Vasorelaxant effect of flavonoids through calmodulin inhibition: Ex vivo, in vitro, and in silico approaches

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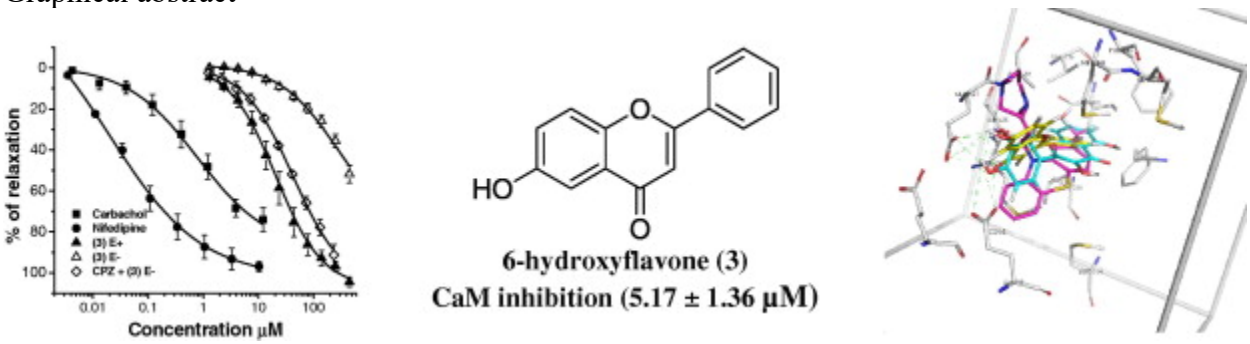
Abstract:

In our search for potential antihypertensive agents, a series of structurally-related flavonoids was screened. Ex vivo and in vitro biological evaluations indicated that compounds **1**–**7** displayed an important vasorelaxant effect on the endothelium-intact (E^+) and -denuded (E^-) aortic rings test. Their in vitro anti-calmodulin (CaM) properties were determined by means of the inhibitory effect on the activation of the calmodulin-sensitive *c*AMP phosphodiesterase (PDE1) assay. Molecular modeling experiments were also performed in order to explore the probable binding site of **1**–**7** with CaM, and the results indicated that they could bind to the protein in the same pocket as trifluoperazine (TFP), a well-known CaM inhibitor.

Graphical abstract



Keywords: Flavonoids | Vasorelaxant effect | Calmodulin inhibition | Docking

Article:

1. Introduction

Hypertension, one of most common cardiovascular diseases, is defined as repeatedly elevated systolic and/or diastolic blood pressure above 140/90 mm Hg.[2] Several antihypertensive drugs used in the treatment of this disease, including diuretics, sympatholytics, vasodilators, and calcium channel and angiotensin blockers; accomplish their activity through four main effectors sites: resistance and capacitance vessels, heart, and kidney.[3] In addition, compounds with significant activity on smooth muscle cells might also act as direct vasodilator agents by means of the production of some second messengers, for example, nitric oxide (NO), cyclic guanosine monophosphate (*c*GMP), prostacyclin PGI_2, or cyclic adenosine monophosphate (*c*AMP). Finally, the protein calmodulin (CaM), a major cellular Ca^{2+}-binding protein which regulates the activity of a series of CaM-dependent enzymes such as NO synthases, phosphodiesterases (PDE's), adenylate cyclases, phosphatases, several kinases, ion channels, calcium-ATPase pumps, among others, represent an important molecular target for the development of new leads that could be used as antihypertensive agents.[4, 5, 6, 7]

Flavonoids are a diverse group of secondary metabolites well-known for having a range of human health-promoting activities such as antiinflammatory, antioxidant, vasodilating, antiallergenic, antiviral, antidiabetic, antidiabetogenic, and anticancer properties.[8, 9, 10, 11, 12] However, this group of compounds has never been studied or used in the therapeutic for the treatment of hypertension.

