Bisindolylmaleimides new inhibitors of CaM protein

Karina Fructuoso-García, Matuz-Mares D1, Isabel Velázquez-López1, and González-Andrade M\*,1

1Facultad de Medicina, Universidad Nacional Autónoma de México, Ciudad de México, C.P 04510, México.

\*Corresponding author. Dr. Martin González-Andrade, 1Facultad de Medicina, Universidad Nacional Autónoma de México, Ciudad de México, C.P 04510, México.

E-mail address: martin@bq.unam.mx

**ABSTRACT**

In the present study, we reported that the BIM also blocksactivity of the solute carrier organic cation transporter (OCT) 1, involved in uptake of mar-keted drugs in the liver, in a PKC-independent manner.

**Keywords**: Biosensors; Calmodulin; Anti-CaM drugs; Docking; Molecular dynamic.

**1. Introduction**

Bisindolylmaleimides (BIMs) are organics compounds derivates from indolocarbazoles, staurosporine formed by a maleimide group and two indole groups bound to it.[1](#_ENREF_1) A series of compounds have been synthesized from BIMs with different substituents in one of the indoles, with the exception of BIM V (Figure 1). This series includes BIM from I to XI, biological activity has been reported for these compounds. BIM I has been reported as an inhibitor of protein kinase C (PKC)[2](#_ENREF_2) and glycogen synthase kinase 3 (GSK3),[3](#_ENREF_3) as well as competitive antagonist at the 5-HT3 receptor.[4](#_ENREF_4) BIM II is a general inhibitor of all PKC.[5](#_ENREF_5) BIMs I, II, III, IV, and V interact and inhibit ABCG2 (a transporter with potential importance in cancer drug resistance).[6](#_ENREF_6) BIM IV in addition to inhibiting the PKC also inhibits the cAMP-dependent protein kinase (PKA).[5](#_ENREF_5) BIM I, BIM II, BIM III, BIM VI, BIM VII, and BIM-VIII inhibit solute carrier organic cation transporter (OCT) 1, involved in uptake of mar-keted drugs in the liver.[7](#_ENREF_7) BIM IX is a potent inhibitors of GSK-3.[3](#_ENREF_3) BIM X It is also considered as an inhibitor of protein kinases (PKs).[8](#_ENREF_8) BIM XI inhibits PKC and prevents T-cell activation and proliferation.[9](#_ENREF_9), [10](#_ENREF_10)



**BIM-I**

**BIM-III**

**BIM-II**

**BIM-VI**

**BIM-IV**

**BIM-V**

**BIM-VIII**

**BIM-VIII**

**BIM-VII**

**CPZ**

**BIM-X**

**BIM-IX**

Figure 1. Structures of BIMs and CPZ.

**2. Results and discussion**

*2.1 CaM-Drug Interaction*

Calmodulin is an intracellular protein which modulates the biological activities of a wide variety of proteins in different metabolic pathways including kinases, phosphatases, synthase, receptors, and calcium channel. For this reason, this protein is a good molecular target for different types of drugs. The main anti-CaM drugs include anti-cancers, antipsychotics, and antidepressants and its side effects include sleepiness, motility disorders, dysrhythmias, and muscular weakness.

*2.2 Determination of the binding affinity of drugs with the biosensor hCaM M124C-mBBr*

To determine the affinity of the drugs using the biosensor *h*CaM M124C-*mBBr* in calcium-saturating conditions (10 M), these were titrated by adding increasing amounts of BIMs to determine the *K*ds. **Figure 3** shows fluorescence spectra of the biosensor with different concentrations of drugs; the differences observed in the fluorescence signal are used to calculate the *K*d, using a one-site binding model equation (Eq. 1, see method section). Table 1 shows the results obtained from the fluorescence titrations of the different compounds. **IMI**, **FLU** and **CPZ** have been previously well reported to bind to a CaM–EGFP fusion protein in the presence of Ca2+, but this system does not respond to **5-HT** [11](#_ENREF_11). In the case of **5-HT** previous studies using a CaM-labeled at position 109, did not observe a response of this biosensor either [12](#_ENREF_12). However, we can perform a complete titration with **5-HT** and obtain a *K*d of 0.71 M (**Figure 3D**), which is within the range expected for most organic molecules. Another neurotransmitter such as **DOP** was analyzed with our biosensor and showed no response (**Figure 1S-A**), agreeing with both CaM-EGFP and with the CaM-labeled at position 109. CaM has specific binding sites for inhibitors and for proteins, so inhibitors share common structural features. In general, the inhibitors of CaM are structures of resonant type, with a zone of character highly hydrophobic and an electronegative pole. The structural differences between **5-HT** and **DOP** comprise primarily in the conjugated system. **DOP** molecule consists of a catechol structure, while **5-HT** is synthesized from the essential amino acid L-Tryptophan; so, its conjugate system is an indole. With respect to the *Vinca alkaloids* **VCT** and **VBT** are anticarcinogenic agents and have been approved for clinical use. Kinetic analysis using CaM-dependent Ca2+-transport ATPase, have reported *K*d in a range of 2-10 M.[13](#_ENREF_13), [14](#_ENREF_14) We obtained Kds of 0.80 and 1.69 M at saturating Ca2+ concentrations for **VCT** and **VBT**, respectively. The *Vinca alkaloids* are complex structures with respect to the classical inhibitors, nevertheless they show similar affinities. The structure-activity relationship of many drugs is associated with affinity for molecular target. In the case of CaM, it is not the exception, drugs with complex structures, hydrophobic zones, resonant systems and oriented dipoles would be the ideal characteristics of a good inhibitor. **AMA** was used as a negative control; this antiviral drug structurally does not exhibit the features described above. **Figure 1S-B** shows that the addition of about 80 M does not change the fluorescence signal of the biosensor.





