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Relaxant effect of 7-ethoxy-4-methyl-2*H*-chromen-2-one by calcium channel blockade: computational and *ex vivo* studies

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ABSTRACT

Current work was conducted in order to determine the underlying mode of relaxant action of 7-ethoxy-4-methyl-2*H*-chromen-2-one (**1**), a coumarin obtained by semisynthesis from umbelliferone (**2**), with significant relaxant effect in a concentration-dependent manner on tracheal rat rings pre-contracted with carbachol (1 µM). In a previous study it was demonstrated that compound **1** and **2** showed significant relaxant effect, being **1** the most active compound (Emax= 100% and EC_{50}= 83 µM) even more active than theophylline [an unspecific phosphodiesterases (PDE's) inhibitor] used as positive control. Moreover, pretreatment with **1** significantly shifted to the right the carbachol-induced contraction. On the other hand, compound **1** (83 µM) produces significant (100%) relaxant effect on the contraction induced by KCl (80 mM) and the $CaCl_2$-induced contraction was significantly reduced by the coumarin **1** as nifedipine does (a L-type calcium channel blocker), used as positive control. Indomethacin (10 µM, unspecific COX inhibitor) significantly reduced **1**-relaxation. Meanwhile, in the presence of isoproterenol (a β-adrenergic agonist), and K^+ channel blockers glibenclamide (10 µM) and 2-AP (100 µM) the relaxant curve was not modified. Compound **1** was docked on an outer cavity located on the extracellular side of the human L-type calcium channel model (affinity energy -6.8 kcal/mol). However, compound **1** was also found on the same location as nifedipine with the same affinity energy (-6.3 kcal/mol) as previously described. Both conformations were stabilized by aliphatic interactions on both binding sites, primordially by a π-π interaction between $F^{IVS6.7}$ and aromatic rings from compound **1**. In conclusion, 7-ethoxy-4-methyl-2*H*-chromen-2-one (**1**) induces a significant relaxant action on rat trachea rings, through L-type calcium channel blockade and, as a second mechanism of action, by a possible intracellular cyclic AMP increasing.

INTRODUCTION

Coumarins are phenolic compounds with a common structure of 2-*H*-1-benzopyran-2-one with multiple biological effects such as anti-inflammatory, anticoagulant, antibacterial, antifungal, antiviral, anticancer, antihypertensive, antitubercular, anticonvulsant, antiadipogenic, antihyperglycemic, antioxidant, and neuroprotective properties (Gouda, 2013; Hoult and Payá, 1996; Khan and Giliani, 2009; Venugopala *et al*., 2013). So, the search for novel molecules based on coumarin scaffold with therapeutic activity represents pharmacological alternatives to treat prevalent diseases such as asthma. In a previous work, our group reported the semisynthesis, *ex vivo* relaxing evaluation and SAR studies of a series of 18 coumarins. The results indicate that 7-ethoxy-4-methyl-2*H*-chromen-2-one (**1**) (Figure 1) was one of the most active compounds, being 2-times more active than theophylline (positive control) (Sánchez-Recillas *et al*., 2013). Moreover, Umbelliferone (**2**) (Figure 1) is a monohydroxylated coumarin that also showed significant pharmacological effects such as antidiabetic, hypolipidemic, anti-inflammatory, anti-allergic, anti-oxidant, and bronchodilator (Hoult and Payá, 1996; Karmase *et al*., 2013; Ramesh and Pugalendi, 2005; Vasconcelos *et al*., 2009). This compound was previously isolated from plant species that are used in traditional medicine as anti-asthmatic (Naeem *et al*., 2012; Ramanitrahasimbola *et al*., 2005). So, current work was designed in order to determine the underlying functional mode of action of compound **1** on tracheal rat rings and, by using Docking studies, to explain its interactions with L-type calcium channel.

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MATERIAL AND METHODS

Chemicals and drugs

Carbamylcholine chloride (carbachol), theophylline, isoproterenol, indomethacin, potassium chloride (KCl), calcium chloride ($CaCl_2$), nifedipine and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). All other reagents were analytical grade from local sources. 7-ethoxy-4-methyl-2*H*-chromen-2-one was previously obtained by semisynthesis from umbelliferone (Sánchez-Recillas *et al* 2013).

