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Mechanism of Relaxant Action of Ethyl 6-amino-5-cyano-2-methyl-4-(pyridin-4-yl)-4H-pyran-3-carboxylate Mainly Through Calcium Channel Blockade in Isolated Rat Trachea

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ABSTRACT

This study aims to investigate the mechanism of relaxant action of Ethyl 6-amino-5-cyano-2-methyl-4-(pyridin-4-yl)- 4H-pyran-3-carboxylate (1) in *in silico* study and ex *vivo* tracheal rat rings pre-contracted with carbachol (1 µM). Compound **1** was more active than theophylline [a phosphodiesterases (PDE's) inhibitor] used as positive control. Moreover, pretreatment with **1** significantly shifted to the right the carbachol-induced contraction and did not allow to reach the maximum effect ($p<0.001$). In addition, compound **1** (96.30 µM) produces significant (100%) relaxant effect on the contraction induced by KCl (80mM), and the $CaCl_2$-induced contraction was completely abolished by **1** as nifedipine does (a L-type calcium channel blocker), used as positive control ($p<0.001$). Meanwhile, in the presence of isoproterenol (a β-adrenergic agonist), propranolol (a β-adrenergic antagonist), and K^+ channel blocker 2-AP the relaxant curve was significantly modified ($p<0.05$). Compound **1** was docked on an outer cavity located on the intracellular side of the human L-type calcium channel model and interacts in the following chains and residues: chain IP (G^{51}, W^{52}, T^{53}, D^{54}), IVP (R^{45}, E^{50}, A^{51}, Q^{53}, D^{54}) and IS6 (W^4, F^7). In conclusion, *ex vivo* and *in silico* approaches suggest that compound **1** induces its relaxant effect mainly by calcium channel blockade, but other mechanisms like potassium channel and cAMP accumulation could be involved.

INTRODUCTION

The increase in intracellular calcium concentration ($[Ca^{2+}]_i$) involves voltage-gated, receptor-operated, store operated, and nonspecific Ca^{2+}-influx, as well as sarcoplasmic reticulum release through channels activated by the phospholipase C (PLC), inositol trisphosphate (IP_3) and CD38/ciclyc ADP ribose (cADPR) phathways (Prakash, 2013; Perez-Zoghbi *et al.*, 2009; Sanderson *et al.*, 2008). Then, mechanisms such as the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA), the bidirectional Na/Ca^{2+} exchanger (NCX), and mitochondrial buffering help limit $[Ca^{2+}]_i$ restore levels after removing the agonist (Mahn *et al.*, 2010; Perez-Zoghbi *et al.*, 2009). Beyond $[Ca^{2+}]_i$, the Ca^{2+} -calmodulin-myosin light chain (MLC) kinase-MLC cascade regulates contractility mediated by actin-miosin interactions (Jude *et al.*, 2008; Berridge 2008). Thus, the regulation of [Ca2+]i in smooth muscle of the airways is a target of interest for research and develop of potential antiasthmatic drugs. Despite the currently available array of antiasmathic therapies, the search for novel chemical entities with new mode of actions, represents an important field of investigation for the development of safely and effectively drugs for the treatment of asthma. In this context, previous research work allowed us to determine the relaxant effect of Ethyl-6-amino-5-cyano-2-methyl-4-(pyridin-4-yl)-4*H*-pyran-3-carboxylate (**1**, Fig. 1) on rat tracheal smooth muscle, and results indicate that it was 1.5 fold more active than theophylline, used as positive control (Alemán-Pantitlán *et al.*, 2016).

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Furthermore, current work was designed in order to determine the underlying functional mode of action of **1** on tracheal rat ring and, by using Docking studies, to explain its interactions with L-type calcium channel.

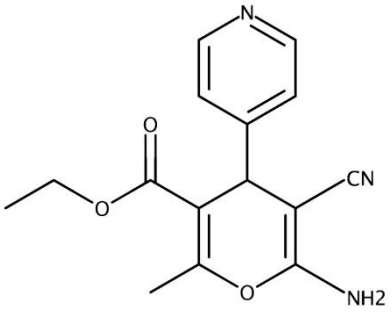


Fig. 1: Chemical structure of compound **1**.