****

**Figure 3.** Fluorescence spectra and titration curves of Ca+2-*h*CaM M124C-*mBBr* with **CPZ** (A), **FLU** (B), **IMI** (C), **5-HT** (D), **VCT** (E), and **VBT** (F). Buffer was 10 mM of potassium acetate pH 5.1 at 37 °C. The absolute changes of maximal fluorescence emission were corrected for light scattering effects and plotted against the ligands to total protein ratio (insets). The continuous line in the insets comes from the fitting of data to the binding model (equation 1 in experimental) to obtain the *K*d.

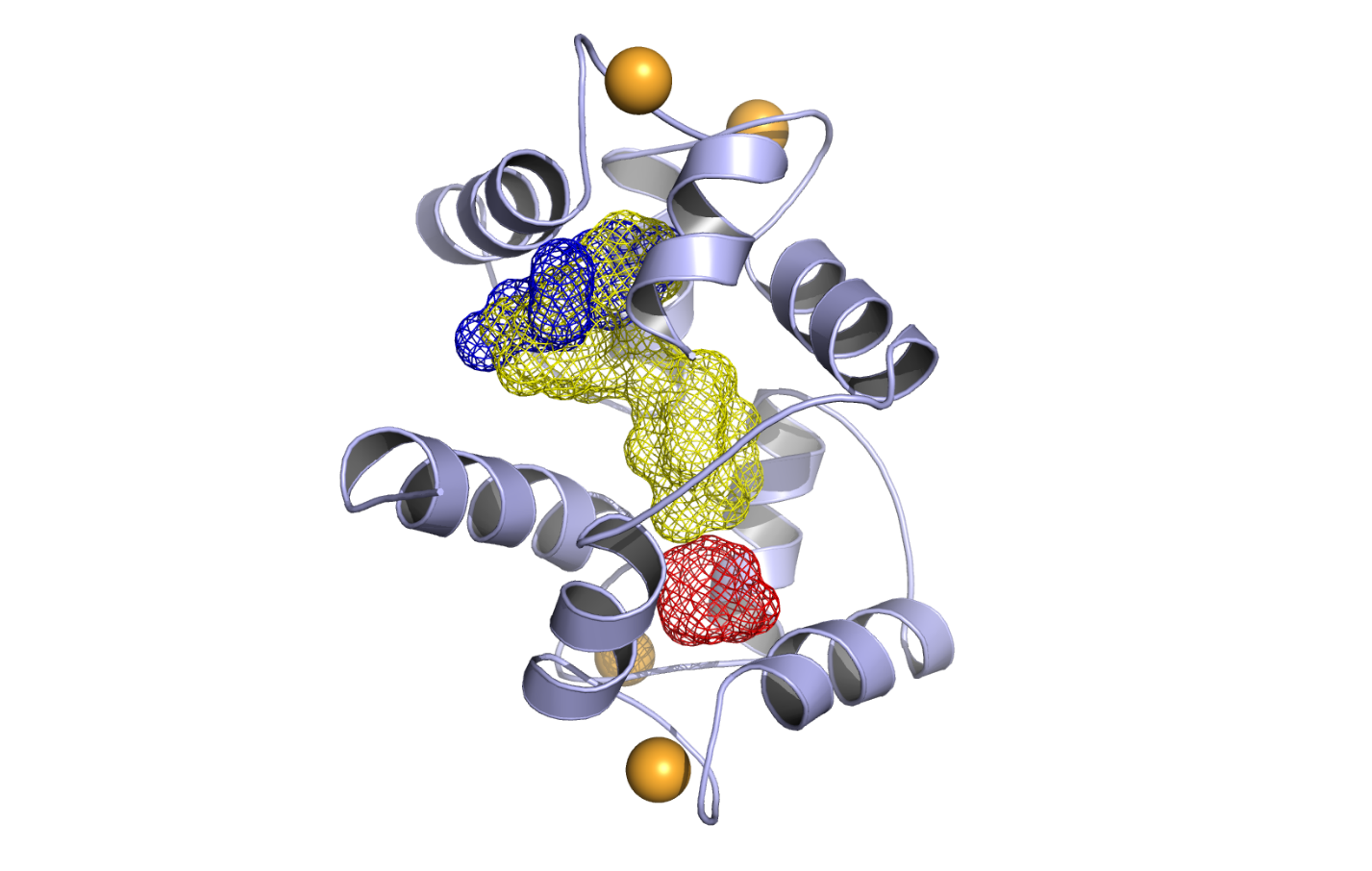
*2.3 Theory studies of complex 4Ca2+-CaM-drugs*

The theoretical studies of 4Ca2+-CaM-Drug complexes help us try to establish a structure-affinity relationship, as well as elucidate the main interactions responsible for the binding affinity of these drugs on the CaM. Today, there are two main tools for conducting theoretical studies of binding of ligands to biomolecules, docking and molecular dynamic studies [15](#_ENREF_15), [16](#_ENREF_16). The first considers the macromolecule as a rigid body and the ligand provides flexibility and mobility to predict a possible binding site. Additionally, estimates a free energy bond and a theoretical *K*i. The second proposes a system closer to reality by controlling parameters such as temperature, pressure, ionic strength and degree of solvation. The processing of the trajectories to analyze the protein-ligand complexes allows us to obtain theoretical thermodynamic data (*H*, *S*, and *G*) associated with the interactions that are carried out in a certain time.