Animals

Healthy male Wistar rats (250–300 g) were used and maintained under standard laboratory conditions with free access to food and water. All animal procedures were conducted in accordance with our Federal Regulations for Animal Experimentation and Care (SAGARPA, NOM-062-ZOO-1999, Mexico), and approved by the Institutional Animal Care and Use Committee based on US National Institute of Health publication (No. 85-23, revised 1985). All experiments were carried out using six animals per group. Animals used were euthanized by cervical dislocation.

Rat trachea ring tests

Trachea was removed from healthy male Wistar rats (250-350 g), maintained under standard laboratory conditions with free access to food and water. The rats were sacrificed by exposure to ether and cervical dislocation.

After the trachea was cleaned out of adhering connective tissue it was cut into 3-5mm length rings. Then, tissue segments were mounted by stainless steel hooks, under an optimal tension of 2g, in 10 mL organ baths containing warmed (37 ºC) and oxygenated (O_2:CO_2, 95:5) Krebs solution (composition, mM: NaCl, 118; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 25.0; EDTA, 0.026; glucose, 11.1, pH 7.4). Changes in tension were recorded by Grass-FT03 force transducers (Astromed, West Warwick, RI, USA) connected to a MP100 analyzer (BIOPAC Instruments, Santa Barbara, CA, USA), as previously described (Estrada-Soto *et al*., 2012). After equilibration, rings were contracted by carbachol (1 μM) and washed every 30 min. for 2h. After pre-contraction with carbachol, the test samples (compound **1** and positive control) were added to the bath in a volume of 100 μL; then cumulative concentration-response curves were obtained for each ring. The relaxant effect of test samples were determined by comparing the muscular tone of the contraction before and after the application of the test materials.

In order to establish the underlying mode of action of **1**, the following *ex vivo* experiments were carried out:

- For the interaction with the cholinergic receptors, concentration-response curves (CRC) were obtained with carbachol (0.006-540 μM) after tissue were incubated with **1** (EC_{50}= 83 μM) during 15 min. Carbachol-contractile effect was determinate comparing the contraction induced by carbachol in absence and presence of **1**.
- For interaction with β_2-adrenergic receptor and cAMP increase, tissues were pre-incubated during 15 min with isoproterenol (10 μM; β_2 adrenergic agonist) and maximal relaxing effect of **1** was compared in absence and presence of isoproterenol.
- For interaction with prostaglandins, tissues were pre-incubated during 15 min with Indomethacin (10 μM; cyclooxigenases inhibitor). Maximal relaxing effect of **1** was compared in absence and presence of Indomethacin.
- To establish a possible interaction of **1** with L-type calcium channel blockade, the tracheal rings were pre-contracted with high KCl (80 mM). Once a plateau was attained, concentration–response curves (CRC) of **1**-induced relaxation were obtained by adding cumulative concentrations of compound to the bath.
- To determine whether the inhibition of extracellular Ca^{2+} influx was involved in **1**-induced relaxation, the experiments were carried out in Ca^{2+}-free Krebs solution. Tracheal rings were washed with Ca^{2+}- free Krebs solution containing KCl (80 mM) (approximately 15 min) and the cumulative concentration–response curve for $CaCl_2$ were obtained in the absence of **1** (control group) or after 15 min incubation with **1** (83 μM). Finally, the contractile effect induced by $CaCl_2$ was compared in absence and presence of **1**.
- In order to know the role of K^+ channels on **1**-induced relaxation, tracheal rings were preincubated with the K^+ channel blockers, glibenclamide (10 μM) and 2-AP (100 μM) for 15 min before carbachol (1 μM) was added, and then **1** was added cumulatively.

In silico docking studies

Marvin was used for drawing and for calculates the accessible solvent area of 7-Ethoxy-4-methyl-2*H*-chromen-2-one (compound **1**) structure (Marvin 6.0.0, 2013, ChemAxon, http://www.chemaxon.com).