MATERIAL AND METHODS

Chemicals and drugs

Carbamylcholine chloride (carbachol), theophylline, isoproterenol, potassium chloride (KCl), calcium chloride ($CaCl_2$), glibenclamide, 2-aminopyridine, nifedipine and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich Co. (St. Louis, MO, US). All other reagents were analytical grade from local sources. Ethyl 6-amino-5-cyano-2-methyl-4-(pyridin-4-yl)-4*H*-pyran-3-carboxylate (**1**) was previously obtained by synthesis (Hernández *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.

Rat tracheal relaxation assay

Trachea was removed from rats, cleaned out of adhering connective tissue, and 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, US) connected to a MP100 analyzer (BIOPAC Instruments, Santa Barbara, CA, US), as described (Sánchez-Recillas *et al.*, 2014a). 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:

a) For the interaction with the cholinergic receptors, concentration response curves (CRC) were obtained with carbachol (0.006-540 μM) after tissues were incubated with **1** (EC_{50}= 96.30 μM) during 15 min. Carbachol-contractile effect was determined comparing the contraction induced by carbachol in absence and presence of **1**.

b) For the interaction with phosphodiesterases (PDE's), tissues were incubated with compound **1** (96.30 μM) during 15 min, then theophylline (inhibitor of PDE's) was cumulatively added to the bath (1.67-550 μM), and concentration response curves (CRC) were obtained. The relaxant effect induced by theophylline was compared in absence and presence of **1**.

c) For interaction with β-adrenergic receptor and cAMP increase, tissues were pre-incubated during 15 min with isoproterenol (10 μM; β2 adrenergic agonist) and propranolol (10 μM; β-adrenergic antagonist) and maximal relaxing effect of **1** was compared in absence and presence of isoproterenol and propranolol.

d) 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, CRC of **1**-induced relaxation were obtained by adding cumulative concentrations of compound to the bath.

e) 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) (15 min) and the cumulative CRC for $CaCl_2$ were obtained in the absence of **1** (control group) or after 15 min incubation with **1** (96.3 μM). Finally, the contractile effect induced by $CaCl_2$ was compared in absence and presence of **1**.

f) In order to explore the role of K^+ channels on -induced relaxation, tracheal rings were preincubated with the K^+ channel blocker 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

The model of the L-type calcium channels was performed by Lipkind and Fozzard (2003), and was kindly given by Prof. Mancilla-Percino (Mancilla-Percino *et al.*, 2010). Nifedipine models of ligands and **1** were built using Marvin

Sketch (6.0.0 Marvin, 2013, ChemAxon, http://www.chemaxon.com). The study of molecular coupling (docking) was performed by using Vina Autodock (Trott and Olson, 2010). The channel was centered at (0,0,0) and was used a mesh size of 22.5 x 22.5 x 22.5 Å with a space in the mesh of 1 Å and exhaustiveness of 50. The systems were prepared using Pymol (Schrodinger, 2010) and Autodock/Vina for Pymol (Seeliger and Groot, 2010). In an effort to improve the statistics of the result obtained, a thousand independent molecular dockings were made using Autodock Vina. Images were made using VMD (Humphrey *et al.,* 1996) and molecular interactions with LigPlus (Laskowski and Swindells, 2011).

Statistics

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

RESULT AND DISCUSSION

Previous results indicate that 1 was one of the most active relaxant compounds of the entire series evaluated (Alemán-Pantitlán *et al.*, 2016), being two-times most active than theophylline (positive control). Thus, we decided to determine the 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 in *in silico* model. Hence, **1**-pretreatment significantly shifted to the right ($p<0.001$) the carbachol-induced contraction, and did not allow reaching carbachol-induced maximum contraction (Fig. 2), suggesting that **1** is acting as a possible functional non-competitive antagonist.

