In silico binding affinity study of calcineurin inhibitors to calcineurin and its close associates

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Received 16 January 2012; revised 25 July 2012; accepted 10 September 2012

Calcineurin (CN) is a calcium regulated serine/threonine protein phosphatase. It is known to be inhibited by cyclosporin A (CsA), tacrolimus (FK506), endothall (ENDO), microcystin (MCYST), okadaic acid (OA) and trifluoperazine (TFP). CsA and FK506 are known inhibitors, which inhibit CN by binding to cyclophilin A (CyPA) and FK506 binding protein 12 (FKBP12), to form CsA-CyPA and FKBP12-FK506 complexes, respectively. These complex molecules finally bind to CN to inhibit its phosphatase activity. The mode of action of the inhibitors and their binding affinity to CN’s subunits and close associates is not very clear. The current study is to dock CN subunits, such as, calcineurin A (CNA), calcineurin B (CNB) and calcineurin AB (CNAB), with known inhibitors as well as close associates of CN like calmodulin (CaM), FKBP12 and CyPA. This was done using Autodock Vina to evaluate their binding free energy (\(\Delta G\)) and binding affinities. The results suggest that inhibition can be brought about by either directly binding to CN and its subunits or associates. The binding affinity of known inhibitors of CN is given as follows: OA>TFP>MCYST>FK506>CsA>ENDO. These inhibitors exhibit very low docked free energy not only with associates of CN, but also with CN subunits. These binding free energies suggest that inhibitors of CN and its associates have different binding affinities and also could exhibit complex inhibition rather than a direct inhibition of CN through a single mechanism.

**Keywords:** Binding affinity, calcineurin, docking, inhibitors

**Introduction**

Calcineurin (CN) is a well preserved Ca\(^{2+}\)-calmodulin activated protein phosphatase\(^3\). It consists of a catalytic subunit CNA and a regulatory subunit CNB. It is a self-regulated enzyme containing an autoinhibitory domain (AID) positioned after that of the calmodulin (CaM)-binding domain, just near the C-terminus of its catalytic subunit\(^2\). CN is usually present in the central nervous system\(^3\) and involved in several functions, such as, T-cell propagation by dephosphorylation of nuclear factor of activated T cells (NFAT)\(^4\), gene expression, in myocardium magnification and regulation of the cyclin dependent kinase 4 (cdk4) in G\(_1\)/G\(_0\) checkpoint of cell cycle\(^5\). CaM is an omnipresent, heat stable, Ca\(^{2+}\)-receptor protein regulated by calcium. It is involved in mediating a number of physiological processes including glycogen and cyclic nucleotide metabolism, secretion, motility\(^6\) and control of cell propagation\(^7\).

In calcium signaling, Ca\(^{2+}\)-bound CaM binds to the CaM-binding domain of CN, releasing the AID from the active site. This makes the phosphatase active site available for binding and dephosphorylation of its substrates. The CaM binding and AID provide an “on-and-off” switch in response to changes in intracellular calcium concentrations. Therefore, the regulation of CN is highly significant in circumstances like organ transplantation and brain related disease. Various CN inhibitors discovered so far include cyclosporin A (CsA), tacrolimus (FK506), endothall (ENDO), microcystin (MCYST), okadaic acid (OA) and trifluoperazine (TFP). Mainly, inhibitors like cyclosporin A (CsA) and FK506 were used as drugs, while other inhibitors required further study of their properties before being considered as potential drugs.

Different levels of CN tend to cause various abnormalities. These range from heart diseases to neurological\(^8\) ailment like Alzheimer’s and psychological illnesses like schizophrenia\(^9-13\). CN inhibition is highly essential to treat these diseases. It is also useful in minimizing graft rejections.
because of its role in T-cell propagation. FKBP12 and CyP are vital immunophilins and reduce immunity by binding with inhibitors to inactivate CN. CN inhibitors can also target downstream of CN signalling.

Known inhibitors inhibit CN regulatory activity, but the mechanism by which they do so is not very clear. Hence, the current study was undertaken to look into the possibility of binding of these inhibitors to CN subunits and its close associates, such as, CaM, CyPA and FKBP12.

Material and Methods

Extraction of Molecules from PDB

3D structure of the protein and ligand molecules were retrieved from protein data bank (PDB). All these retrieved molecules with their ID are listed in Table 1. Preparation of the docking molecules and experiments were performed on the Windows system Core 2 Duo, 2 GB RAM.