*2.4 Docking*

The docking studies indicate that the CaM has several probable sites of union of the different drugs. This has been well established by both structural studies (x-ray diffraction) and molecular modeling. What is important in this study is to consider the structural differences of the drugs to establish a stoichiometry and with it have a good structural model to initiate studies of molecular dynamics. **Table 1** and **Figure 4** show the results obtained for the eight compounds studied. **Figure 4** shows the probable binding sites, which depend on the size of the drug, its flexibility and its chemical properties (functional groups). The estimated union parameters (estimated final energy of binding (EFEB) and *K*i, **Table 1**) indicate that all ligands have good bonding possibilities including the negative control drugs (**AMA**), even though it has a higher *K*i. **Figure 5A** shows the correlation between experimental and docking data, showing a good correlation.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Table 1. Experimental and theoretical binding properties of Ca2+-CaM-Ligands complexes.** | | | | | | | |
|  | **Experimental studies** | | **Docking studies** | | **DM studies** | | |
| **Complex** | **Gexp**  **(kcal mol-1)** | ***K*d1**  **(M)** | **EFEB**  **(kcal mol-1)** | **2**  **(M)** | ***H***  **(kcal mol-1)** | *****S***  **(kcal mol-1)** | **Gcal**  **(kcal mol-1)** |
| Ca2+-CaM -**CPZ** | -8.19 | 0.97x10-6 | -8.24 | 0.90 x10-6 | -22.27±2.5 | -17.75±7.7 | -4.52±1.8 |
| Ca2+-CaM -**FLU** | -8.05 | 1.24x10-6 | -8.29 | 0.84 x10-6 | -26.94±3.64 | -20.11±6.87 | -6.82±1.45 |
| Ca2+-CaM -**IMI** | -7.05 | 6.71x10-6 | -8.11 | 1.13 x10-6 | -38.03±6.1 | -17.12±7.2 | -20.91±9.5 |
| Ca2+-CaM -**VBT** | -7.86 | 1.69x10-6 | -8.07 | 1.22 x10-6 | -54.35±9.1 | -34.72±7.5 | -19.62±2.7 |
| Ca2+-CaM -**VCT** | -8.31 | 0.80x10-6 | -8.09 | 1.18 x10-6 | -45.96±5.6 | -32.18±7.4 | -13.78±6.4 |
| Ca2+-CaM -**5-HT** | -8.38 | 0.71x10-6 | -7.56 | 2.84 x10-6 | -21.38±2.7 | -16.52±3.4 | -4.85±0.4 |
| Ca2+-CaM -**AMA** | - | - | -7.74 | 2.13 x10-6 | +133.30±13.8 | +15.60±5.16 | +117.69±21.2 |
| Ca2+-CaM -**DOP** | - | - | -7.21 | 2.96 x10-6 | -11.85±3.5 | -13.60±5.24 | +1.74±0.8 |
| 1 Apparent dissociation constants at 298.15 K  2 Theory inhibitor constants at 298.15 K | | | | | | | |

**Figure 4.** Structural model of then probable binding sites Ca2+-CaM-Ligands. Blue, yellow, and red mesh are the probable sites of binding of ligands to the CaM.

*2.5 Molecular Dynamics Simulations*

Unlike docking studies, molecular dynamics (MD) simulations represent the movement of all the atoms of a system, where they vibrate and move in a defined time. Integrating Newton's equations of motion, under a potential empirical field that approximates the energies of molecular systems representing atoms as charged spheres, and links as springs with special properties. The different components of the systems have been parameterized so that they can predict statistical properties of the physicochemical systems, while providing detailed information of their interactions, and can estimate the energy contribution of different parameters which can be compared with experimental data; in our case, of the 4Ca2+-CaM-Drug interaction. A key element for conducting MD studies is the starting structure of the CaM-Drug complex. The initial structures were obtained from docking studies and available experimental information of some compounds. Another key point is the optimal time to perform an MD, which will depend on the particle system being studied. **Figure 2S** shows the changes in system energy along the simulation process of the 4Ca2+-CaM-CPZ complex, which gives us the guideline to establish the simulation times for this system. In addition to this complex, the MD were performed at different times in the range of 10-100 ns (with 10 ns intervals) and subsequently analyzed. **Table 1S** and **Figure 3S** show that from 50 ns the *H* is stable and both the *S* and the *G* are cyclically compensated. Taking advantage of the computing power of today, it was decided to make 100 ns of MD for all the complexes.

We calculated *G* of all complexes using the method normal mode analysis, which calculates the enthalpy and entropy contributions, respectively. This strategy, used to estimate the *G,* allowed us to separate the energetic contributions (enthalpy-entropy compensations) associated with molecular recognition in the binding of drugs to 4Ca2+-CaM. **Table 1** shows the theoretical thermodynamic parameters of the MD trajectories. The *G*Theo as in the docking studies has a good correlation with the experimental data (**Figure 5B**). However, unlike the docking results, the negative control (**AMA**) and **DOP** show positive Gs which are fully consistent with the experimental results. The enthalpic component contributes in greater proportion to the *G* in Ca2+-CaM-Drug complexes (except for Ca2+-CaM-**DOP**), which is expected for this type of interactions. The visualization of the trajectories of the DMs (Movie 1-8) show that the complexes are maintained over time. Only for the case of the **AMA** (Movie 7. 1a29-AMA.mp4), it moves away from the CaM; which may explain the positive values of the thermodynamics parameters. **Figure 6**, shown the root mean square deviations (RMSDs) vs Time for 100 ns MD simulation; RMSDs show little fluctuation (1-3 Å) over time for all complexes, except for the open form of the CaM (1CLL) and for the Ca2+-CaM-**DOP** complex (up to 12 Å). These data are in harmony with the calculated energies and with the *K*ds of the complexes. The large RMSD observed for the CaM in its “open” conformation can be attributed to the dynamics of the protein itself, which fluctuates between the “open” and “closed” form and when a ligand bind stabilizes a conformation, in this case, as it lacks ligands, the forms “open” tends to fluctuate more. For the Ca2+-CaM-**DOP** complex, the **DOP** does not bind to the protein, fails to stabilize a conformation and possibly the CaM explores new conformation (Movie 8, 1a29-DOP.mp4), which translates into a high RMSD.

