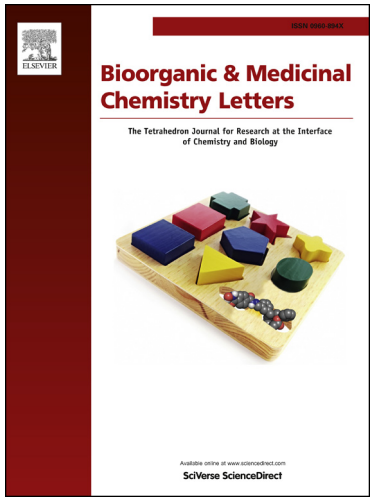

Structure guided design of potential inhibitors of human calcium-calmodulin dependent protein kinase IV containing pyrimidine scaffold

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Running head: Potential Inhibitors of CAMKIV

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

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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 ligand in active-site cavity of the CAMKIV structure correlating such interactions with a probable inhibition mechanism. Finally, three active pyrimidine substituted compounds (**molecule 1–3**) have been successfully synthesized and characterized by ^{1}H & ^{13}C NMR. Molecule 3 is showing very high binding-affinity for the CAMKIV, with a binding constant of $2.2x10^{8}$, M^{-1} (±0.20). All three compounds are nontoxic to HEK293 cells upto 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.

Keywords: Calcium-Calmodulin Dependent Protein Kinase IV; Drug Target; Molecular Docking; Fluorescence Binding Study; High affinity ligands; Cancer and Neurodegenerative diseases

Introduction

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

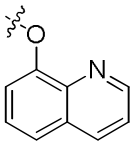
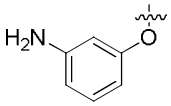
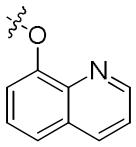
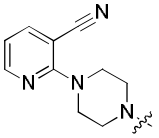
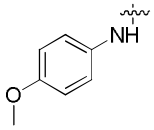
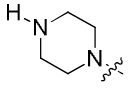
Recently, we have shown that curcumin, a natural anticancer agent, is showing a significant binding-affinity to the CAMK4 [24]. To proceed further for designing a specific inhibitor of CAMKIV, this is an attempt to synthesize few potential compounds that contain a pyrimidine scaffold, with high binding-affinity. 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 **(Figure 1A and 1B).** 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 **(Figure 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 Waal's 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.

Table 1: Structures and free energy value of the synthesized compounds.

Molecule	R	R′	ΔG Kcal/mol
1	 2	 5	-5.6
2	 3	 6	-7.38
3	 4	 7	-7.62

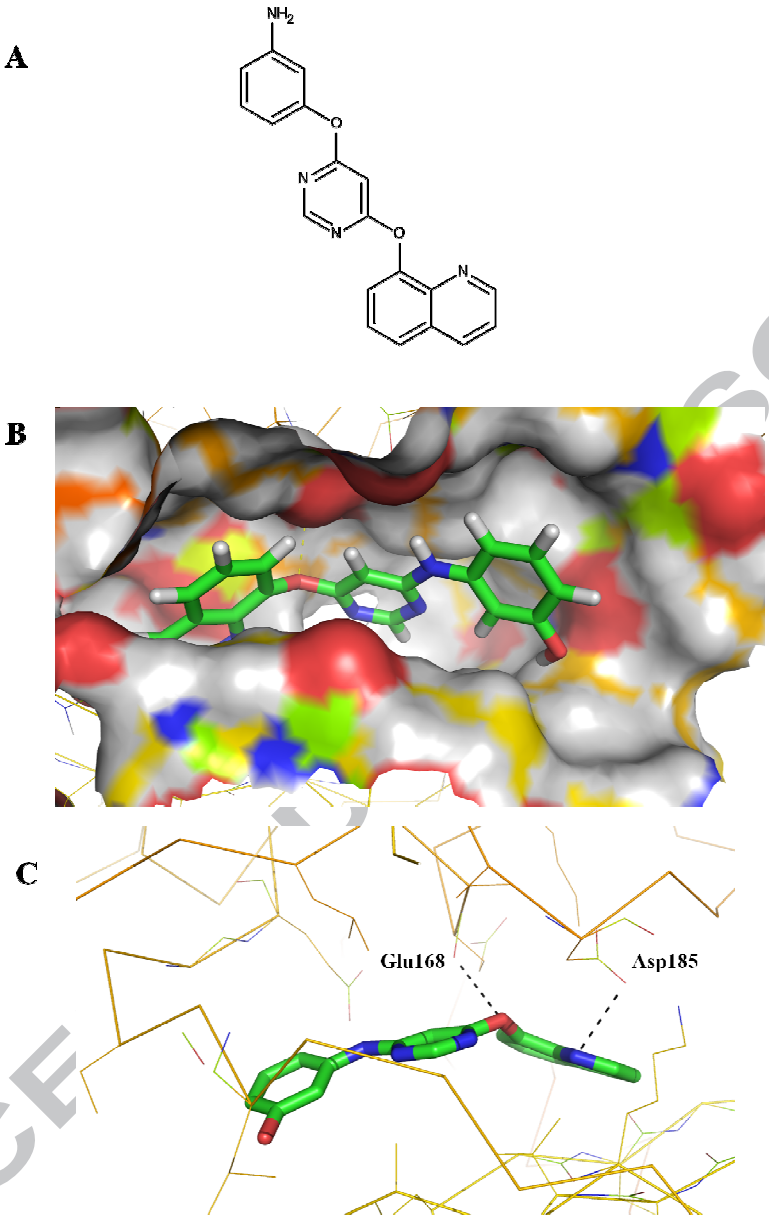


Figure 1

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 **(Figure 2A)**. Molecule 2 is expected to bind at the active site of CAMKIV **(Figure 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 **(Figure 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.