Docking studies were performed on human L-type calcium channel as published elsewhere (Hernández *et al*., 2013). Protein-ligand interaction diagrams were obtained by LigPlot+ (Laskowski and Swindells, 2011) (double bond were not shown for compound **1** as obtained by LigPlot). VMD was used for all other figures (Humphrey *et al*., 1996). All residues numbers were adopted from Pandey *et al*., 2012.

Statistics

Data were expressed as mean ± S.E.M. and statistical significance was evaluated by using an ANOVA followed by Tukey's test. *P* values less than 0.05 were considered to denote statistical significance.

RESULT AND DISCUSSION

In our current program for the discovery of natural and/or semisynthetic bioactive compounds as potential antiasthmatic drugs, we are focusing in the study of natural occurring coumarins and their semisynthetic derivatives based on their wide spectra of

pharmacological activities, including relaxant and anti-inflammatory actions (Amin *et al*., 2011; Bansal *et al*., 2013). In this context, current work is the first attempt to show the underlying mechanism of relaxant action of **1** in trachea rat rings.

Compound **1** showed significant concentration-dependent relaxant effect on the contraction induced by carbachol (1 µM) in rat trachea rings (Fig. 2A) (Sánchez-Recillas *et al*., 2014). The relaxant effect of **1** was more active than theophylline, used as positive control.

Moreover, pretreatment with **1** significantly shifted to the right the carbachol-induced contraction (Fig 2B). On the other hand, compound **1** (83 µM) produces significant (100%) relaxant effect on the contraction induced by KCl (80 mM) and the $CaCl_2$-induced contraction was significantly reduced by the coumarin **1** as nifedipine does (a L-type calcium channel blocker), used as positive control (Fig. 3A-B). Indomethacin (10 µM, unspecific COX inhibitor) significantly reduced relaxations induced by **1** (Fig. 4). Meanwhile, in the presence of isoproterenol (Fig. 4) (a *β*-adrenergic agonist), and K^+ channel blockers glibenclamide (10 µM) and 2-AP (100 µM) the relaxant curve was not modified (Fig. 5).

As compound **1** was capable to relax the contraction induced by KCl and to inhibit the $CaCl_2$-induced contraction, we think that **1**-relaxation is related with an interference of a common pathway which several receptor agonists exert such as the augment of free cytosolic Ca^{2+} levels (Flores-Soto *et al*., 2013). In this context, in smooth muscle cells there are two kinds of Ca^{2+} channels: voltage-dependent Ca^{2+} channels (high KCl induced contraction is due to membrane depolarization, leading to increased Ca^{2+} influx through voltage-dependent channels) and receptor operated Ca^{2+} channels (contraction induced by carbachol in Ca^{2+}-free medium is due to intracellular Ca^{2+} release, through sarcoplasmic reticulum Ca^{2+} channels activated by IP_3) (Racké and Matthiesen, 2004; Racké *et al*., 2006). Coumarin **1** was capable to inhibit contractility induced by KCl (80 mM) suggesting that **1** might obstruct both voltage-dependent and receptor operated Ca^{2+} channels. Moreover, we found that this compound significantly inhibited $CaCl_2$ induced contraction in tracheal rings, in Ca^{2+}-free medium containing 80 mM KCl, supporting the idea that **1** possesses a Ca^{2+} entry blocking activity (Flores-Soto *et al*., 2013; Siddiqui et al., 2013).

On the other hand, **1**-relaxant effect was significantly reduced in the presence of indomethacin, which suggests a second mode of action related with intracellular cAMP concentration increasing.

Since the relaxant effect of **1** was related with the calcium channel blockade, we investigated *in silico* the putative interaction of compounds **1** with L-type calcium channel. Coumarin **1** was docked on human L-type calcium channel model. Lowest affinity energy docked on a different configuration as those found on our previous work (Figure 6). As noted on Figure 7, compound **1** was docked on an outer cavity located on the extracellular side of the human L-type calcium channel model (affinity energy -6.8 kcal/mol). However, **1** was also found on the same location as nifedipine does with the same affinity energy (-6.3 kcal/mol).