****

**Figure 5**. Correlations between experimental and theoretical data on Ca2+-CaM-Drug interactions. A) EExp vs EFEBTheo and B) EExp vs GTheo. GExp was calculated using the Van ´t Hoff equation from the Kd, R (1.9872 cal/mol K) and T (298.15 K). Estimated Energy Free Binding (EFEB) was calculated through a semi-empirical force field with AutoDock 4.2. Binding free energies theory (GTheo) by MM/PBSA from the MD trajectories.



**Figure 6**. The RMSD & Time plot for 100 ns MD simulation. (A) shows the differences between closed complex: Ca2+-CaM-**AMA**,Ca2+-CaM-**DOP**, Ca2+-CaM-**CPZ**, Ca2+-CaM-**FLU**, Ca2+-CaM-**IMI**, Ca2+-CaM-**5-HT**, Ca2+-CaM-**VTB**, and Ca2+-CaM-**VCT**; and the open conformation (1CLL).

**3. Conclusions**

In this paper, we present experimental and theoretical studies of the binding of seven anti-CaM compounds currently used in clinical therapy, except for **5-HT**. The binding experiments indicate affinity ranges between 0.71 and 6.71 M, which are consistent with those previously reported with other methods (indirect and direct), except for **5-HT** which is reported for the first time its title and its *K*d with a biosensor. The sensitivity of the biosensor *h*CaM M124C-*MBBr* allows monitoring different compounds to the classic inhibitors as is the case of **5-HT** and to dissent between pharmacophores. The affinities of the compounds may be related to the different side effects and their intensity. On the other hand, the combination of theoretical studies such as docking and MD is an excellent complement to the experimental data. In our case, both studies correlate in an acceptable way with the experimental results and help us establish structure-activity relationships, which are useful data in the development and design of new CaM inhibitors. The docking results provide general binding information for the CaM-drug complex, which is used to perform the MD studies. The time interval of the DM is in accordance with the analysis that is wanted and the own system, for our case from 50 ns the stable complexes are maintained. In conclusion, studies of the different compounds, both experimentally and theoretically, have different degrees of affinity for CaM and this may be related to potency and pharmacological action, as well as to the side effects of the drugs described in this work.

**4. Experimental protocols**

*4.1. Chemistry*

The biosensor (*h*CaM M124C-*mBBr*) was obtained using the methodology described above by González-Andrade, M. and *col*. [17](#_ENREF_17). The drugs and metabolites were acquired from Sigma (St. Louis, MO) and Santa Cruz Biotechnology, Inc. (Dallas Texas, USA). All other reagents were of analytical reagent grade and were purchased from Sigma (St. Louis, MO).

*4.2* *Steady-state fluorescence*

All measurements were conducted with an ISS–PC1 spectrofluorometer (ISS, Champaign, IL) with sample stirring at 37 ºC. The *h*CaM M124C-*mBBr* (1 M) was incubated in buffer (10 mM of potassium acetate [pH 5.1] and 10 M of CaCl2). Fluorescence emission spectra were acquired with excitation and emission slit widths of 4 and 8 nm, respectively. The excitation wavelength was 381 nm, and emission wavelengths of 415 to 550 nm were measured. The fractional degree of saturated *h*CaM M124C-*mBBr* with ligand (*y*) was calculated by changes in fluorescence on ligand binding according to *y* = (F-F0) / (F∞-F0), where F∞ represents the fluorescence intensity at saturation of the ligand, *y* is plotted as a function of the protein/ligand relation (L), and the apparent dissociation constants (*K*d) and stoichiometric (S) were obtained by fitting to the equation:



where *y* represents the fractional degree of fluorescence intensity at 470 nm, *K*d is the apparent dissociation constant for the ligands, *L* is the protein/ligand relation and *S* is the stoichiometric. The data were analyzed using the OriginPro version 9.0 64-bit SR2 program (OriginLab, Northampton, MA).

*4.3 Preparation of initial coordinate files*

The coordinates corresponding to the structure of CaM were obtained from the Protein Data Bank (PDB, http://www.rcsb.org). The CaM-ligands complexes, the X-ray structure of CaM with calcium and TFP named 1A29.pdb (1A29, close form of the CaM) refined at 2.7 Å were chosen [18](#_ENREF_18), [19](#_ENREF_19). The ligands were obtained from the PDB co-crystillized structure, and when the crystals were not available, their structures were constructed using HyperChem 8 software. All structures of the ligands were minimized using Gaussian 09, revision A.02 (Gaussian Inc., Wallingford, CT) at DTF B3LYP/3-21G level of theory. Partial charges and force field parameters of the inhibitors were generated automatically using the *antechamber* program in AMBER 14 [20](#_ENREF_20).