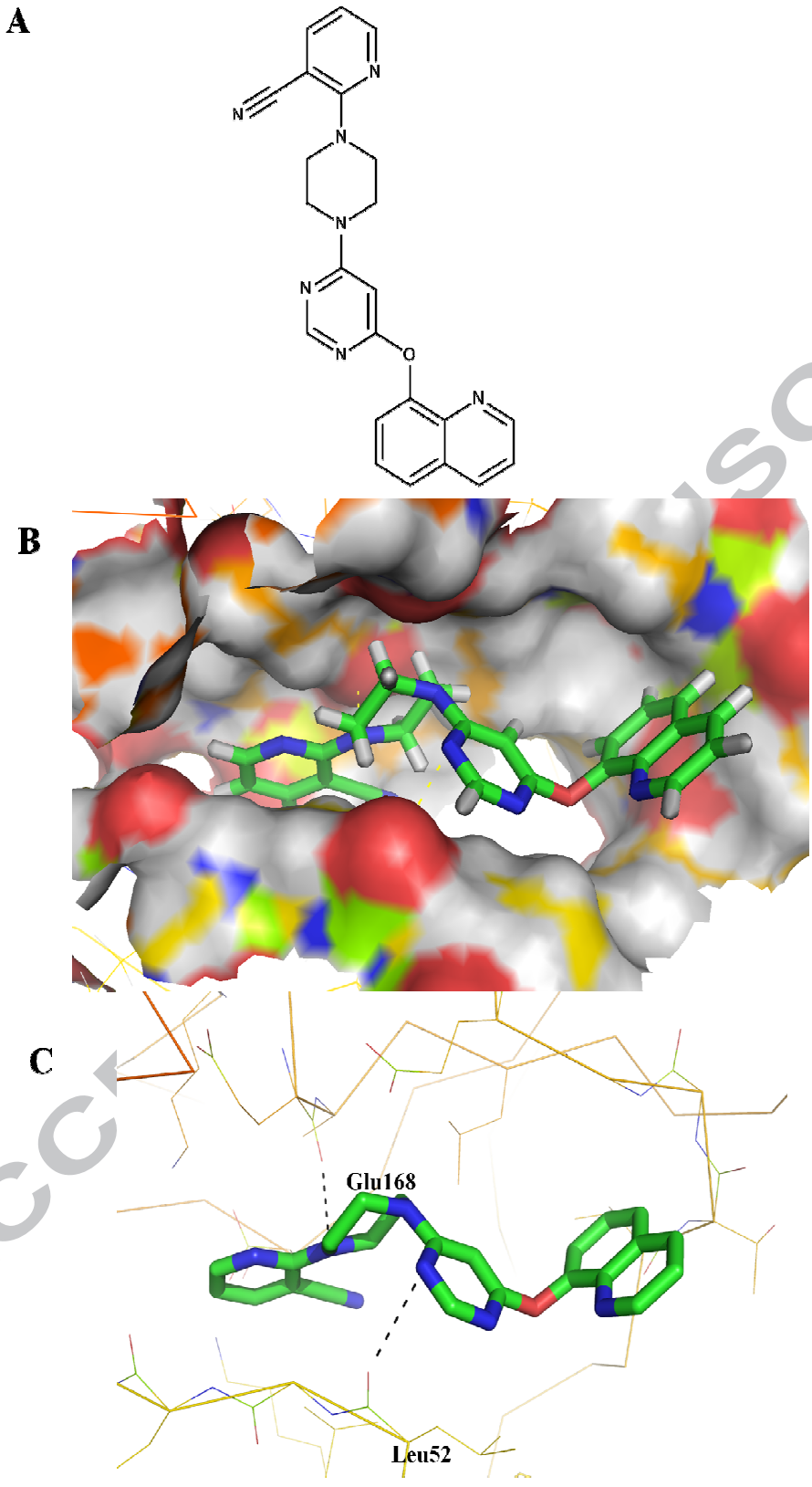


Figure 2

In order to get better ligand, we have decreased the size of ligand and designed molecule 3 for further study **(Figure 3A),** which completely enters a deep inside the active site cavity of CAMKIV **(Figure 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 **(Figure 3B).** Asp185 holds the molecule at the active site by forming H-bond of 2.75 Å length with the nitrogen of pyrimidine **(Figure 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 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.