In an attempt to find novel antihypertensive drugs from medicinal plants, here we report the ex vivo and in vitro vasorelaxant effect of a series of structurally-related flavonoids, using the aorta rat rings pre-contracted model. In addition, to provide detailed information about the possible mechanism of action of these metabolites through the Ca^{2+}-CaM complex inhibition, a series of in vitro enzymatic and in silico experiments were conducted.

2. Results and discussion

A series of seven known flavonoids, flavone (**1**), 3-hydroxyflavone (**2**), 6-hydroxyflavone (**3**), 7-hydroxyflavone (**4**), chrysin (**5**), quercetin (**6**), and naringenin (**7**) (Fig. 1), were subjected to a series of ex vivo and in vitro vasorelaxant and CaM-Ca^{2+}-PDE assays, as well as in silico docking experiments. Flavonoids **1**−**6** showed a significant vasorelaxant activity, in a concentration-dependent manner, on the contraction activity induced by noradrenaline (0.1 μM, NA) (Fig. 2). Compounds **1**, **3**, and **4** were the most potent, and revealed a partial endothelium-dependent effect (Fig. 2 and Table 1). The latter suggests that endothelium-derived factors such as NO or prostacyclin PGI_2 are involved on the activity of these compounds.[11] In contrast, an endothelium-independent relaxation is related with a smooth muscle cells activity, which interferes on contraction processes such as α-adrenoceptors antagonism, calcium channel blockade, potassium channel opening, *c*AMP or *c*GMP increment, or Ca^{2+}-CaM complex activity inhibition.[7, 13]

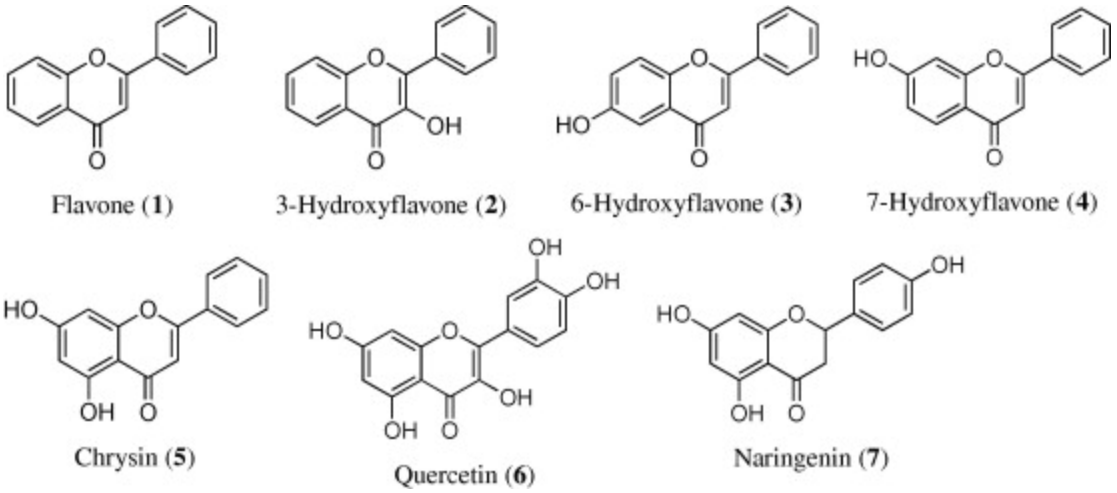


Figure 1. Structurally-related flavonoids studied.