Both conformations were stabilized by aliphatic interactions on both binding sites as noted on Figure 7. As can see, **1** conformation in the extracellular side of human L-type calcium channel model was stabilized primordially by a π-π interaction between $F^{IVS6.7}$ and aromatic rings from **1** as shown on Figure 8. The smaller accessible solvent area (ASA) of compound **1** (~ 417 $Å^2$) compared to nifedipine (~ 523 $Å^2$) allowed it to bind to inaccessible sites for nifedipine. However, nifedipine have a nitro and ester groups that may stabilize it with their interactions with the calcium channel.

Finally, both binding sites might be possible targets for compound **1** but their precise location must be studied in more detail by mutagenesis experiments to discriminate on this sites.

CONCLUSION

In conclusion, 7-ethoxy-4-methyl-2*H*-chromen-2-one (**1**) induces a significant relaxant action on rat trachea rings through L-type calcium channel blockade and, as a second mechanism of action, by a possible intracellular cyclic AMP increasing.

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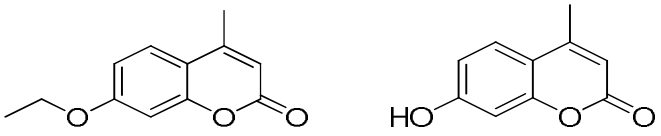
ABBREVIATIONS

°C: degree centigrade, µL: microliter, µM: micromolar, 2-AP: 2-aminopyridine, AMP: adenosine monophosphate, ASA: accessible solvent area, Ca^{2+}: calcium ion, $CaCl_2$: calcium chloride, cAMP: cyclic adenosine monophosphate, CO_2: carbon dioxide, COX: cyclooxygenase, CRC: concentration-response curves, DMSO: dimethyl sulfoxide, EC_{50}: effective concentration medium, EDTA: ethylenediaminetetraacetic acid, E_{max}: maximum effect, g: gram, h:hour, IP_3: inositol 1,4,5-trisphosphate, K^+: potassium ion, Kcal: kilocalorie, KCl: potassium chloride, KH_2PO_4: monopotassium phosphate, $MgSO_4$: magnesium sulfate, mL: milliliter, mM: millimolar, mm: millimeter, NaCl: sodium chloride, $NaHCO_3$: sodium bicarbonate, O_2: oxygen, PDE's: phosphodiesterases, S.E.M: standard error of the mean, π-π: pi-pi interactions.



(1) **(2)**

Fig. 1: Compounds studied.