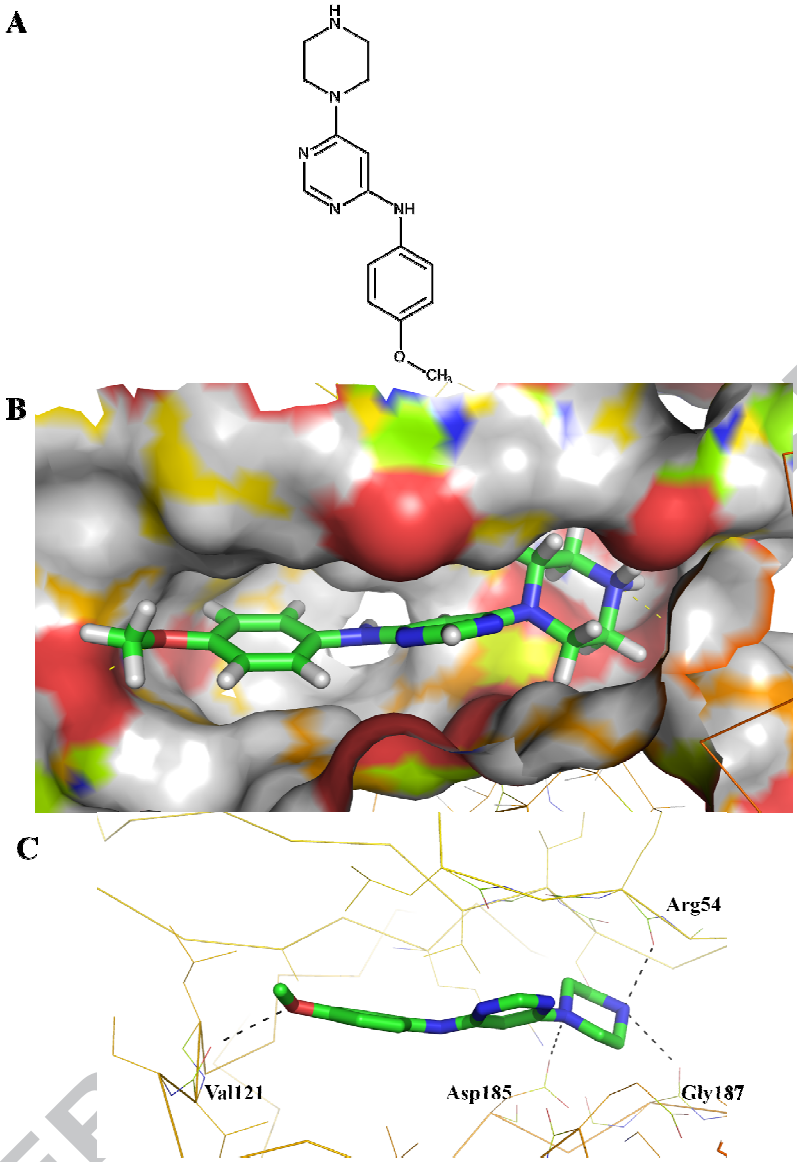
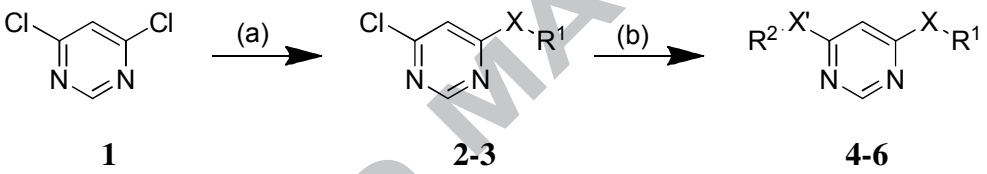


Figure 3

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 amine or phenol in presence of base as (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

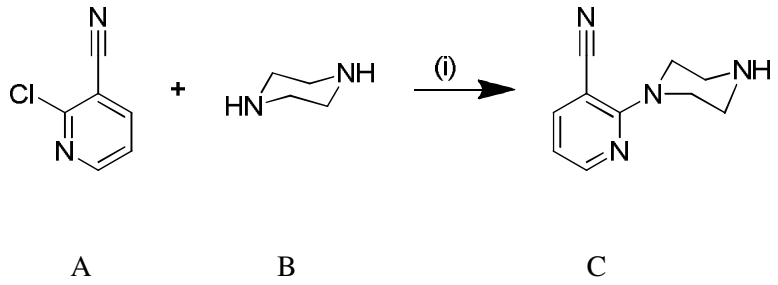
DMF at higher temperature to give **Molecule 1, 2 and 3** as disubstituted pyrimidines **(Scheme 1).** The first as well as second substitution showed temperature dependent nucleophilic aromatic substitution reactions in which the first chlorine was replaced at room temperature with 1 equivalent of nucleophile and 1-1.5 equivalents of appropriate base in DMF. While the second chlorine replaced at higher temperature with 1-2.5 equivalents of nucleophile and 1.2-1.5 equivalents of appropriate base in dry DMF in dry nitrogen atmosphere. All the synthesized compounds **(2-3, molecule 1, 2, 3)** were purified by column chromatography using EtOAc/Pet. ether as eluent and characterized by various spectroscopic techniques.



1 **2-3** **4-6**

Scheme 1: Reagents and conditions: (a) Appropriate base, DMF, r.t, 2-10hrs. (b) Appropriate base, Dry DMF, Reflux, overnight. 1= 4,6-Dichloropyrimidine scaffold; RR´= Aryl group; XX´= Different nucleophiles.