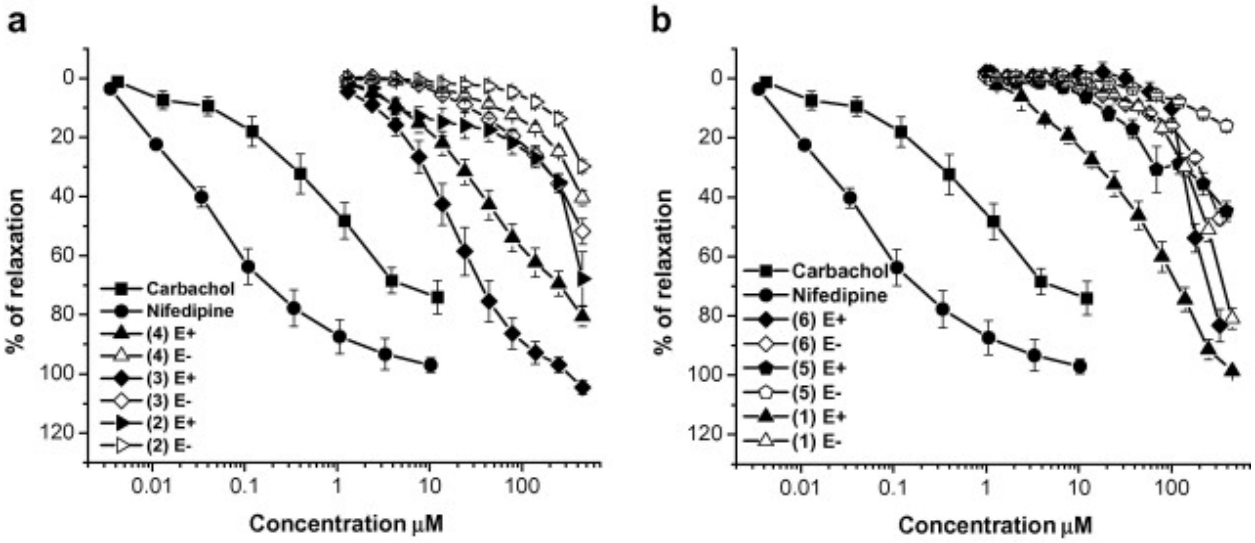


Figure 2. Concentration-dependent vasorelaxant effect of compounds **1**–**7** on the endothelium-intact (E^+) or -denuded (E^-) aortic rings test.

In previous works, Ajay and co-workers described that compounds **1** and **6** showed an endothelium-dependent vasorelaxant effect, with an NO and prostacyclin PGI_2 increase production.[14] Also, compound **7** induced vasorelaxant effect through NO production, PDE inhibition, and K^+ channel opening.[14, 15, 16, 17] On the other hand, Calderone and co-workers demonstrated that compounds **3** and **4** induced a vasorelaxant effect, in a concentration-dependent manner, on an endothelium-independent model, attributed to a K^+ channel opening.[18] In this context, compound **3**, the most active compound in the functional vasorelaxant assay, was subjected to a series of experiments to determine its mechanism of action. The pre-incubation of endothelium-denuded aortic rings with chlorpromazine (CPZ), a well-known CaM inhibitor used as a positive control, significantly increase the activity of **3**, which is showed as a left shift in the vasorelaxant effect curves (Fig. 3). This effect suggests a possible Ca^{2+}-CaM complex inhibition by **3**, and is consistent with the significant endothelium-independent relaxation on aortic rings pre-contracted with NA, previously observed by hesperidin, which inhibited the formation of the

complex CaM-Ca^{2+}-PDE1 and -PDE4 isolated from bovine aorta with IC_{50} values of 74 and 70 μM, respectively.[7]

Table 1. Vasorelaxant effect and anti-CaM properties of compounds **1**–**7**

Compound	Ex vivo vasorelaxant effect				Ca^{2+}-CaM-PDE1 inhibition	
	IC_{50} (μM)		E_{max} relaxation (%)		IC_{50} (μM)[c]	Potency[d]
	Cambiar por E^{+a}	Cambiar por E^{-b}	E^{+a}	E^{-b}		
1	46.00	203.00	98.64	80.97	102.28 ± 26.90	0.34[e]
2	203.60	248.30	67.77	29.80	55.20 ± 16.87	0.36[f]
3	20.80	112.37	104.57	51.84	5.17 ± 1.36	3.89[f]
4	37.80	167.04	80.59	40.39	37.10 ± 5.74	0.54[f]
5	75.70	106.90	44.75	16.00	33.43 ± 6.46	1.03[e]
6	162.50	222.47	83.23	43.18	12.97 ± 1.35	2.82[e]
7	ND[g]	ND[g]	ND[g]	ND[g]	22.01 ± 3.02	0.91[f]
Carbachol[h]	0.79	ND[g]	88.62	ND[g]	ND[g]	ND[g]
Nifedipine[i]	ND[g]	0.02	ND[g]	102.17	ND[g]	ND[g]

