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Structure guided design of potential inhibitors of human calcium–calmodulin dependent protein kinase IV containing pyrimidine scaffold

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ABSTRACT

Calmodulin dependent protein kinase IV (CAMKIV) belongs to the serine/threonine protein kinase family and considered as an encouraging target for the development of novel anticancer agents. The interaction and binding behavior of three designed inhibitors of human CAMKIV, containing pyrimidine scaffold, was monitored by in vitro fluorescence titration and molecular docking calculations under physiological condition. In silico docking studies were performed to screen several compounds containing pyrimidine scaffold against CAMKIV. Molecular docking calculation predicted the binding of these ligands in active-site cavity of the CAMKIV structure correlating such interactions with a probable inhibition mechanism. Finally, three active pyrimidine substituted compounds (molecules **1**–**3**) have been successfully synthesized and characterized by ^{1}H and ^{13}C NMR. Molecule **3** is showing very high binding-affinity for the CAMKIV, with a binding constant of 2.2×10^{8}, M^{-1} (±0.20). All three compounds are nontoxic to HEK293 cells up to 50 μM. The cell proliferation inhibition study showed that the molecule **3** has lowest IC_{50} value (46 ± 1.08 μM). The theoretical and experimental observations are significantly correlated. This study reveals some important observations to generate an improved pyrimidine based compound that holds promise as a therapeutic agent for the treatment of cancer and neurodegenerative diseases.

Calcium–calmodulin dependent kinase IV (CAMKIV) is a member of Ser/Thr family and associated with numerous cellular activities such as cell signaling, cell cycling, apoptosis, differentiation or proliferation, immune and inflammatory responses.[1–4] It is also involved in the regulation of transcription factors through phosphorylation of the cAMP response element-binding protein (CREB).[5–8] Recently, it was shown that CAMKIV is increased and required during Th17 cell differentiation, and its increased levels leads to the stimulation Th17-inducing cytokines.[9] Extracellular stimuli like change in calcium concentration, inflammatory response or hormonal stimuli cause the stimulation of CAMKIV.[3,10,11] On the other hand, overexpression or mutation in CAMKIV inhibits the autophosphorylation activity which leads to the development of neurodegenerative diseases, and its transformation into oncogenic kinase eventually increase the cancer risk.[12] Recent studies proven that CAMKIV is directly associated with the hepatic[13] and epithelial ovarian cancers.[14]

All these findings suggest that CAMKIV may be considered as a potential drug target for the neurodegenerative diseases and cancer.[15] Despite of its potential role in the cellular physiology and a close association with numerous diseases, a little attempt was taken for designing any ligand/inhibitor of CAMKIV.[16–18] Corcoran and Means[19] have shown that KN-93, an inhibitor of CAMKs had a dramatic effect on post-tetanic potentiation. Other CAMK inhibitors such as KN-93 and KN-62 can induce the differentiation of multiple leukemic myeloid cells (e.g., HL-60 and NB4).[20,21] Recent studies have clearly indicated that inhibiting CAMKIV could help treat systemic lupus erythematosus.[17,22,23] Inhibition of CAMKIV in MRL/lpr mice causes a significant suppression of nephritis and skin disease due to a remarkable decrease in the expression of costimulatory molecules CD86 and CD80 on B cells, and suppression of IFNγ and tumor necrosis factor α production.[22] Koga et al.,[9] showed that CAMKIV knockout or inhibition with a small molecule

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have decreased the severity disease. Hence, development of few specific CAMKIV inhibitors and extending it to the development of drug molecules is highly promising.

Recently, we have shown that curcumin, a natural anticancer agent, is showing a significant binding-affinity to the CAMKIV.[24] To proceed further for designing a specific inhibitor of CAMKIV, this is an attempt to synthesize few potential compounds that contain a pyrimidine scaffold, with high binding-affinity. We constructed a ligand library of 100 compounds ends up with three synthesizable lead molecule which are showing a significant binding affinity to the CAMKIV, as confirmed by molecular docking and fluorescence binding studies. All three molecules were successfully synthesized and characterized using ^{1}H and ^{13}C NMR and showing a considerable binding affinity to the CAMKIV in vitro, that may be further utilized for the design of potential drug molecules for the associated diseases.

Crystal structure of human CAMKIV (residues 15–340) has been deposited in the protein data bank with PDB code 2W4O. However, there are many atoms are missing. Hence, we modeled the structure of CAMKIV as described earlier.[24] We have successfully docked all three molecules in the active-site cavity of the CAMKIV.[25] A reasonably high docking score was obtained, indicating a strong binding affinity of these molecules with the CAMKIV. The structure of CAMKIV is divided into five specific domains namely, autoinhibitory, nucleotide-binding (305–321), serine/threonine phosphatase 2A (PP2A)-binding (306–323), CaM-binding domain (322–341) (all four comprise the regulatory site) and the protein kinase domain (46–300), the active site of the enzyme. Thr200, Ser12 andSer13 is the site of its phosphorylation.[26] Asp164 is an essential residue for the catalysis and acts as a proton acceptor. While, Lys75 is the ATP-binding site.[26]

Molecule **1** is present in the hydrophobic cavity of active-site. However, some portion of this molecule is protruded out of the protein molecule (Fig. 1A and B). Two hydrogen bonds are formed by molecule **1** to the Glu168 and Asp185 of the CAMKIV accompanied by several van der Waals interactions, clearly indicating the formation of a stable complex (Fig. 1C). A strong interaction of molecule **1** to the CAMKIV which have the most favorable binding energy and clarifies the hydrogen-bonding and van der Waals interactions with the important amino acids, further suggest that molecule **1** as a potential ligand for the CAMKIV. This pharmacological interaction is useful for better understanding of ligand binding mechanisms and the potential use of molecule **1** as a therapeutic agent.