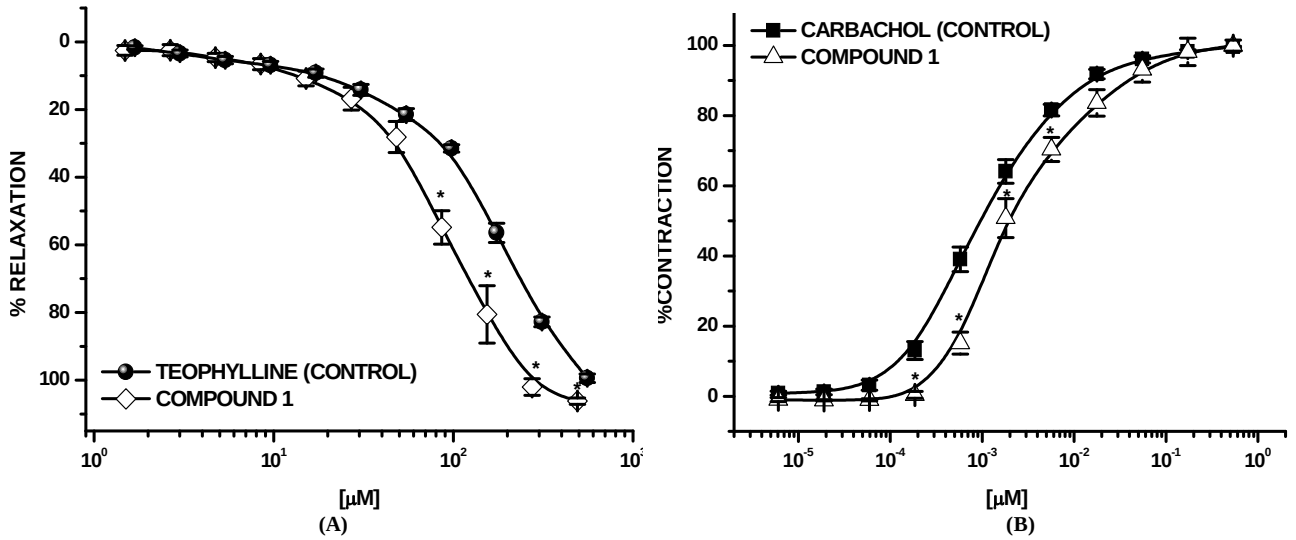


Fig. 2: (**A**) Concentration-Response curve of the relaxant effect of compound **1** and theophylline on trachea rat rings pre-contracted with carbachol 1 μM and **B**) inhibitory effect of compound **1** on the concentration-response curve of the contraction induced by carbachol. All results are expressed as the mean ± SEM of six experiments. (*P< 0.05).

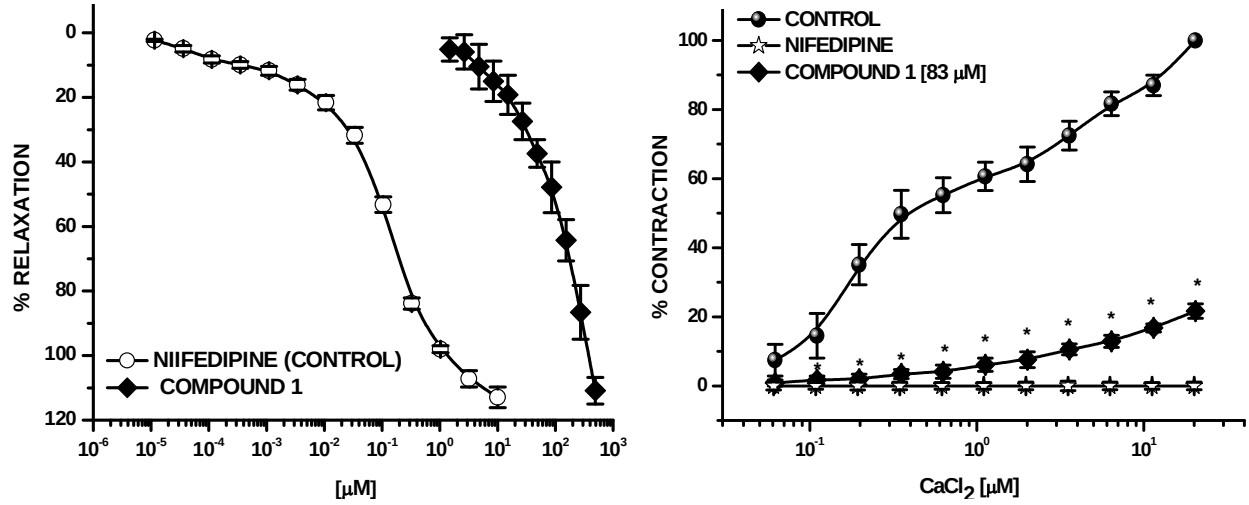


Fig. 3: A) Inhibitory effects of **1** on the contraction induced by KCl (80 mM) in rat tracheal rings and **B**) Inhibitory effect of **1** on the cumulative-contraction curve dependent on extracellular Ca^{2+} influx induced by 80 mM KCl in Ca^{2+}-free solution. Results are presented as mean±S.E.M., n=6, *p<0.05 compared with control.

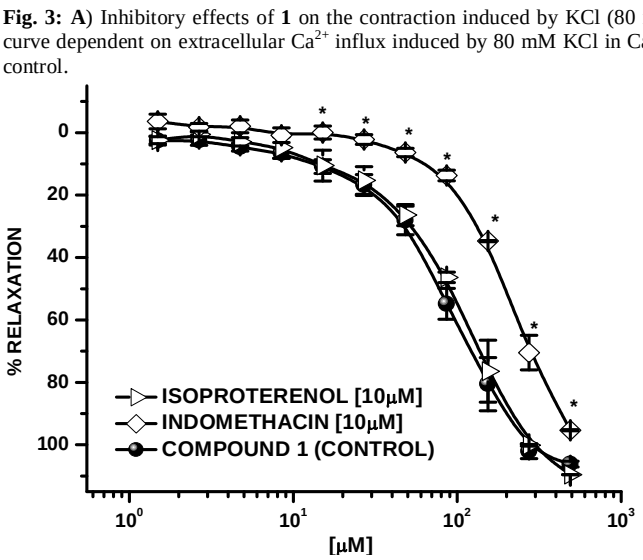


Fig. 4: Effects of isoproterenol and indomethacin treatments on **1**-induced relaxation in tracheal rings precontracted by carbachol 1 μM. Results are presented as mean±S.E.M., n=6.