Preparation of Molecules for Docking

Molecules retrieved from PDB did not have complete charge assigned to them. Hence, before docking, the polar hydrogens were added to the macromolecules and then assigned the partial atomic charges using Autodock. The non-polar and polar hydrogen atoms were merged. For ligands, Gasteiger charges were added, the non-polar hydrogens were merged and also rotatable bonds were determined based on the nature of ligand molecule. TORSDOF was used to calculate the change in free energy (∆G) caused by the loss of torsional degree of freedom upon binding. The atomic fragmental volume and the atomic solvation parameters were used here to calculate the energy contribution of desolvation of the macromolecules by ligand binding. Here, peptide backbone bonds were constructed. Bonds between selected atoms and all the active bonds are made rotatable.

Grid maps were generated and spacing was adjusted to 1.0 Å to enable ligand binding. Grid dimension was adjusted to 40x40x40 points. AutoDock uses interaction maps for docking. Prior to the actual docking run, these maps were calculated by AutoGrid. For each ligand atom type, the interaction energy between the ligand atom and the receptor was calculated for the entire binding site, which is discretized through a grid. The protein was embedded in a 3D grid and a probe was placed at each grid point. Interaction energy of the protein was assigned at each grid point and the affinity grid and electrostatic potential for each atom of the ligand was calculated. Electrostatic interaction was evaluated by interpolation.

Docking Protein and Ligand Molecule

Automated docking software AutoDock Vina was used to evaluate binding affinity of ligands with CNA, CNB, CNAB, CaM, CyPA and FKBP12. Docking energy of all six ligand molecules, such as, CsA, FK506, MCYST, ENDO, OA and TFP were evaluated by using empirical free energy functions and Lamarkian genetic algorithm. These calculate the binding free energy (∆G) based on the different electrostatic, Van der Waal, hydrogen bonding and desolvation effects.

Results and Discussion

The concept of free energy (∆G) is used to determine the binding affinity of protein-ligand complex in docking studies. The negative or low value of ∆G indicates the strong binding affinity between protein-ligand complex and that the ligand is in the most favourable conformation. Hence, in the present study, authors have determined the binding free energy as shown in Table 2, which reflects the binding affinity of the different ligands to a CN protein molecule by calculating intermolecular and torsional free energies using AutoDock Vina.

Docking studies of CsA with CN and CN subunits show that CsA has higher binding affinity with CNB as compared with CNAB and CNA. Further, docking studies on CN associates reveal that CsA has higher binding affinity with CaM as compared to the binding affinity with FKBP12 and CyPA. A comparison of the binding affinities of CsA with CNB

| Table 1—CN, its associates and inhibitors retrieved from respective PDB ID molecules from Protein data bank |
|-----------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| PDB ID         | Calcineurin subunits, associates and inhibitors                                                                                                                                                                                                                                                                                                                                                   |
| 1MF8           | Calcineurin A, calcineurin B, calcineurin AB, cyclosporin A, cyclophilin A                                                                                             |
| 1FKJ           | FKBP12-FK506                                                                                                                                                                                                                          |
| 3H61           | Endothall                                                                                                                                                                                                                 |
| 1EVB           | Microcystin                                                                                                                                                                                                                           |
| 1U32           | Okadaic acid                                                                                                                                                                                                                           |
| 3HR4           | Calmodulin                                                                                                                                                                                                                 |
| 3KO0           | Trifluoperazine                                                                                                                                                                                                                           |
and CaM indicates that CsA has stronger binding affinity with CaM.

Similarly, docking studies of FK506 with CN and CN subunits reveal that it has higher binding affinity with CN-subunit, CNB as compared with subunits CNA and CNAB. Moreover, docking results with CN associates show that FK506 has highest binding affinity with FKBP12 as compared with CaM and CyPA. A comparison of binding affinities of FK506 with CNB and FKBP12 shows that FK506 has the highest affinity with FKBP12, and the result corroborates with the findings of Duyne et al.\textsuperscript{32} These findings indicate that CN may be inhibited either directly by inhibitors or by forming a complex with CN associates.