[a] E^+: endothelium-intact aortic rings.
[b] E^-: endothelium-denuded aortic rings.
[c] Values as means ± SEM, $n = 6$, $p < 0.05$.
[d] Potency was obtained by the formula: IC_{50} (μM) CPZ/IC_{50} (μM) compound, assuming a value of 1.00 for CPZ.
[e] IC_{50} μM of CPZ = 34.57 ± 1.59.
[f] IC_{50} μM of CPZ = 20.1 ± 3.13.
[g] ND: Not determined.
[h] Positive control for experiments with E^+.
[i] Positive control for experiments with E^-.

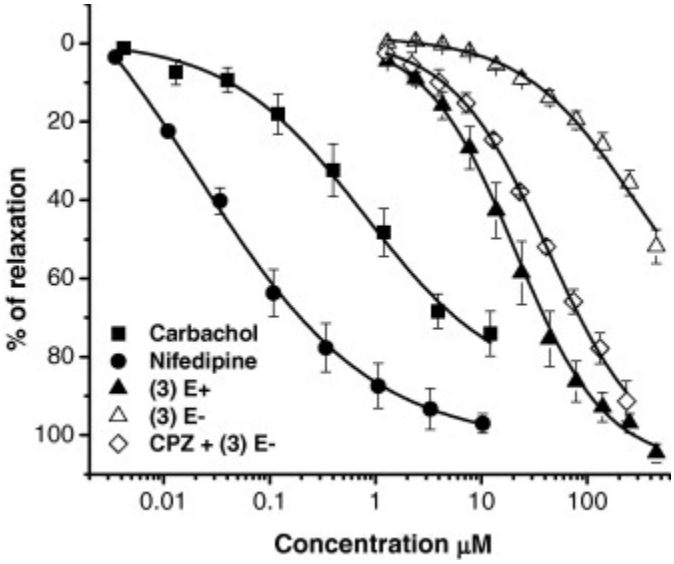


Figure 3. Concentration-dependent vasorelaxant effect of compound **3** on the endothelium-intact (E^+) or -denuded (E^-) aortic rings pretreated with CPZ (1 μM).

Thus, the effect of **1**–**7** on a human recombinant-CaM was initially assessed in vitro, using the CaM-sensitive *c*AMP phosphodiesterase (PDE1) as a monitoring enzyme.[19, 20] This functional experiment is commonly employed to detect CaM antagonists.[20, 21, 22] The results showed in Table 1 revealed that all flavonoids inhibited the activation of PDE1 in a concentration-dependent manner; compounds **3** (IC_{50} = 5.17 ± 1.36 μM) and **6** (IC_{50} = 12.97 ± 1.35 μM) were

the most active, while **5** (IC_{50} = 33.43 ± 6.46 μM) and **7** (IC_{50} = 22.01 ± 3.02 μM), displayed a moderate activity comparable to that of CPZ.

CaM binding sites has been subjected to several in silico studies. Recently, Li and collaborators reported the interaction of the phosphorylated flavonoid chrysin with CaM using docking and molecular dynamic analyses.[23] Two different favorable binding modes were observed; the most favored binding pocket consisted of the amino acids Ala57, Asp58, Pro66, Glu67, Thr70, Arg74, Met76, Lys77, Asp78, Thr79, Asp80, and Glu82, and the molecule was tethered to the CaM by several hydrogen bonds. The putative interaction of several xanthones and alkaloids has also been studied, and the binding sites predicted were close-related to those for the classical CaM inhibitors trifluoperazine (TFP) and CPZ.[19, 20, 24, 25] In this context, in order to gain better understanding of the interaction of flavonoids **1**–**7** with CaM, molecular docking studies were performed. Briefly, the validated docking protocol employed adequately predicts the binding mode of TFP, which was removed from the active site and docked back into pocket, in the conformation found in its CaM crystal structure (Protein Data Bank code: 1A29).[20, 23] CPZ docking was also validated.