To prepare 2-(piperazin-1-yl)nicotinonitrile (C), a solution of Piperazine (3g), and K_2CO_3 (4.8 g, 1 eq.) in dioxane (15mL) was added 2-chloro-3-cyanopyridine (4.8 g, 1 eq.) and was stirred to reflux at 100-110 °C for 4-5 hrs **(Scheme 2).** The resulting precipitate was filtered off and concentrated at reduced pressure to obtain the crude product which was purified was silica gel column chromatography MeOH:$CHCl_3$ (5:95 v/v) to give compound **C** as yellow solid (3g, 46%) [27].



A B C

Scheme 2: Reagents and conditions: (i). K_2CO_3, Dioxane, reflux, 4-5 hrs.
A= 2-Chloro-3-cyanopyridine, B= Piperazine.

To further validate in silico binding observations, we performed fluorescence binding studies of synthesized molecules to the CAMKIV [28]. We have successfully cloned, expressed and purified the kinase domain of CAMKIV (Method is described in the biology section). 2.5 mL solution containing 4 μM of CAMKIV was titrated by the successive addition of 1.0 mM stock solution of ligands and the concentrations were varied from 0 to 132 μM. Titrations were done manually by using micro-injector. An excitation wavelength (Ex) of 280 nm was chosen in the experiment and emission spectra were recorded in the range of 300 to 400 nm.

The effect of molecule 1, molecule 2 and molecule 3 on the fluorescence emission spectra of CAMK4 (pH = 7.4) at 298 K are shown in **Figures 4A, 5A and 6A,** respectively. These data were used for calculation of binding affinity of these molecules to the CAMKIV as provided in the **Table 2.** As shown in these figures, the peak at fluorescence emission maxima of CAMKIV is observed at around 343 nm, is gradually quenched by the increasing concentration of the ligands, while the ligands has no intrinsic fluorescence in this range. These observations clearly indicates that a strong interaction exist between designed molecules (Molecule 1, 2, and 3) and CAMKIV. The relationship between decrease in fluorescence intensity and the concentration of ligands can be described by the Stern–Volmer equation following Eq. (2), the apparent *K*a is the

binding constant and *n* is the number of binding sites per protein molecule can be expressed for ligand-CAMKIV system [29, 30].

Table 2: Binding affinity of designed compounds measured by fluorescence

**mean errors are given in the parenthesis for three measurements.*

S.NO	Name of compound	Binding Constant*	No. of Binding site
1	Molecule 1	$1.6x10^5$, M^{-1} (±0.12)	1.0
2	Molecule 2	$4.6x10^5$, M^{-1} (±0.16)	1.0
3	Molecule 3	$2.2x10^8$, M^{-1} (±0.20)	2.0

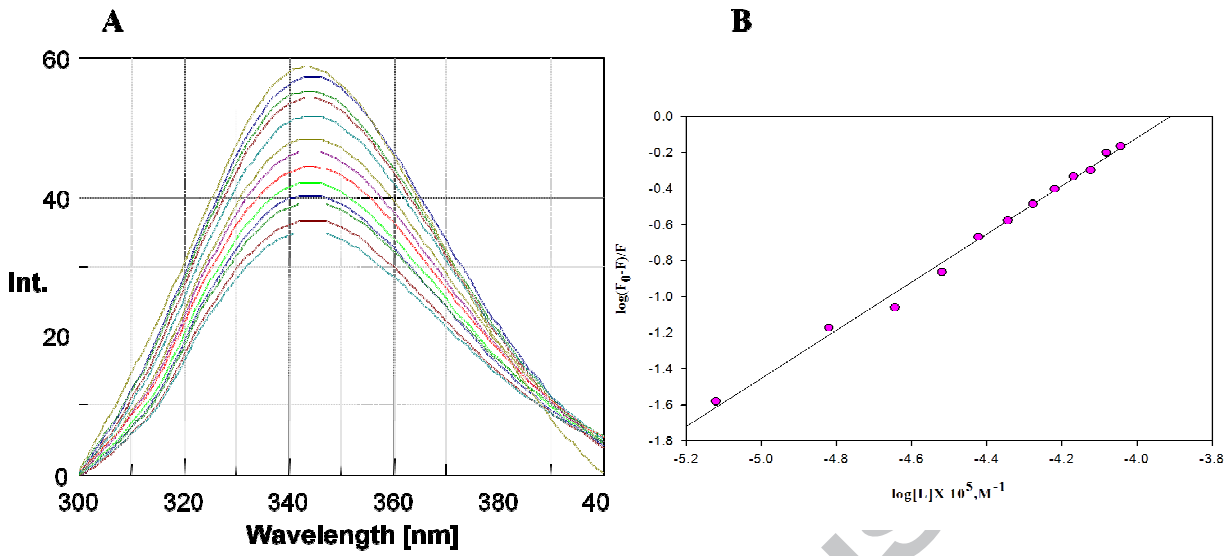


Figure 4.