Molecular docking studies were carried out with a slightly bigger ligand (molecule **2**) to further identify the binding mode inside the active site cavity of CAMKIV (Fig. 2A). Molecule **2** is expected to bind at the active site of CAMKIV (Fig. 2B). However, it does not enters completely to the cavity. Amino acids Glu168 and Lue52 of CAMKIV form H-bonds with the molecule **2** supported by several hydrophobic interactions (Fig. 2C). The docked ligand forms a stable complex with human CAMKIV with a binding affinity (ΔG in kcal/mol) value of −7.38 (Table 1). These preliminary results suggest that molecule **2** might exhibit inhibitory activity against human CAMKIV, and may have further therapeutic application.

In order to get better ligand, we have decreased the size of ligand and designed molecule **3** for further study (Fig. 3A), which completely enters a deep inside the active site cavity of CAMKIV (Fig. 3B). This ligand is expected to bind efficiently to the active site residues of protein with weak non-covalent interactions, most prominent of which are H-bonding, π–π stacking and alkyl–π interactions (Fig. 3B). Asp185 holds the molecule at the active site by forming H-bond of 2.75 Å length with the nitrogen of pyrimidine (Fig. 3C). Similarly, Gly187 holds the molecule **3** by forming H-bond with the nitrogen of pyrimidine. However, Val121 is forming H-bond with the oxygen atom of molecule **3**. Several

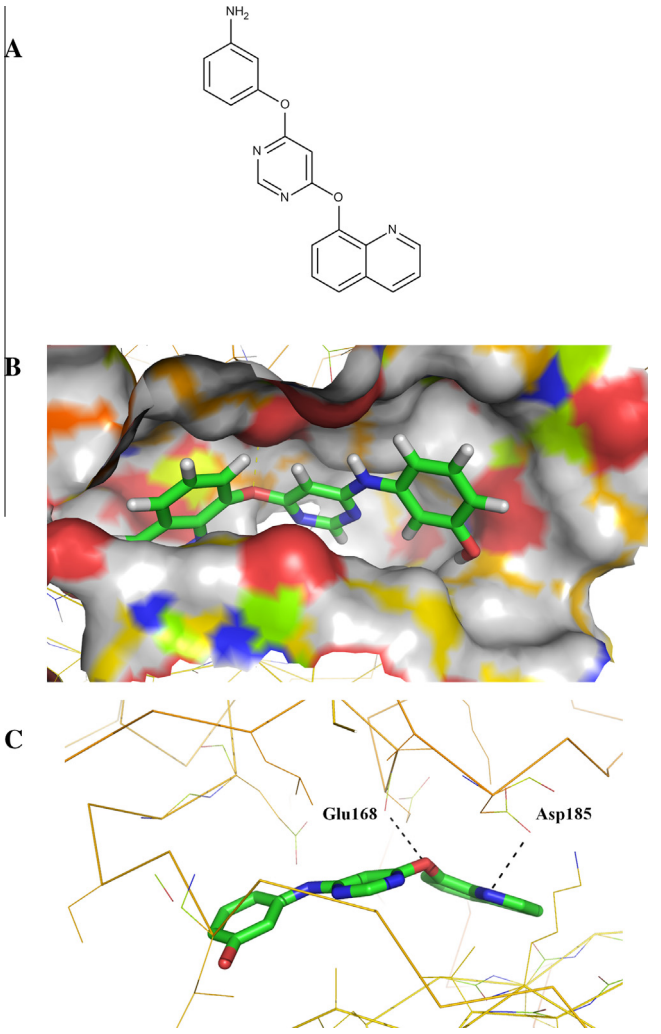

Figure 1. Binding of compound **1** with the CAMKIV. (A) Structure of 3-((6-(quinolin-8-yloxy)pyrimidin-4-yl)oxy)aniline (molecule **1**) (B) Showing surface view of ligand (stick model) present in the active site cavity of the CAMKIV. (C) Showing interaction of ligand (stick model) with the protein atoms (line model).

hydrophobic residues also play role in stabilizing the ligand–macromolecular complex by forming an alkyl–π interaction with central pyrimidine ring. Binding free energy (ΔG in kcal/mol) was found to be −7.62, as predicted by Pardock module of *Sanjeevini*, suggests a very high binding-affinity between the ligand with CAMKIV. These results draw us to the conclusion that this molecule might exhibit inhibitory activity against the CAMKIV. However biological tests need to be done to validate the computational predictions.

Key steps in the synthesis of compounds shown in Table 1 involved nucleophilic aromatic substitution of 4,6-dichloropyrimidine with various nucleophiles depicted in Scheme 1. Firstly, 4,6-dichloropyrimidine **1** was treated with different primary amies or phenols in the presence of a base (K_2CO_3 or DIEA) in DMF at room temperature to give compounds **2**–**3** as monosubstituted pyrimidines in good yields. The formed mono-substituted pyrimidine was further treated with different nucleophile such as amino-phenol with appropriate base (K_2CO_3, KOH or DIEA) in dry DMF at higher temperature to give Molecules **1**, **2** and **3** as disubstituted pyrimidines (Scheme 1). The first as well as second substitution showed temperature dependent nucleophilic aromatic substitution reactions in which the first chlorine was replaced at room temperature with 1 equiv of nucleophile and 1–1.5 equiv of