MCYST-LR is a known inhibitor of protein phosphatases\textsuperscript{33}. However, there is no evidence to show that it can inhibit CN activity \textit{per se}. The docking studies of MCYST-LR with CN and CN subunits indicate that MCYST-LR has the highest binding affinity with CNAB in comparison to CNA and CNB. Further, docking results with CN associates indicate that it has the highest binding affinity with CaM in comparison to CyPA and FKBP12. On comparison of these results, it is clear that MCYST-LR has stronger binding affinity with CaM as compared with CNAB.

Binding-affinity studies of ENDO with CN and its subunits show that it has highest binding affinity to CNAB as compared to CNA and CNB; and with CN associates, it has stronger binding affinity with CyPA as compared to CaM and FKBP12. A comparison of binding affinity studies of ENDO with CNAB and CyPA reveals that it has highest binding affinity to CNAB.

OA has been shown to inhibit protein phosphatase 1 and 2\textsuperscript{34}. Docking studies of OA with CN and its subunits indicate that it has high binding affinity towards CNB; the docking site of CNB with OA is shown in Fig. 1. Moreover, the docking of OA with CN associates reveals that it has strong binding affinity towards CaM. Docking analysis of OA with CNB and CaM reveals that OA has the highest binding affinity towards CNB.

TFP is a CaM antagonist and it prevents binding of CaM to CN, which leads to decrease in CN phosphatase activity\textsuperscript{35}. Among CN, CN subunits and CN associates, the docking study of TFP indicates that it has the highest and equal affinity towards CNB and CaM, respectively.

Since CN inhibitors exhibit binding affinity not only with CN but also with CN associates, the average docking energies of all inhibitors were also determined for CN, CN subunits and its associates (Table 3). Present study indicates that OA exhibits the highest average binding affinity with CN, CN subunits and CN associates. In conclusion, OA exhibits highest binding affinity to CN as compared to any other inhibitor studied.

\begin{table}[h]
\centering
\small
\begin{tabular}{|c|c|c|c|c|c|c|c|}
\hline
Combination targets/Inhibitors & CsA & FK506 & MCYST & ENDO & OA & TFP \\
\hline
CNA & -4.9 & -6.8 & -5.6 & -5.1 & -7.5 & -7.4 \\
CNB & -6.6 & -6.9 & -5.7 & -4.7 & -9.1 & -7.8 \\
CNAB & -6.2 & -6.6 & -7.2 & -5.6 & -8.2 & -7.4 \\
CyPA & -5.5 & -6.1 & -6.7 & -5.3 & -7.9 & -6.7 \\
FKBP12 & -5.6 & -9.7 & -6.7 & -5.2 & -7.8 & -7.3 \\
CaM & -6.8 & -7.7 & -7.3 & -4.6 & -8.4 & -7.8 \\
\hline
\end{tabular}
\caption{Binding free energy ($\Delta G$) of different inhibitors with CN subunits and its associates at kcal/mol}
\end{table}
Conclusion
Docking softwares have been frequently used in drug development to study the binding affinity of inhibitor molecules to target proteins. Known inhibitors CsA and FK506 are used as drugs to treat CN related disorders. It is difficult to study the binding affinity of protein-ligand complex molecule in vivo and in vitro in large number. The crystal structures of all protein and inhibitor molecules and docking softwares enable us to study their affinity for each other. The current study predicts the binding affinity of all known inhibitors with CN, its subunits, and its close associates using Autodock Vina.

Conclusions can be drawn from the binding affinity study of protein-ligand complex molecule. In the present study, binding affinities show that all inhibitors have binding affinity with CN, its subunits and associates. Binding affinity of CsA is found greater with CaM, suggesting that major inhibition of CN is not only mediated through CyPA, but also through CaM. FK506 exhibits more binding affinity with CN associate, FKBP12. MCYST appears to be a direct inhibitor of CNAB rather than it acts through CN associates. OA and ENDO appear to be the broad spectrum inhibitors and inhibit CN and its associates; between these two, OA shows greater affinity. In conclusion, all six inhibitors exhibit different types of affinity with CN and its associates.

From the present study, it can be concluded that of all the six known inhibitors, OA could be the strongest inhibitor for CN, collectively. However, further in vitro studies are required for enhanced knowledge on the mechanism of inhibitory action.

Acknowledgement
Authors would like to thank Dr Kureekal Ramesh, Jain University, Bangalore for his kind support in using Autodock software. They are also grateful to Bangalore University for providing the necessary facilities and the funding support under the Bangalore University Internal Research Fund (BUIRF).

Reference