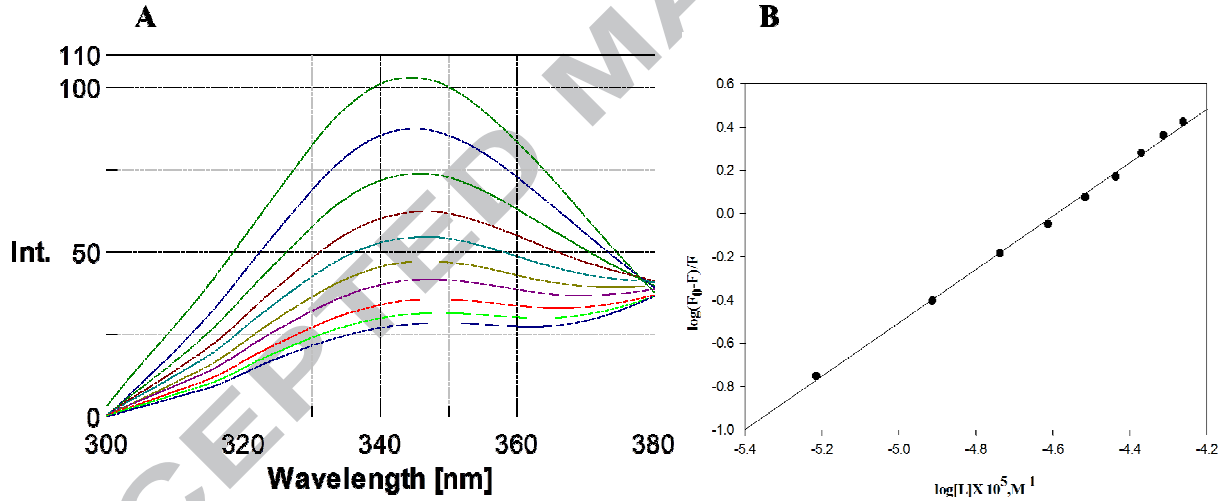


Figure 5.

Figure 4A, 5A and 6A clearly indicate that all three molecules quench the fluorescence emission of CAMKIV with higher binding affinity and experiences a static quenching process. The data of fluorescence intensity at 343 nm were plotted against [ligand] to calculate the binding-affinity and the number of binding sites **(Figure 4B, 5B and 6B).** It is interesting to note that molecule 1 and 2 are having binding affinity value as $1.6x10^5$, M^{-1}(±0.12) and $4.6x10^5$, M^{-1} (±0.16),

respectively. Both these molecules having a single binding site to the CAMKIV. However, molecule 3 shows a significantly very high binding affinity for the CAMKIV ($2.2x10^8$, M^{-1} (±0.20)) and the number of binding site is two. All these observations clearly indicate that molecule 3 is one of the very efficient ligand, and can be further exploited for the development of novel pharmacophore for the CAMKIV associated diseases.

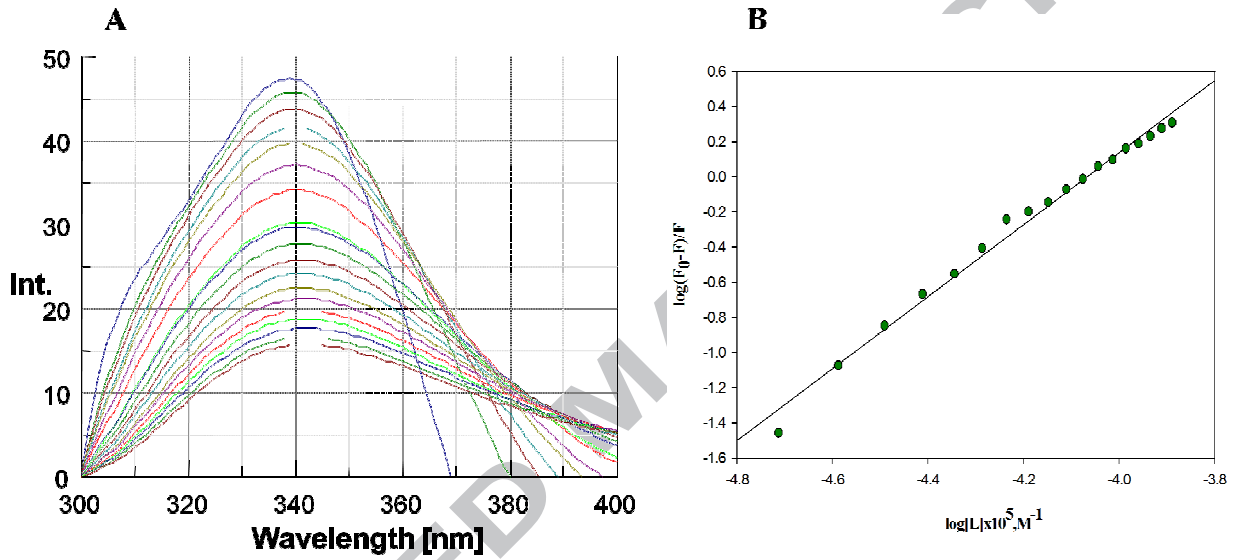


Figure 6

We started with a large number of diphenyl derivatives of pyrimidine. Out of 100 compounds, we selected top 10 hits of docking results for further study. However, we did not successfully synthesized all 10 compounds. Finally, three molecules were successfully synthesized and their chemical analysis is reported in the chemistry section. These three molecules are showing a considerable binding affinity to the CAMKIV.

