the pathology of a disease.

4. CONCLUSIONS

MARK4 is involved in several malignancies, including, diabetes mellitus, cancer and neurodegeneration. Repurposing approved drugs provides an alternative approach to developing safe and effective therapeutics against rapidly emerging diseases. Here, we employed a structure-based rational virtual screening process to find potential inhibitors of MARK4. After structural and pharmacological aspect analyses, the virtual screening suggested empagliflozin and linagliptin as possible inhibitors of MARK4. Both drugs possess appreciable affinity and specificity toward the binding pocket of MARK4 and its crucial active residues. Further investigation of all-atom MD simulations for 250 ns on MARK4 and its ligand-bound complexes promises stable MARK4−drug complexes throughout the simulation trajectories. Kinase assay further demonstrated linagliptin and empagliflozin as potent MARK4 inhibitors with IC_{50} of 7.63 and 7.10 μM, respectively. Taken together, we repurpose that empagliflozin and linagliptin can be used as potential MARK4 inhibitors for therapeutic management against MARK4-mediated malignancies after required validations.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c06634.

Molecular descriptors for ligands; MMGBSA energy affinity for MARK4-empagliflozin and MARK4-linagliptin; and a list of antidiabetic drugs used in the virtual screening process (PDF)

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A.A.: conceptualization, writing-review, and editing, project administration, data analysis, and investigation; A.A.: visualization and resources; M.A.: data validation, data analysis; writing-review and editing; D.D.G.: data curation, data validation, resources, visualization, software, writing-review, and editing; N.M.A.: editing, formal analysis, and data curation; S.E.S.: data curation and methodology; W.A.A.: funding acquisition, data validation, and methodology; K.M.A.: writing-review and editing and data analysis; A.S.: conceptualization, data analysis, methodology, visualization, software, and writing-review and editing.

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Notes

The authors declare no competing financial interest.

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