*4.4 Docking*

Docking was conducted using PDB X-ray structure of the CaM with the ligand TFP (1A29.pdb). The crystal structure was rebuilt and refined, we performed a final all-atom refinement of CaM with the idealization application of the Rosetta3.1 release.[21](#_ENREF_21) All compounds were built using the HyperChem 8.0 release program and optimized geometrically using the Gaussian 09 program, revision A.02 (Gaussian Inc., Wallingford, CT) at DTF B3LYP/3-21G level of theory. The protein and ligands were further prepared using the utilities implemented by AutoDockTools 1.5.4 (<http://mgltools.scripps.edu/>). The protein was adding polar hydrogen atoms, Kollman united-atom partial charges and to the ligands computing Gasteiger-Marsilli formalism charges**,** rotatable groups which were assigned automatically as were the active torsions. Blind docking was carried out using AutoDock4 version 4.2 software (<http://autodock.scripps.edu/>) [22-24](#_ENREF_22), using the default parameters the Lamarkian genetic algorithm with local search, number of individuals in population (150), maximum number of energy evaluations (2.5 million), maximum number of generations (27 000), rate of gene mutation (0.02), rate of crossover (0.8) and 100 runs for docking. Electrostatic grid maps were generated for each atom type in the ligands using the auxiliary program AutoGrid4 part of the software AutoDock4. The initial grid box size was 60 Å × 60 Å × 60 Å in the x, y, and z dimensions. In order to refine the docking analyses, they were performed in a smaller grid box, with 30 Å × 30 Å × 30 Å dimensions, placed in the ligand. The analysis of the docking was made with AutoDockTools using cluster analysis and program PyMOL [25](#_ENREF_25).

*4.5 Molecular dynamics simulation*

The coordinates of the ligands, resulting from the docking study, were processed with antechamber (a set of auxiliary programs for molecular mechanic studies) in order to generate suitable topologies for the LEaP module from AMBER 14 [26-28](#_ENREF_26). Each structure and complex were subjected to the following protocol: hydrogen’s and other missing atoms were added using the LEaP module with the parm99 parameter set, Na+ counterions were added to neutralize the system, the complexes were then solvated in an octahedral box of explicit TIP3P model water molecules localizing the box limits at 12 Å from the protein surface. MD simulations were performed at 1 atm and 298 K, maintained with the Berendsen barostat and thermostat, using periodic boundary conditions and particle mesh Ewald sums (grid spacing of 1 Å) for treating long-range electrostatic interactions with a 10 Å cutoff for computing direct interactions. The SHAKE algorithm was used to satisfy bond constraints, allowing employment of a 2 fs time step for the integration of Newton’s equations as recommended in the Amber package [27](#_ENREF_27), [29](#_ENREF_29). Amber f99SB force field [28](#_ENREF_28), [30](#_ENREF_30), [31](#_ENREF_31) parameters were used for all residues and Gaff force field [20](#_ENREF_20), [32](#_ENREF_32) parameters were used for the ligands. All calculations were made using graphics processing units (GPU) accelerated MD engine in AMBER (pmemd.cuda), program package that runs entirely on CUDA-enabled GPUs [33](#_ENREF_33). The protocol consisted in performing an optimization of the initial structure, followed by 50 ps heating step at 298 K, 50 ps for equilibration at constant volume and 500 ps for equilibration at constant pressure. Several independents 100 ns MD simulations were performed. Frames were saved at 100 ps intervals for subsequent analysis

*4.6 Binding free energies calculated by molecular mechanics/poisson boltzmann surface area (MM/PBSA)*

This method involves a combination of molecular mechanic’s energy with implicit solvation models to calculate binding free energies. In MM/PBSA [34](#_ENREF_34), [35](#_ENREF_35), binding free energy (ΔGbind) between a ligand (L) and a target (T) to form a complex is calculated as:

where Δ*E*MM, Δ*G*Sol and −*T*Δ*S* are the changes of the gas phase molecular mechanics energy, the solvation free energy and the conformational entropy upon binding, respectively. Δ*E*MM comprises Δ*E*Internal (bond, angle and dihedral energies), Δ*E*Electrostatic (electrostatic energies), and Δ*E*Vdw (van der Waals energies). Δ*G*Solv is the sum of electrostatic solvation energy (polar contribution) -Δ*G*PB- and non-electrostatic solvation component (non-polar contribution) -Δ*G*SA-. The polar contribution is calculated using the Poisson-Boltzmann surface area model, while the non-polar energy is estimated from the solvent accessible surface area (*SASA*). The conformational entropy change (-*TS*) was computed by normal mode analysis from a set of conformational snapshots taken from the MD simulations [34](#_ENREF_34), [36](#_ENREF_36), [37](#_ENREF_37).

All calculations were made using a system HP Cluster Platform 3000SL, supercomputer “MIZTLI” with a processing capacity of 118 TFlop/s. It has 5,312 Intel E5-2670 processing cores, 16 NVIDIA m2090 cards, a total RAM of 15,000 Gbytes and a mass storage system of 750 Terabytes (<http://www.super.unam.mx/>).

**Abbreviations**

CaM, calmodulin protein; 5-HT, serotonin; *h*CaM M124C-mBBr, fluorescent-engineered human calmodulin; VCT, vincristine; CPZ, chlorpromazine; FLU, fluoxetine; VBT, vinblastine: IMI, imipramine; AMA, amantadine; DOP, dopamine; SSRIs, selective serotonin-reuptake inhibitors; CNS, central nervous system; EFEB, estimated final energy of binding; MD, molecular dynamics; RMSDs, root mean square deviations; 1CLL, open form of the CaM; 1A29, close form of the CaM.