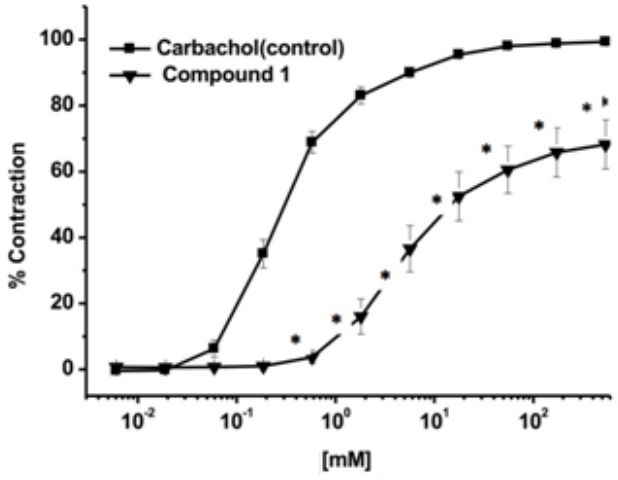


Fig. 2: Inhibitory effect of compound **1** on the concentration-response curve of the contraction induced by carbachol. All results are expressed as the mean ± S.E.M. of six rats.

In addition, compound **1** (1.06-350 µM) produces significant (100%) relaxant effect on the contraction induced by KCl (80 mM) (Fig. 3) and the CaCl2-induced contraction was significantly reduced by compound **1** ($p<0.001$) (Fig. 4). Thus, Since **1**-induced a non-competitive antagonism effect, offers the idea that bioactive **1** is not directly interacted with muscarinic receptor (Racké *et al*., 2006).

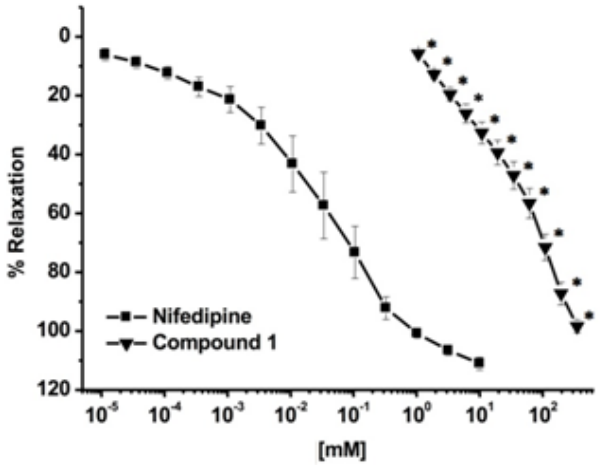


Fig. 3: Relaxant effect of compound **1** on the contraction induced by KCl (80 mM) in rat tracheal rings. Results are presented as mean ± S.E.M.of six rats.

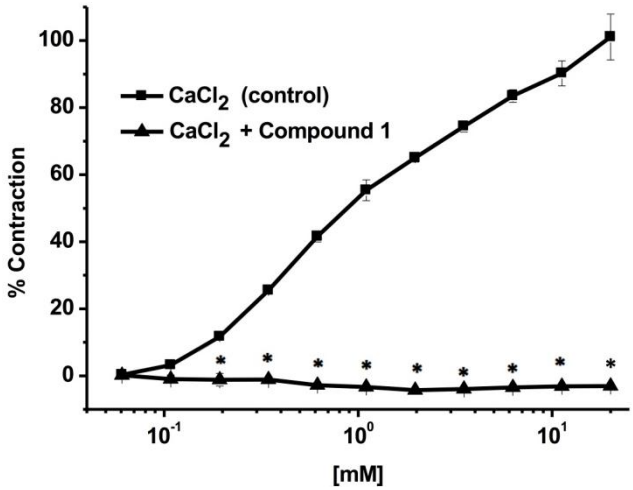


Fig. 4: Inhibitory effect of compound **1** on the cumulative-contraction curve dependent on extracellular Ca^{2+} influx induced by 80 mM of KCl in Ca2+-free solution. Results are presented as mean ± S.E.M. of six rats.