For compounds **1**–**7**, the best docking pose revealed a well fitting into the same pocket of TFP and CPZ, the active cavity of CaM (Fig. 4a). The calculated free binding energies values (EFEB) for **1**−**7**, are in the range of −5.5 and −7.0 kcal/mol (Table 2), and are comparable with those of CPZ (−6.9 kcal/mol), but higher than TFP (−10.5 kcal/mol). In addition, the following preliminary structure-in silico activity could be done: (a) compounds bearing substituent at position C-3 of the C ring of the flavone core have the lowest calculated free energies (**2** and **6**); (b) compounds bearing one or none substituent at positions 3, 5 or 7 displayed similar free energies (**1**–**4**), having **4** the relatively lowest free energy; and (c) compounds having two hydroxyl groups at positions 5 and 7 in the A ring displayed similar free energies (**5** and **7**). For instance **3** and **6**, with IC_{50} values of 5.17 ± 1.36 and 12.97 ± 1.35 μM, respectively, exhibited the lowest free energy values of −6.24 and −7.01 kcal/mol, respectively, being both compounds the most potent. Besides the fact that the lipophilic and hydrophilic residues in this active site are one of the most important characteristics in favor to their electrostatic interaction with the receptor, and, as shown in Figure 4b, compounds **3** and **6** are inserted into a pocket surrounded by the residues Glu7, Glu11, Phe92, Ile100, Leu105, Met109, Met124, Ile125, Glu127, Val136, Phe141, Met144, Met145, and Ala128. In addition, hydrogen bonds have been also formed with the receptor at Glu7, Glu11, and Glu127 (<5 Å distance). Finally, hydrophobic π–π interaction are also seen between the dihydroxyphenyl or aromatic ring of **3** and **6**, with Phe92 and Phe141 residues (Fig. 4b), as well as the hydrophobic interactions with the Met residues. In overall, the same behavior has been demonstrated for the classical drugs TPZ and CPZ.