**Acknowledgment**

This work was supported by grants from DGAPA-UNAM (PAPIIT-IA204116) and DGTIC-UNAM (LANCAD-UNAM-DGTIC-313). The authors are very grateful to Mrs. Maria Josefina Bolado Garza from the División de Investigación, Facultad de Medicina, UNAM for the review and edition of the manuscript; and to doctor D. Baker for supplying Rosetta software. We are indebted to Dirección General de Cómputo y de Tecnologías de Información y Comunicación, UNAM, for providing the resources to carry out computational calculations through Miztli System.

**Conflict of interest**

The authors declare no any conflict of interest.

**Supplementary data**

Supplementary data related to this article can be found at:

**ReferencesReferences ans Notes**

1. Faul, M. M.; Winneroski, L. L.; Krumrich, C. A., A New, Efficient Method for the Synthesis of Bisindolylmaleimides. *J Org Chem* **1998**, 63, 6053-6058.

2. Toullec, D.; Pianetti, P.; Coste, H.; Bellevergue, P.; Grand-Perret, T.; Ajakane, M.; Baudet, V.; Boissin, P.; Boursier, E.; Loriolle, F.; et al., The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C. *J Biol Chem* **1991**, 266, 15771-81.

3. Hers, I.; Tavare, J. M.; Denton, R. M., The protein kinase C inhibitors bisindolylmaleimide I (GF 109203x) and IX (Ro 31-8220) are potent inhibitors of glycogen synthase kinase-3 activity. *FEBS Lett* **1999**, 460, 433-6.

4. Coultrap, S. J.; Sun, H.; Tenner, T. E., Jr.; Machu, T. K., Competitive antagonism of the mouse 5-hydroxytryptamine3 receptor by bisindolylmaleimide I, a "selective" protein kinase C inhibitor. *J Pharmacol Exp Ther* **1999**, 290, 76-82.

5. Davis, P. D.; Hill, C. H.; Lawton, G.; Nixon, J. S.; Wilkinson, S. E.; Hurst, S. A.; Keech, E.; Turner, S. E., Inhibitors of protein kinase C. 1. 2,3-Bisarylmaleimides. *J Med Chem* **1992**, 35, 177-84.

6. Robey, R. W.; Shukla, S.; Steadman, K.; Obrzut, T.; Finley, E. M.; Ambudkar, S. V.; Bates, S. E., Inhibition of ABCG2-mediated transport by protein kinase inhibitors with a bisindolylmaleimide or indolocarbazole structure. *Mol Cancer Ther* **2007**, 6, 1877-85.

7. Mayati, A.; Bruyere, A.; Moreau, A.; Jouan, E.; Denizot, C.; Parmentier, Y.; Fardel, O., Protein Kinase C-Independent Inhibition of Organic Cation Transporter 1 Activity by the Bisindolylmaleimide Ro 31-8220. *PLoS One* **2015**, 10, e0144667.

8. Deane, F. M.; Lin, A. J. S.; Hains, P. G.; Pilgrim, S. L.; Robinson, P. J.; McCluskey, A., FD5180, a Novel Protein Kinase Affinity Probe, and the Effect of Bead Loading on Protein Kinase Identification. *ACS Omega* **2017**, 2, 3828-3838.

9. Birchall, A. M.; Bishop, J.; Bradshaw, D.; Cline, A.; Coffey, J.; Elliott, L. H.; Gibson, V. M.; Greenham, A.; Hallam, T. J.; Harris, W.; et al., Ro 32-0432, a selective and orally active inhibitor of protein kinase C prevents T-cell activation. *J Pharmacol Exp Ther* **1994**, 268, 922-9.

10. Bit, R. A.; Davis, P. D.; Elliott, L. H.; Harris, W.; Hill, C. H.; Keech, E.; Kumar, H.; Lawton, G.; Maw, A.; Nixon, J. S.; et al., Inhibitors of protein kinase C. 3. Potent and highly selective bisindolylmaleimides by conformational restriction. *J Med Chem* **1993**, 36, 21-9.

11. Dikici, E.; Deo, S. K.; Daunert, S., Drug detection based on the conformational changes of calmodulin and the fluorescence of its enhanced green fluorescent protein fusion partner. *Analytica Chimica Acta* **2003**, 500, 237-245.

12. Douglass, P. M.; Salins, L. L. E.; Dikici, E.; Daunert, S., Class-selective drug detection: Fluorescently-labeled calmodulin as the biorecognition element for phenothiazines and tricyclic antidepressants. *Bioconjugate Chem* **2002**, 13, 1186-1192.

13. Gietzen, K.; Wuthrich, A.; Bader, H., Effects of microtubular inhibitors on plasma membrane calmodulin-dependent Ca2+-transport ATPase. *Mol Pharmacol* **1982**, 22, 413-20.

14. Watanabe, K.; West, W. L., Calmodulin, activated cyclic nucleotide phosphodiesterase, microtubules, and vinca alkaloids. *Fed Proc* **1982**, 41, 2292-9.

15. Campagna-Slater, V.; Therrien, E.; Weill, N.; Moitessier, N., Methods for docking small molecules to macromolecules: a user's perspective. 2. Applications. *Curr Pharm Des* **2014**, 20, 3360-72.