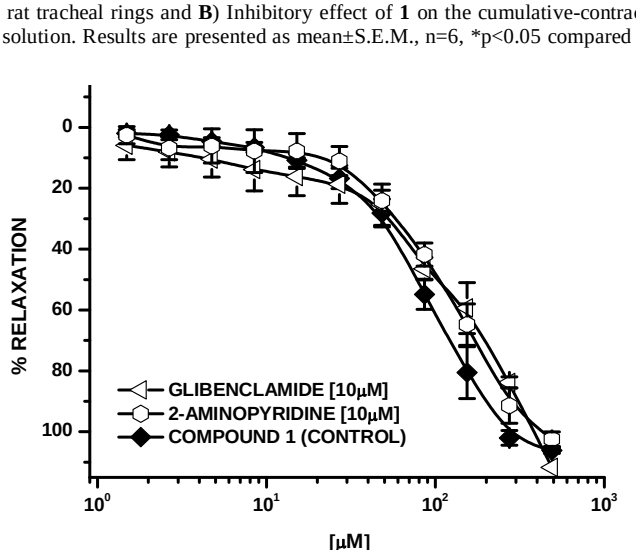


Fig. 5: Effects of glibenclamide and 2-AP treatments on **1**-induced relaxation in tracheal rings precontracted by carbachol 1 μM. Results are presented as mean±S.E.M., n=6.