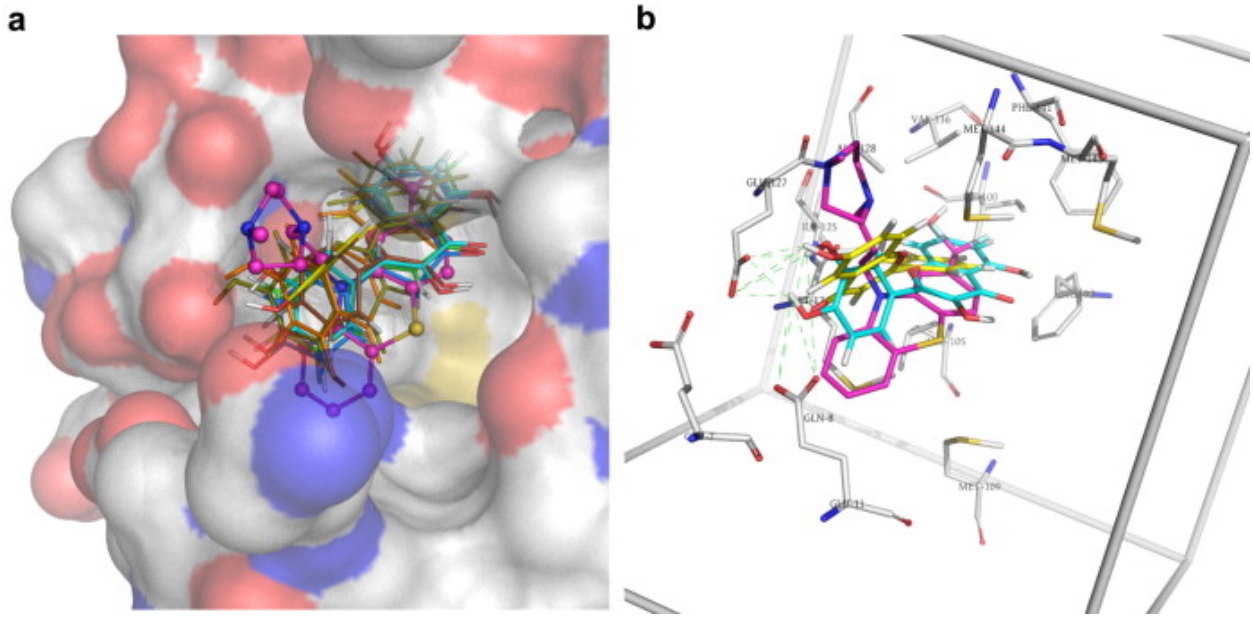


Figure 4. (a) Best docking score conformations of compounds **1**–**7** (green, brown, yellow, olive, orange, cyan, and blue, respectively, sticks) into CaM (surface). TFP X-ray crystal pose is shown as balls and sticks (magenta). (b) Residues involved in the interaction of compounds **3** (yellow) and **6** (cyan) with CaM (carbon atoms in grey, oxygen in red, nitrogen in blue, and sulfur in pale yellow sticks). Dashed green lines are the intermolecular hydrogen (H)-bonds. Images were created by PyMol l.3.

Table 2. Calculated free binding energies (EFEB) with autodock 4.0 for compounds **1**–**7**, CPZ and TFP

Compound	EFEB (kcal/mol)
1	−6.08
2	−6.07
3	−6.24
4	−6.08
5	−5.74
6	−7.01
7	−5.55
TFP	−10.49
CPZ	−6.90

3. Conclusions

In summary, a series of structurally-related flavonoids studied in the present investigation showed an important vasorelaxant effect on the endothelium-denuded aortic rings test. Their anti-CaM properties demonstrated by both ex vivo and in vitro experiments, were confirmed by the in silico analysis, allowed us to establish that this category of secondary metabolites have a potential for further development as novel antihypertensive agents.

4. Experimental

4.1. Chemicals

All chemicals were ACS grade; flavone (**1**), 3-hydroxyflavone (**2**), 6-hydroxyflavone (**3**), 7-hydroxyflavone (**4**), chrysin (**5**), quercetin (**6**), and naringenin (**7**) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). For the ex vivo and in vitro experiments all compounds were dissolved with DMSO, and then diluted with distilled water.

4.2. Animals

All the animals were conducted according to the Mexican Official Norm for Animal Care and Handing (NOM-062-ZOO-1999) and in compliance with International Guidelines on Care and Use of Laboratory Animals. Furthermore, clearance for conducting the studies was taken from the Ethics Committee for the Use of Animals in Pharmacological and Toxicological Testing. Wistar rats weighing between 200 and 300 g were fed a standard rodent diet *ad libitum* with free access to water and maintained under standard laboratory conditions (12 h light/dark cycle, 25 °C and humidity of 45–65%).

4.3. Rat aorta ring assay

The vasorelaxant activity was performed using a modified standard protocol of Saponara and co-workers.[16] All animals were sacrificed by cervical dislocation and the thoracic aorta was removed, cleaned, and cut in about 3–5 mm length rings. In addition, for some aortic rings the endothelium layer was removed by manual procedures. Then, each piece of tissue was suspended in a tissue chamber containing Krebs solution at 37 °C, continuously gassed with O_2/CO_2 (9:1). Tissues were placed under a resting tension of 3.0 g and allowed to stabilize for 60 min. The contractions were recorded with an isometrical force transducer Grass FT 03 (Astromed, West Warwick, RI), connected to a MP100 Manager Biopac System polygraph (Biopac Instruments, Santa Barbara, CA). After the stabilization period the tissues were stimulated with NA (0.1 μM) during 10 min and they were washed with fresh Krebs solution. This procedure was repeated three times at 30 min intervals before starting the experiments. The absence or presence of endothelium layer was confirmed by the lack of the relaxant response induced by carbachol (1 μM) in the last contraction to assess viability. Finally, all tissues were contracted with NA and test samples (pure compounds or positive control) were added to the bath in quarter-log cumulative concentrations (evaluation period). The relaxant effect of the samples was determined by its ability to induce a maximal vascular contraction before and after their addition.