16. Gonzalez-Andrade, M.; Rodriguez-Sotres, R.; Madariaga-Mazon, A.; Rivera-Chavez, J.; Mata, R.; Sosa-Peinado, A.; Pozo-Yauner, L. D.; Arias, O., II, Insights into molecular interactions between CaM and its inhibitors from molecular dynamics simulations and experimental data. *J Biomol Struct Dyn* **2015**, 1-14.

17. González-Andrade, M.; Figueroa, M.; Rodríguez-Sotres, R.; Mata, R.; Sosa-Peinado, A., An alternative assay to discover potential calmodulin inhibitors using a human fluorophore-labeled CaM protein. *Anal. Biochem.* **2009**, 387, 64-70.

18. Vertessy, B. G.; Harmat, V.; Bocskei, Z.; Naray-Szabo, G.; Orosz, F.; Ovadi, J., Simultaneous binding of drugs with different chemical structures to Ca2+-calmodulin: crystallographic and spectroscopic studies. *Biochemistry* **1998**, 37, 15300-10.

19. Chattopadhyaya, R.; Meador, W. E.; Means, A. R.; Quiocho, F. A., Calmodulin structure refined at 1.7 A resolution. *Journal of molecular biology* **1992**, 228, 1177-92.

20. Wang, J.; Wang, W.; Kollman, P. A.; Case, D. A., Automatic atom type and bond type perception in molecular mechanical calculations. *Journal of molecular graphics & modelling* **2006**, 25, 247-60.

21. Wedemeyer, W. J.; Baker, D., Efficient minimization of angle-dependent potentials for polypeptides in internal coordinates. *Proteins* **2003**, 53, 262-72.

22. Morris, G. M.; Goodsell, D. S.; Halliday, R. S.; Huey, R.; Hart, W. E.; Belew, R. K.; Olson, A. J., Automated docking using a Lamarckian genetic algorithm and an empirical binding free energy function. *Journal of Computational Chemistry* **1998**, 19, 1639-1662.

23. Huey, R.; Morris, G. M.; Olson, A. J.; Goodsell, D. S., A semiempirical free energy force field with charge-based desolvation. *J Comput Chem* **2007**, 28, 1145-52.

24. Moitessier, N.; Henry, C.; Maigret, B.; Chapleur, Y., Combining pharmacophore search, automated docking, and molecular dynamics simulations as a novel strategy for flexible docking. Proof of concept: docking of arginine-glycine-aspartic acid-like compounds into the alphavbeta3 binding site. *J Med Chem* **2004**, 47, 4178-87.

25. DeLano, W. L., Use of PYMOL as a communications tool for molecular science. *Abstracts of Papers of the American Chemical Society* **2004**, 228, U313-U314.

26. D.A. Case, J. T. B., R.M. Betz, D.S. Cerutti, T.E. Cheatham, III, T.A. Darden, R.E. Duke, T.J. Giese, H. Gohlke, A.W. Goetz, N. Homeyer, S. Izadi, P. Janowski, J. Kaus, A. Kovalenko, T.S. Lee, S. LeGrand, P. Li, T. Luchko, R. Luo, B. Madej, K.M. Merz, G. Monard, P. Needham, H. Nguyen, H.T. Nguyen, I. Omelyan, A. Onufriev, D.R. Roe, A. Roitberg, R. Salomon-Ferrer, C.L. Simmerling, W. Smith, J. Swails, R.C. Walker, J. Wang, R.M. Wolf, X. Wu, D.M. York and P.A. Kollman *AMBER 2015*, 2015.

27. D.A. Case, T. A. D., T.E. Cheatham, III, C.L. Simmerling, J. Wang, R.E. Duke, R. Luo, R.C. Walker, W. Zhang, K.M. Merz, B. Roberts, S. Hayik, A. Roitberg, G. Seabra, J. Swails, A.W. Goetz, I. Kolossvai, K.F. Wong, F. Paesani, J. Vanicek, R.M. Wolf, J. Liu, X. Wu, S.R. Brozell, T. Steinbrecher, H. Gohlke, Q. Cai, X. Ye, J. Wang, M.-J. Hsieh, G. Cui, D.R. Roe, D.H. Mathews, M.G. Seetin, R. Salomon-Ferrer, C. Sagui, V. Babin, T. Luchko, S. Gusarov, A. Kovalenko, and P.A. Kollman, In; University of California, San Francisco, 2012.

28. Case, D. A.; Cheatham, T. E., 3rd; Darden, T.; Gohlke, H.; Luo, R.; Merz, K. M., Jr.; Onufriev, A.; Simmerling, C.; Wang, B.; Woods, R. J., The Amber biomolecular simulation programs. *Journal of Computational Chemistry* **2005**, 26, 1668-88.

29. Walker, R. C.; Crowley, M. F.; Case, D. A., The implementation of a fast and accurate QM/MM potential method in Amber. *Journal of Computational Chemistry* **2008**, 29, 1019-31.

30. Lindorff-Larsen, K.; Piana, S.; Palmo, K.; Maragakis, P.; Klepeis, J. L.; Dror, R. O.; Shaw, D. E., Improved side-chain torsion potentials for the Amber ff99SB protein force field. *Proteins* **2010**, 78, 1950-8.

31. Thomas, A. S.; Mao, S.; Elcock, A. H., Flexibility of the bacterial chaperone trigger factor in microsecond-timescale molecular dynamics simulations. *Biophysical journal* **2013**, 105, 732-44.

32. Wang, J.; Wolf, R. M.; Caldwell, J. W.; Kollman, P. A.; Case, D. A., Development and testing of a general amber force field. *Journal of Computational Chemistry* **2004**, 25, 1157-74.