Meanwhile, the relaxant effect could be produced by blocking a common step which is necessary to produce cholinergic contraction, such as the augment of $[Ca^{2+}]_i$. It is well known that, in smooth muscle cells, two classes of Ca^{2+} channels exist: 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+} release, through sarcoplasmic reticulum Ca^{2+} channel activated by IP_3) (Montaño and Bazan-Perkins, 2005; Siddiqui *et al.*, 2013; Racké *et al.,* 2006). Therefore, our results suggest that **1** induced its relaxant effect by the interference with the Ca^{2+} influx into the smooth

muscle cells, since compound **1** was capable to relax the contraction induced by KCl and abolished the $CaCl_2$-induced contraction. Furthermore, we believe that 1 acts as calcium-channel blocker, which result in a decrease in intracellular calcium concentration, and therefore reflected in the relaxation of tracheal smooth muscle (Flores-Soto *et al*, 2013; Sanchez-Recillas *et al.*, 2014b; Medeiros *et al.*, 2011). On the other hand, in the presence of isoproterenol (β-adrenergic agonist) the relaxant curve was significantly displaced to the left ($p<0.001$), which indicates a possible synergic effect on β-adrenergic receptor and/or a potential accumulation of cAMP by guanilate cyclase activation. Likewise, preincubation with propranolol (β-adrenergic antagonist) (Fig. 5), also modified the relaxant curve induced by **1**, corroborating later asseveration (Dowell *et al.*, 2014).

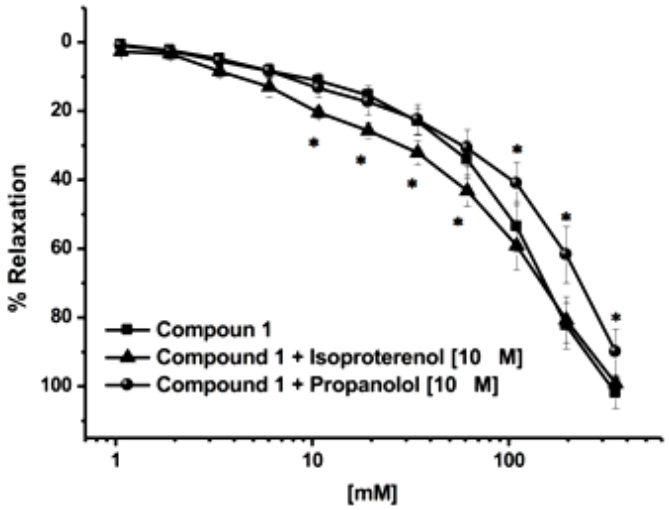


Fig. 5: Effects of isoproterenol (10 µM; β2 adrenergic agonist) and propranolol (10 µM; β-adrenergic antagonist) treatments on 1-induced relaxation in tracheal rings precontracted by carbachol 1 µM. Results are presented as mean ± S.E.M. of six rats.

On the other hand, our finding shows that 2-AP (10 µM) provokes a shifted to the right the relaxant curve of 1 ($p<0.001$), which suggest a potential potassium channel-opening mode of action. Finally, pre-incubation of glibenclamide (10 µM) did not produce any change in the concentration-response relaxant curve induced by 1 (Fig. 6), which allowed us to discard the ATP sensitive potassium channels (KATP) opening in the relaxant effect (Perez-Zoghbi *et al*., 2009).

In addition, compound **1** did not modify the relaxant curve induced by theophylline (Fig. 7), a non-specific inhibitor of phophodiesterases which are responsible for converting cAMP into AMP, suggesting that 1 did not induce an augment of intracellular cAMP as relaxant mechanism of action.

Once the relaxant effect of **1** was demonstrated and related with the calcium channel blockade, we decided to investigate the *in silico* putative interactions of active compound with L-type calcium channel (LTCC). For this, nifedipine (a well know L-type calcium channel blocker) and compound **1** were docked on human LTCC model. In this context, docking results for nifedipine gave four possible sites with different affinity energies ranging from -6.36+/-0.16 to -5.55+/-0.07 kcal/mol. Binding sites and energies are shown in Fig. 8.