MTT assay has gained high attention in cell proliferation studies. MTT is a tetrazolium salt, which can be easily reduced to a colored formazan product with the help of reducing enzyme

present only in metabolically active or dividing cells [31]. The enzyme succinate dehydrogenase and cytochrome oxidase present in mitochondria of active cells that help in the reduction of MTT [32]. Thus under a particular condition reduction of MTT and subsequent formation of formazan is proportional to the number of viable or active cells present in the medium [31, 32]. With the help of MTT-assay we have analyzed the cytotoxicity and cell-proliferation inhibition activity of designed inhibitors [33]. Comparison of the cell viability is shown in **Figure 7A** which clearly revealed that molecule 3 nearly up to 50 μM, and molecules 1 and 2 are nearly up to 25 μM, was non-toxic to normal cells. Above this concentration, the reference and the tested inhibitors both became toxic to the normal cells.

We further performed the anti-cancerous activity of these inhibitors on human cancer cells and found an appreciable activity against cancer cell line. From the percentage viability we have calculated the inhibitory concentration (IC_{50}) of each inhibitor. It is evident from **Figure 7B** that these synthesized compounds display a dramatic anticancer activity for human hepatic cancer cell-line. As shown in **Table 3** for HuH7 cells, the molecule 1 and 2 show higher IC_{50} values as compared to the molecule 3. Calculation shows that the molecule 3 inhibits the proliferation of cancerous cells almost two times more than the molecule 1 and 2. These results are comparable to curcumin, a natural anticancer compound used a positive control in this study. Our findings also suggest that these inhibitors are selective and proven 8-10 times more active against cancerous cells then normal one **(Figure 7).** Moreover, on HuH7 cells the molecule 3 shows the better anti-proliferative activity in comparison to molecule 1 and 2, which was also complimented by our binding and docking studies. The results of our *in vitro* and *in silico* studies to a certain extent validate our strategies to design novel and potential inhibitors against CAMKIV.

Table 3: IC_{50} (μM) values of designed inhibitors on HuH7 and HEK293 cells

Name of compound	IC_{50} (HuH7 cells) in μM	IC_{50} (HEK293 cells) in μM
[a]Curcumin	48± 1.11	>400
Molecule 1	116± 1.12	400 ± 1.10
Molecule 2	104±1.77	>400
Molecule 3	46± 1.08	>400

[a]positive control

In conclusion, CAMKIV is an important member of calcium-triggered signaling cascade, involved in the regulation of transcription and microtubule dynamics. Its abnromal expression is directly associated with varieties of cancer and neurodegenerative diseases. Three potential ligands of CAMKIV were suggested by docking simulation studies, were successfully synthesized and characterized. We further shown that all three molecules bind efficiently to the active site of CAMKIV. Molecule 1 and 2 can bind to CAMKIV with stoichiometry ratio of 1:1, however, molecule 3 in stoichiometry ratio of 2:1. The CAMKIV-ligand complex is stabilized by several non-covalent interactions offered by residues in vicinity to the active site. We further shown that molecule 3 is showing very good anticancerous activity on HuH7 cell line. No doubt, these results are important in the field of pharmacy, pharmacology and biochemistry, and are expected to shed some light on the binding property of CAMKIV with the ligands. These findings may further explored for the drug design and discovery of potential drug molecules for the diseases associated with the altered expression of CAMKIV.

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Conflict of interest

Authors declare no conflict of interest regarding any financial and personal relationships with other people or organizations that could inappropriately influence (bias) this work.

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25. The docking and scoring of ligands with CAMKIV protein was accomplished using Pardock module of Sanjeevini drug design suite which is based on physico-chemical descriptors. It is a rigid docking module which accomplishes a series of computational steps such as preparation of reference protein complex and ligand in a force-field compatible manner as an input file. Docking of ligand molecules at the active-site using all atom energy based Monte Carlo algorithm, minimizes and scores the docked complex. The docked complexes are further minimized using the parallel version of sander module of AMBER, predicted binding free energy of docked poses are obtained using Bappl/BapplZ scoring function. The crystal structure of complex, CAMKIV protein in complex with 4-Amino(sulfamoyl-phenylamino)-triazole-carbothioic acid (2,6-difluoro-phenyl)-amide) (PDB ID 2W4O) was utilized as reference for building new model and docking studies. The energy minimized structure of 100 ligands were docked at the active site out which three potential ligands were chosen for further synthesis (details are provided separately in the chemistry section) and binding studies.