Next, in order to obtain evidence of the vasorelaxant mode of action exerted by compound **3**, an additional analysis with CPZ, a well know CaM inhibitor, was assessed using 1 μM of CPZ in the presence or absence of **3**.

4.4. Phosphodiesterase activity

Phosphodiesterase activity was measured according to the method described by Figueroa and co-workers.[20] CaM (0.08 μg) was incubated with 0.015 units of PDE1 from bovine brain during 30 min in 40 μL of assay solution containing 0.063 units of 5′-nucleotidase (*Crotalus atrox* venom from Sigma), 45 mM Tris–HCl, 5.6 mM magnesium acetate, 45 mM imidazole, 2.5 mM calcium chloride, and 10 μM BSA, pH 7.0. Test compounds were then added to the

assay medium at 0.5, 1, 2, 3, 4, 7, 13, 20, 32, 50, and 65 μM in ACN/water (1:1), and the samples incubated during 30 min; thereafter 10 μL of 10.8 mM *c*AMP were added to start the assay. After 15 min, the assay was stopped by the addition of 190 μL of malachite green solution. The amount of inorganic phosphate released, measured spectrophotometrically at 700 nm, correlated with the activity of the PDE1. All the results are expressed as the mean of at least six experiments ± SEM. The IC_{50} (concentration inhibiting by 50% the activity of the enzyme) values were determined by non-linear regression analysis by fitting to hyperbolic inhibition.

4.5. Molecular docking

Docking calculations for compounds **1**–**7** into the crystal structure of CaM (PDB code: 1A29; refined at 2.74 Å) were performed using the autodock 4.0 program.[23] Ligands structures were constructed using the Spartan 08 software (Wavefunction Inc., Irvine, CA). Hydrogen atoms were added and geometry optimization was performed using the PM3 force field. The Lamarckian genetic algorithm used to simulate the ligand–receptor docking was first validated with the positive controls (TFP and CPZ) dockings of co-crystallized, in both protonated and unprotonated forms. These results were in complete agreement with our previous report (data not shown).[20, 24] Grid dimensions were 60 Å × 60 Å × 60 Å, with a spacing of 0.375 Å between the grid points. The docking protocol applied was the following: initial population size of 100, random starting position and conformation, translation step ranges of 0.2 Å, rotation step ranges of 50 Å, elitism of 0.1, mutation rate of 0.02, crossover rate of 0.8, local search frequency of 0.06, and 2.5 million energy evaluations. All the rotatable bonds of ligands were allowed to rotate during docking simulations. Cluster analysis was performed on the docked results using a root mean square (RMS) deviation tolerance of 2.0 Å. All the docking results were sorted by the lowest binding energy of the most populated cluster using autodock tools,[23] and the top hit from each evaluation was selected for further analysis. Optimization of the docked complexes was done by energy minimization up to a gradient of 0.01 kcal/mol/Å using the PM3 force field, to further refine the autodock output. Binding site residues were selected by a distance for the ligand not higher that 6 Å.

4.6. Statistical and data analysis

Data are expressed as the mean of experiments ± SEM for the number (n = 6) of animals used. Graphics were plotted and experimental data were adjusted by the nonlinear, curve fitting program Microcal® Origin 8.0 (OriginLab, Northampton, MA). Statistical analysis was performed by one way analysis of variance (ANOVA) followed by Duncan's and post-hoc Tukey's tests. p-Values less than 0.05 were considered to be statistically significant.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.10.063. These data include MOL files and InChiKeys of the most important compounds described in this article.

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