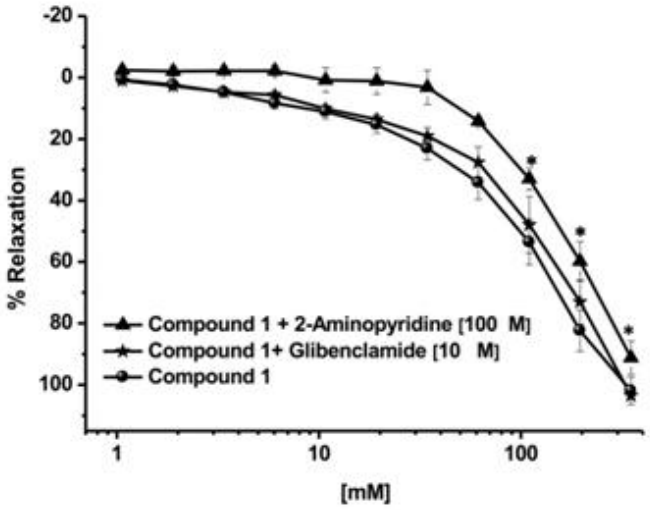


Fig. 6: Effects of glibenclamide (10 µM) and 2-AP (100 µM) treatments on 1-induced relaxation in tracheal rings precontracted by carbachol 1 µM. Results are presented as mean ± S.E.M. of six rats.

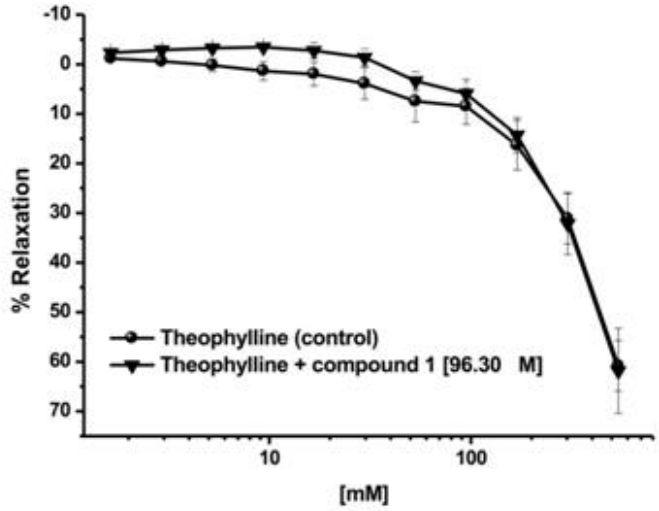


Fig. 7: Effect of compound **1** on the concentration-response curve of the relaxation induced by theophylline. All results are expressed as the mean ± S.E.M. of six rats

Each binding site was analyzed individually and identifying their corresponding interactions between nifedipine and LTCC model. Fig. 9 shows the structures that were found in each binding site and their interactions. Some of the binding sites calculated in current work were reported previously; however, they were not classified as it was done in this study, as follows: binding site C (Hernández *et al.*, 2013), binding site B (Sánchez-Recillas *et al.*, 2014b), binding sites B and D (Pandey *et al.*, 2012), and binding site D (Lipkind and Fozzard 2003).

Even when the energetic differences were small, results showed that nifedipine might be bound in different places for the calcium channel cavities broadening the search for more specific compounds than nifedipine. Lowest affinity energy site is characterized by closed contacts of residues of four distinct chains: IIIP (F^{49}, E^{50}, P^{53}), IVP (C^{46}, A^{51}, Q^{53}, W^{52}), IVS5 (M^{26}) and IVS6 (I^{4}, F^{7}, I^{8}, F^{11}). Only two residues of IVP chain form hydrogen bonds with nifedipine, E^{50} and Q^{53}, respectively. As noted, Figure 9B showed that no correlation exist between the number of hydrogen bonds created and the affinity energy. Specifically, B5 site interacts with IIIP (F^{49}, E^{50}, P^{53}), IVP (I^{4}, Q^{50}, A^{51}, W^{52}, Q^{53}, E^{54}, C^{46}), IVS5 (M^{26}) and IVS6 (F7). Taken in account latter results, and by using the same methodology as for nifedipine, it was found that compound **1** docked primordially (99.9%) on a site nearby nifedipine binding sites A and B (Fig. 9). The average affinity energy for **1** was -6.49 +/- 0.04 kcal/mol. Binding site and those amino acids interacting with 1 are shown in Fig. 10. In Fig. 10B is shown that compound **1** is interacting with the calcium channel model in the following chains and residues: chain IP (G51, W52, T53, D54), IVP (R45, E50, A51, Q53, D54), and IS6 (W4, F7). Even when there is only one residue interacting with **1** by hydrogen bond (IVP Q53), the rest of residues interact by van der Waals forces stabilizing the ligand in the calcium channel. Docking results found in this work showed that **1** might be able to bind to the calcium channel with a subtle affinity greater than nifedipine does. In conclusion, *ex vivo* and *in silico* approaches suggest that compound **1** induces its relaxant effect mainly by calcium channel blockade.