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28. Fluorescence measurements were carried out in the Jasco Spectrofluorimeter (Model FP-6200) using 5 mm quartz cuvette. Experiment was carried out at 25 ± 0.1 °C maintained by an external thermostated water circulator. Stock solutions (5mg/ml) of synthesized compounds 1, 2, 3 were prepared in the DMSO and then diluted to 1 mg/ml in the 50 mM phosphate pH 7.4 buffer containing 150 mM NaCl (PBS). All compounds were further dialyzed in the PBS before the experiment. Intrinsic fluorescence emission spectra of CAMKIV in the presence of different concentrations of synthesized compounds were performed in the PBS at 25 °C. For this purpose excitation wavelength was fixed at 280 nm and emission was at 300-400nm. The excitation and

emission slit widths (5 nm each) and scan rate (100 nm/min) were constantly maintained for all experiments. A typical emission peak was observed at 343 nm. Final spectrum was collected after deducting their corresponding blank. Three independent experiments were performed for each compound and their average was taken for data analyses. A decrease in the fluorescence intensity due to increasing concentrations of compounds were used as a parameter for calculating the binding constant K and number of binding site n using modified Stern-Volmer equations with slight modification.

$$\log(Fo-F)/F = \log Ka + n\log[Q] \qquad (1)$$

Where, Fo is the fluorescence intensity of protein and F is the fluorescence intensity of ligand, Ka is the binding constant and n is the number of binding sites, Q represents quenching constant. For the ligand-protein complex, the values for Ka and n can be derived from the intercept and slope.

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33. Exponentially growing human heptoma cells (HuH7) and human embryonic kidney cells (HEK293) were seeded in a 96-well micro titer plate (cell count of 7000 cells per well). For checking cell proliferation, on the next day cells were treated with different concentrations (5 µM-150 µM) of inhibitors. Curcumin was used as a positive control. At the end of 48 hr incubation, the mixture was removed from the cells and after washing with PBS, 100µl fresh Dulbecco's modified Eagle's medium and 20 µl MTT (from 5mg/ml stock) was added to each well. The plates were incubated further for 4-5 hr at 37°C. After the incubation period is over, the bulk of the residual medium was removed using a multichannel pipette carefully without disturbing the formazan crystals formed behind. After that 100 µl DMSO (dimethyl sulphoxide) was added to each well for dissolving the formazan crystals. The plates were then agitated for 15-20 minutes on an orbital plate shaker, after which they were read immediately on Titerplate reader (BioRad) at 570 nm. The relative cell viabilities (in percentage) were calculated with that of the control by comparing the viability of the treated cells.

Figure Legends:

Figure 1: Binding of Compound 1 with the CAMK4. **(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 CAMK4. **(C).** Showing interaction of ligand (stick model) with the protein atoms (line model).

Figure 2: Binding of Compound 2 with the CAMK4. **(A).** Structure of 2-(4-(6-(quinolin-8-yloxy)pyrimidin-4-yl)piperazin-1-yl)nicotinonitrile (Molecule 2) **(B).** Showing surface view of ligand (stick model) present in the active site cavity of the CAMK4. **(B).** Showing interaction of ligand (stick model) with the protein atoms (line model).

Figure 3: Binding of Compound 3 with the CAMK4. **(A).** Structure of N-(4-methoxyphenyl)-6-(piperazin-1-yl)pyrimidin-4-amine (Molecule 3) **(B).** Showing surface view of ligand (stick model) present in the active site cavity of the CAMK4. **(C).** Showing interaction of ligand (stick model) with the protein atoms (line model).

Figure 4. Fluorescence binding study of compound 1 with the CAMK4. **(A)**. Fluorescence spectra of CAMKIV (4 μM) in the increasing concentrations of ligand 8, 16, 24, 32, 40, 48, 56, 64, 72, 80, 88, 96 μM (from top to bottom) at pH 7.4. The protein was excited at 280nm and emission spectra was collected in the range of 300-400 nm) **(B).** Modified Stern–Volmer plot for tryptophan quenching of CAMKIV by compound 1 used for the calculation of binding affinity.

Figure 5. Fluorescence binding study of compound 2 with the CAMK4. **(A).** Fluorescence spectra of CAMKIV (4 μM) in the increasing concentrations of ligand 0, 6, 12, 18,

24, 30, 36, 42, 48, 54 μM (from top to bottom) at pH 7.4. The protein was excited at 280nm and emission spectra was collected in the range of 300-400nm) **(B).** Modified Stern–Volmer plot for tryptophan quenching of CAMKIV by compound 2 used for the calculation of binding affinity.

Figure 6. Fluorescence binding study of compound 3 with the CAMK4. **(A).** Fluorescence spectra of CAMKIV (4 μM) in the increasing concentrations of ligand 0, 20, 26, 32, 38, 44, 50, 56, 62, 68, 74, 80, 86, 92, 98, 104, 110, 116, 120, 126, 132 μM (from top to bottom) at pH 7.4. The protein was excited at 280nm and emission spectra was collected in the range of 300-400nm) **(B).** Modified Stern–Volmer plot for tryptophan quenching of CAMKIV by compound 3 used for the calculation of binding affinity.

Figure 7: **(A).** Cytotoxicity results of synthesized inhibitors (molecule 1, 2, and 3) on the viability of HEK293 cells. A total of 7000 cells in triplicates were exposed to the indicated concentration of inhibitors for 48 h and subjected to MTT assay. **(B).** Effect of synthesized inhibitors (molecule 1, 2, and 3) on the proliferation of HuH7 cells. A total of 7000 cells in triplicates were exposed to the indicated concentrations of curcumin and inhibitors for 48 h and subjected to MTT assay. Data represent three independent sets of experiments and results are shown as the mean ± S.D. Curcumin is used as a positive control.

