Bisindolylmaleimides new inhibitors of CaM protein

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**ABSTRACT**

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

las interacciones a nivel molecular de una serie de compuestos denominados Bisindolilmaleimida, como inhibidores de la proteína CaM. Los compuestos Bisindolilmaleimida son prototipos de fármaco derivado de la Estaurosporina, la cual es un alcaloide con actividad para el tratamiento del cáncer. Los compuestos II, IV, VII, X y XI de Bisindolilmaleimida son propuestos y reportados como nuevos inhibidores de la proteína calmodulina por primera vez. Para lo anterior se utilizaron herramientas de modelaje molecular (Acoplamiento molecular y Dinámicas Moleculares) para realizar los estudios teóricos y un dispositivo biotecnológico (un biosensor fluorescente de la proteína CaM -hCaM M124C-mBBr), para determinar la unión experimental de estos compuestos. Los resultados obtenidos nos indican que esta serie de compuestos unen a la proteína calmodulina con una afinidad entre 193-248 nM, una orden de magnitud menor que la mayoría de los inhibidores clásicos. Por otro, los estudios teóricos respaldan los resultados experimentales y nos aportan detalles moleculares complementarios de la interacción entre la proteína y la serie BIS. Se concluye que estos compuestos pueden ser excelentes prototipos para ser considerarlos nuevos inhibidores de la calmodulina.

**Keywords**: Biosensors; Calmodulin; Bisindolylmaleimides; 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, except BIM V (Figure 1). This series includes BIM from I to XI, and 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 a 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 the uptake of marketed drugs in the liver.[7](#_ENREF_7) BIM IX is a potent inhibitor 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)

CaM is a ubiquitous protein, highly conserved, and considered the main calcium signaling protein in eukaryotic cells. This protein controls various physiological processes such as cell motility, cytoskeleton architecture and function, cell proliferation, apoptosis, autophagy, ion transport, osmotic control, protein phosphorylation/dephosphorylation, reproductive processes, muscle contraction, and gene expression [11](#_ENREF_11), [12](#_ENREF_12). CaM is a small, acidic protein comprising of 148 amino acids (16.7 kDa) [13](#_ENREF_13).

A direct detection tool developed by our group is the calmodulin biosensor (*h*CaM-M124C-*mBBr*), which we have used to detect the binding of various ligands [12](#_ENREF_12), [14-18](#_ENREF_14). On the other hand, theoretical computational studies such as Molecular Dynamics (MD) [19](#_ENREF_19),



**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**





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**Figure 3.** Fluorescence spectra and titration curves of Ca+2-*h*CaM M124C-*mBBr*

|  |  |  |
| --- | --- | --- |
| **Table 1. Experimental binding properties of Ca2+-CaM-Ligands complexes.** | | |
|  | **Experimental studies** | |
| **Complex** | ***K*d1**  **(nM)** | **stoichiometry** |
|  |  |  |
| Ca2+-CaM -**BIS-II** | 257.8 ± 5.5 | 2.5 ± 0.2 |
| Ca2+-CaM -**BIS-IV** | 223.8 ± 3.7 | 3.7 ± 0.2 |
| Ca2+-CaM -**BIS-VII** | 186.2 ± 4.1 | 4.4 ± 0.1 |
| Ca2+-CaM -**BIS-X** | 205.2 ± 3.8 | 4.3 ± 0.1 |
| Ca2+-CaM -**BIS-XI** | 239.0 ± 5.0 | 3.4 ± 0.1 |
| Ca2+-CaM -**CPZ** | 492.2 ± 4.6 | 3.4 ± 0.1 |
|  |  |  |

*Docking*

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Table 1. Theoretical binding properties of Ca2+-CaM-Ligands complexes.** | | | | | |
|  | **Docking studies** | | | | |
| **Complex** | **i2 (nM)** | | | |
| Site | 1 | 2 | 3 | 4 |
| Ca2+-CaM -**BIS-II** | 109.79 | 161.58 |  |  |
| Ca2+-CaM -**BIS-IV** | 1710 | 3390 | 7450 |  |
| Ca2+-CaM -**BIS-VII** | 2.14 | 26.95 | 52.43 | 263.87 |
| Ca2+-CaM -**BIS-X** | 20.36 | 59.39 | 71.65 | 217.1 |
| Ca2+-CaM -**BIS-XI** | 9.66 | 55.96 | 75.12 | 191.71 |
| Ca2+-CaM -**CPZ** | 715.65 | 1169 | 1640 |  |
|  |  |  |  |  |
|  | | | | | |

*Molecular Dynamics Simulations*



**R2=0.703**

**Figure 5**. Correlations between experimental and theoretical data on Ca2+-CaM-Drug interactions. A) GExp 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.

**3. Conclusions**

**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*. [18](#_ENREF_18). 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 [20](#_ENREF_20), [21](#_ENREF_21). 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 [22](#_ENREF_22).

*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.[23](#_ENREF_23) 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/>) [24-26](#_ENREF_24), 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 [27](#_ENREF_27).

*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 [28-30](#_ENREF_28). 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 [29](#_ENREF_29), [31](#_ENREF_31). Amber f99SB force field [30](#_ENREF_30), [32](#_ENREF_32), [33](#_ENREF_33) parameters were used for all residues and Gaff force field [22](#_ENREF_22), [34](#_ENREF_34) 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 [35](#_ENREF_35). 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 [36](#_ENREF_36), [37](#_ENREF_37), 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 [36](#_ENREF_36), [38](#_ENREF_38), [39](#_ENREF_39).

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/>).

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**Conflict of interest**

The authors declare no any conflict of interest.

**Supplementary data**

Supplementary data related to this article can be found at:

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