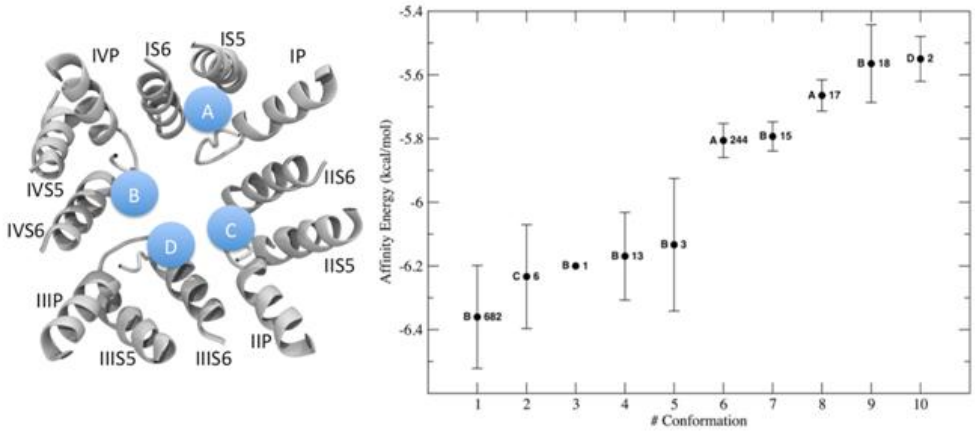
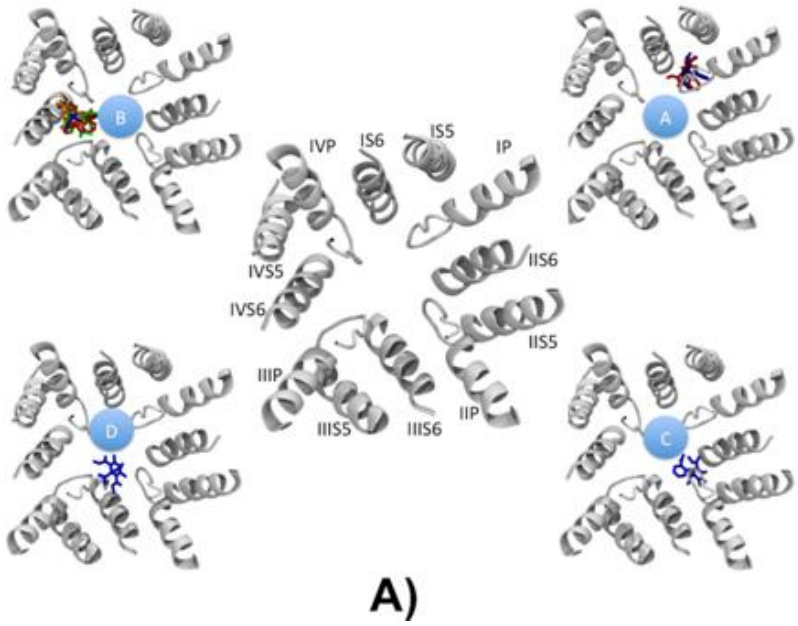


Fig. 8: Binding sites and affinity energies found on 1000 independent docking studies. In the graph, letters on the left side correspond to the binding site and the number on the right to the number of conformations found by AutodockVina.