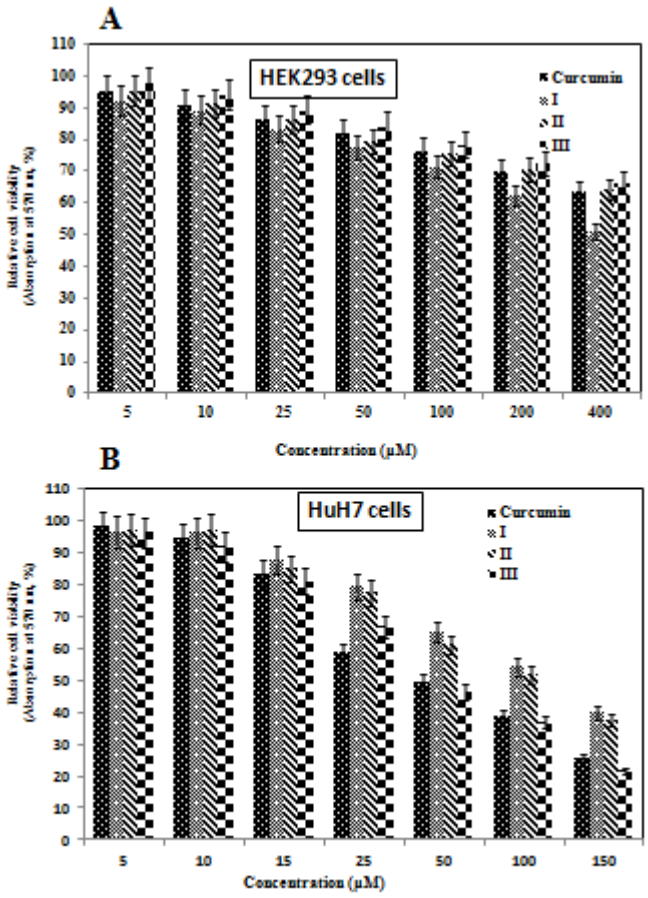


Figure 7

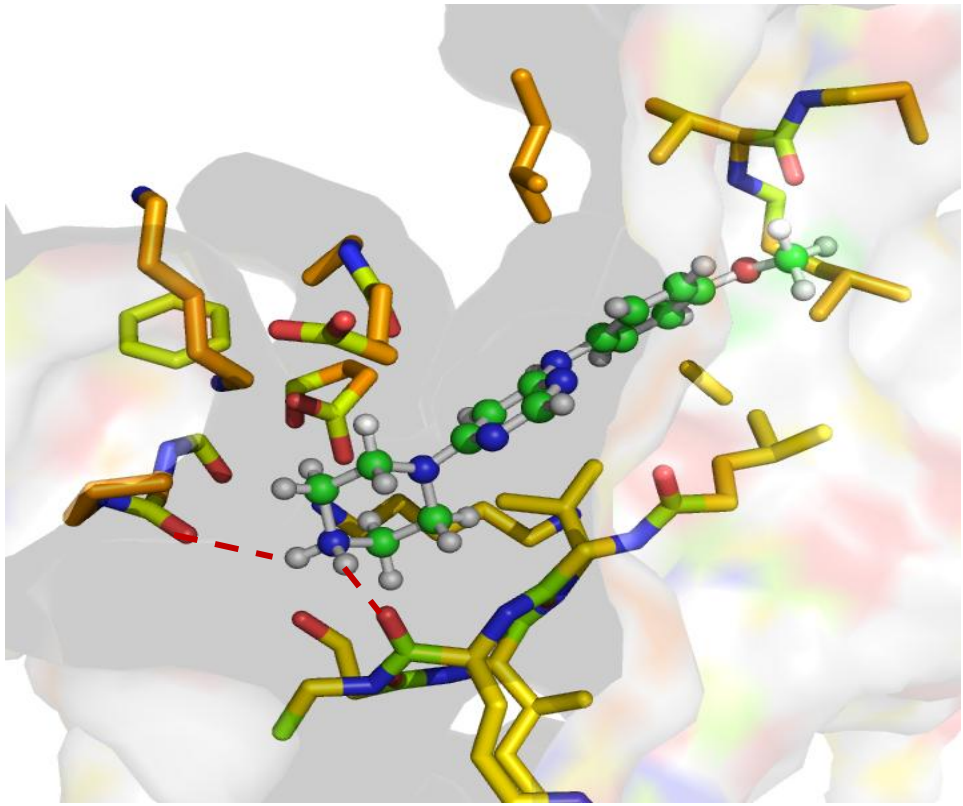
*Graphical Abstract (for review)

Structure Guided Design of Potential Ligand of Human Calcium-Calmodulin Dependent Protein Kinase IV Containing Pyrimidine Scaffold

Huma Naz, Ehtesham Jameel, Nasimul Hoda, Ashutosh Shandilya , B. Jayaram, Faizan Ahmad, Asimul Islam, and Md. Imtaiyaz Hassan*

Highlights

•Three potential ligands were docked in the active site cavity of CAMKIV.
•Several non-covalent interactions are formed between ligands and CAMKIV.
•All three ligands were synthesized and characterized successfully.
•Molecule 3 is showing very high affinity for the CAMKIV in the nm range.



Molecule 3 in the active site cavity of CAMKIV Showing various non-covalent interaction