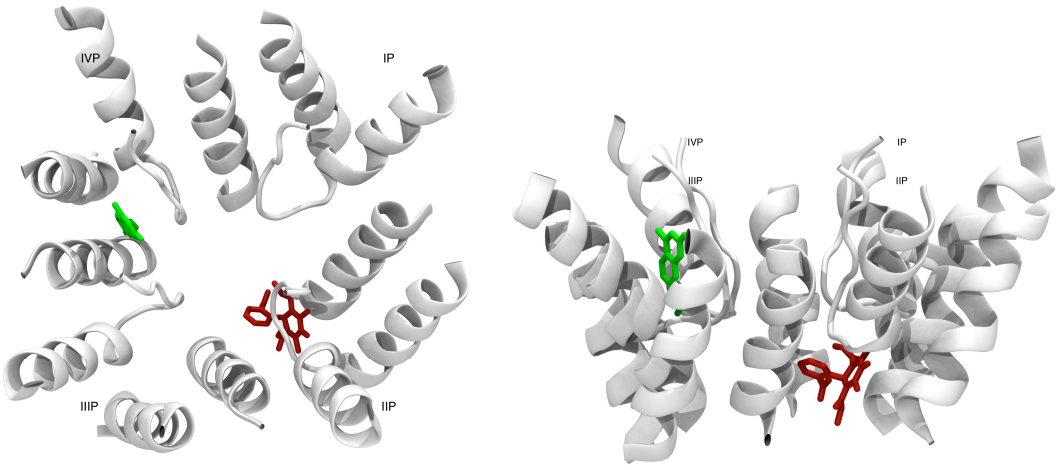
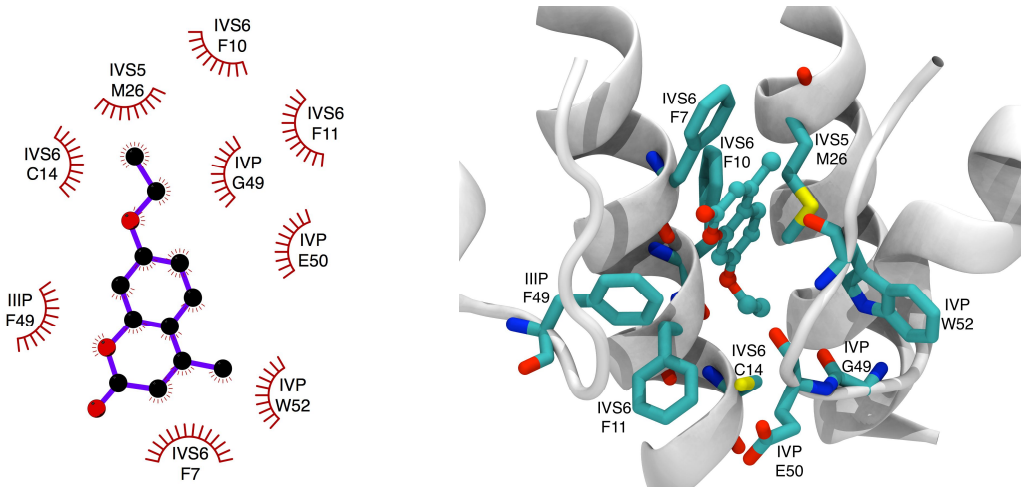
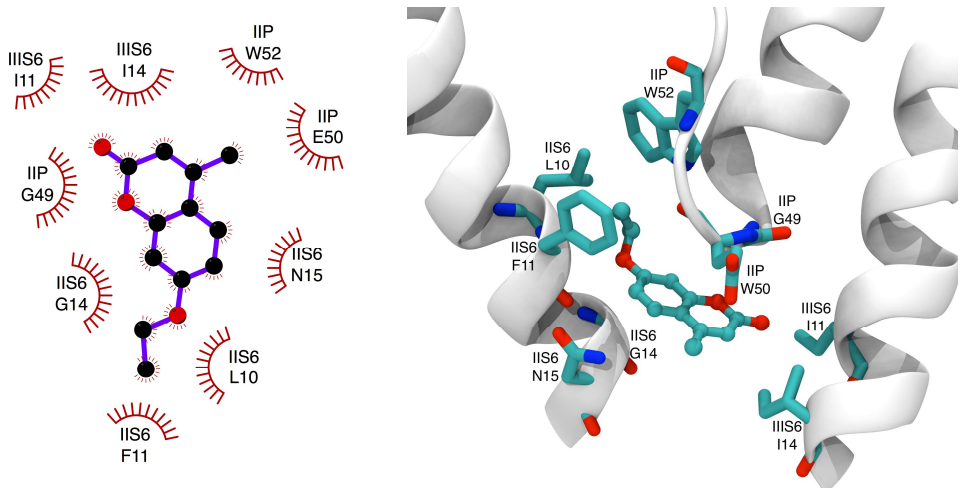


Fig. 6: 7-Ethoxy-4-methyl-2*H*-chromen-2-one (**1**, green) and nifedipine (red) locations on human L-type calcium channel model.



Extracellular binding site (-6.8 kcal/mol)



Intracellular binding site (-6.3 kcal/mol)

Fig. 7: Possible binding sites for 7-Ethoxy-4-methyl-2*H*-chromen-2-one (**1**) on human L-type calcium channel model.

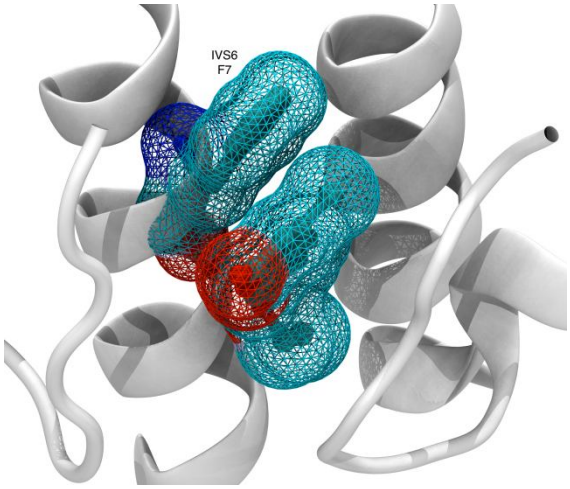


Fig. 8: π-π interaction between $F^{IVS6.7}$ and 7-Ethoxy-4-methyl-2*H*-chromen-2-one (**1**) that might stabilize the ligand conformation.

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