33. Salomon-Ferrer, R.; Gotz, A. W.; Poole, D.; Le Grand, S.; Walker, R. C., Routine Microsecond Molecular Dynamics Simulations with AMBER on GPUs. 2. Explicit Solvent Particle Mesh Ewald. *J Chem Theory Comput* **2013**, 9, 3878-88.

34. Treesuwan, W.; Hannongbua, S., Bridge water mediates nevirapine binding to wild type and Y181C HIV-1 reverse transcriptase--evidence from molecular dynamics simulations and MM-PBSA calculations. *Journal of molecular graphics & modelling* **2009**, 27, 921-9.

35. Zhou, Z.; Madura, J. D., Relative free energy of binding and binding mode calculations of HIV-1 RT inhibitors based on dock-MM-PB/GS. *Proteins* **2004**, 57, 493-503.

36. Kollman, P. A.; Massova, I.; Reyes, C.; Kuhn, B.; Huo, S.; Chong, L.; Lee, M.; Lee, T.; Duan, Y.; Wang, W.; Donini, O.; Cieplak, P.; Srinivasan, J.; Case, D. A.; Cheatham, T. E., 3rd, Calculating structures and free energies of complex molecules: combining molecular mechanics and continuum models. *Accounts of chemical research* **2000**, 33, 889-97.

37. Hou, T.; Wang, J.; Li, Y.; Wang, W., Assessing the performance of the MM/PBSA and MM/GBSA methods. 1. The accuracy of binding free energy calculations based on molecular dynamics simulations. *Journal of chemical information and modeling* **2011**, 51, 69-82.

**Figure and Table supplementary**



**Figure 1S**. Fluorescence spectra of Ca+2-*h*CaM M124C-*mBBr* adding known concentrations of DOP and AMA.

**Figure 2S**. Progress of the molecular dynamics simulation of the 4Ca2+-CaM-CPZ complex. Total energy of the system vs time. The steps of the simulation comprise 50 ps of heating, 50 ps of density, 500 ps of equilibrium, and 100 ns of molecular dynamics.



**Figure 3S**. Tendency of the thermodynamic parameters of the system CaM-CPZ complex to different time. G, H, and S.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Table 1S. Analysis of binding free energies of CaM-CPZ complex calculated by molecular dynamic at different time.** | | | | | | | |
| **DM studies** | | | | | | | |
| **Time (ns)** | **Evdw**  **(kcal mol-1)** | **Eele**  **(kcal mol-1)** | **GPB**  **(kcal mol-1)** | **GSA**  **(kcal mol-1)** | ***H***  **(kcal mol-1)** | *****S***  **(kcal mol-1)** | **Gcal**  **(kcal mol-1)** |
| 10 | -31.2±2.7 | -3.3±1.8 | 10.6±3.9 | -4.3±0.3 | -28.2±2.8 | -18.1±2.4 | -10.1±3.2 |
| 20 | -33.0±3.1 | -4.4±1.1 | 12.0±3.4 | -4.5±0.4 | -29.8±3.2 | -17.2±2.0 | -12.6±2.9 |
| 30 | -35.0±4.2 | -5.0±1.0 | 12.5±3.1 | -4.2±0.5 | -32.3±4.7 | -18.6±3.2 | -13.6±4.9 |
| 40 | -36.3±4.4 | -5.4±0.9 | 13.1±3.0 | -4.9±0.5 | -32.6±4.9 | -18.9±2.7 | -13.6±3.7 |
| 50 | -36.7±4.2 | -5.7±0.7 | 13.2±2.8 | -5.0±0.5 | -34.3±4.7 | -21.5±3.5 | -12.8±5.0 |
| 60 | -36.8±3.9 | -5.7±0.6 | 13.2±2.7 | -5.0±0.5 | -34.3±4.4 | -18.1±3.4 | -16.2±4.8 |
| 70 | -37.2±3.8 | -5.3±0.7 | 13.0±2.6 | -5.1±0.4 | -34.6±4.2 | -22.1±1.9 | -12.4±3.5 |
| 80 | -37.2±3.7 | -4.5±1.4 | 12.4±3.1 | -5.1±0.4 | -34.4±4.0 | -18.4±0.2 | -15.9±2.2 |
| 90 | -37.4±3.6 | -3.9±1.7 | 12.0±3.2 | -5.1±0.4 | -34.4±3.9 | -21.0±6.7 | -13.4±3.4 |
| 100 | -37.4±3.5 | -3.4±1.9 | 11.5±3.3 | -5.1±0.4 | -34.4±3.7 | -19.4±5.0 | -14.9±5.9 |

**Movie 1 1a29-CPZ.** Trajectory of the MD of the 4Ca2+-CaM-CPZ complex

**Movie 2 1a29-FLU.** Trajectory of the MD of the 4Ca2+-CaM-FLU complex

**Movie 3 1a29-IMI.** Trajectory of the MD of the 4Ca2+-CaM-IMI complex

**Movie 4 1a29-VBT.** Trajectory of the MD of the 4Ca2+-CaM-VBT complex

**Movie 5 1a29-VCT.** Trajectory of the MD of the 4Ca2+-CaM-VCT complex

**Movie 6 1a29-5HT.** Trajectory of the MD of the 4Ca2+-CaM-5HT complex

**Movie 7 1a29-AMA.** Trajectory of the MD of the 4Ca2+-CaM-AMA complex

**Movie 8 1a29-DOP.** Trajectory of the MD of the 4Ca2+-CaM-DOP complex