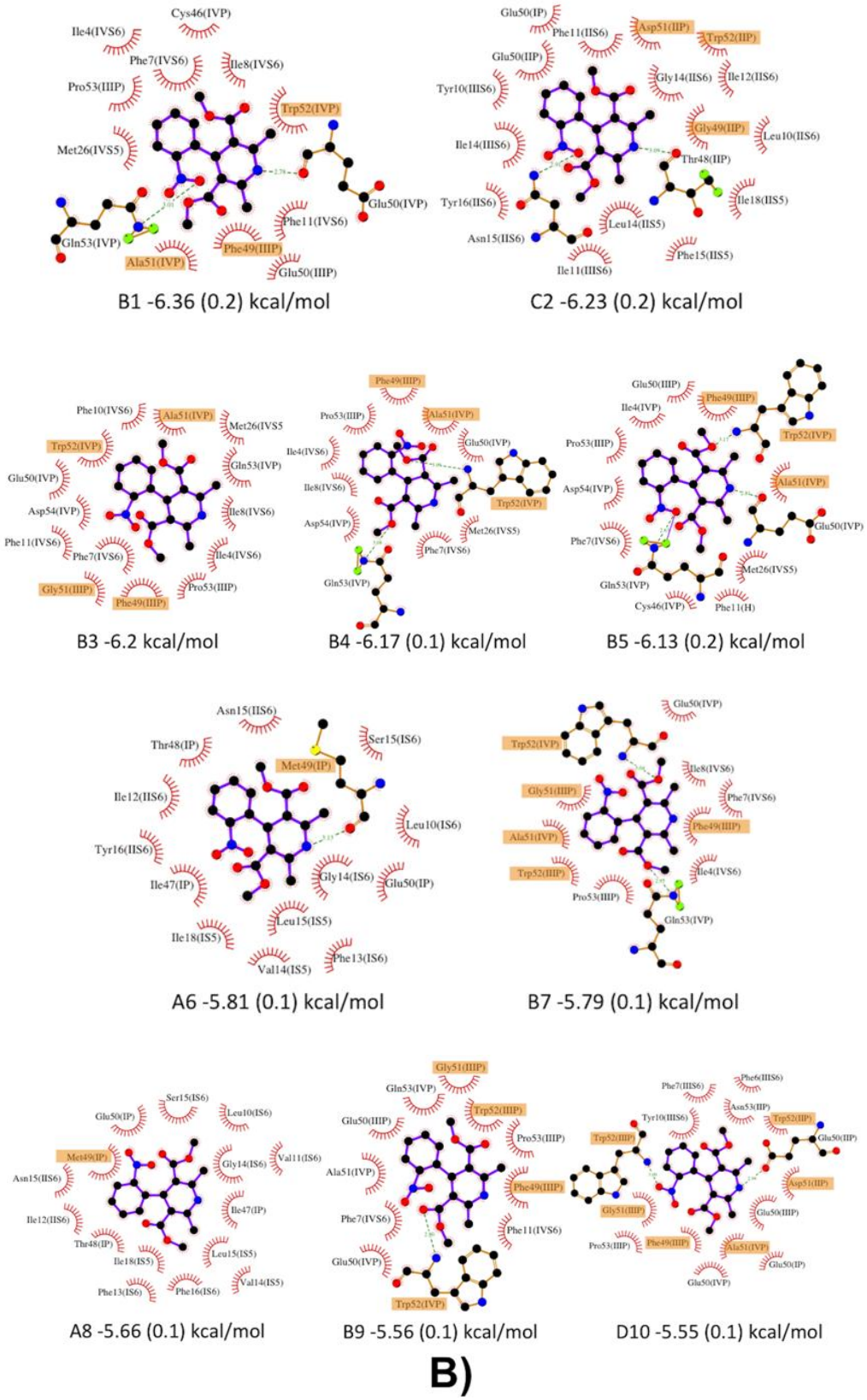


Fig. 9: Binding sites found by AutodockVina for nifedipine and calcium channel studies. A) Binding sites found in current study. B) Nifedipine/calcium channel interactions for each energy group generated by Ligplus (Laskowski and Swindells, 2011). Residues orange highlighted were found previously as disease related by mutagenesis (Pandey *et al.*, 2012). Letter corresponds to the binding site and the number to the conformation in the plot in Fig. 8. The standard deviation for each conformation is in parenthesis.

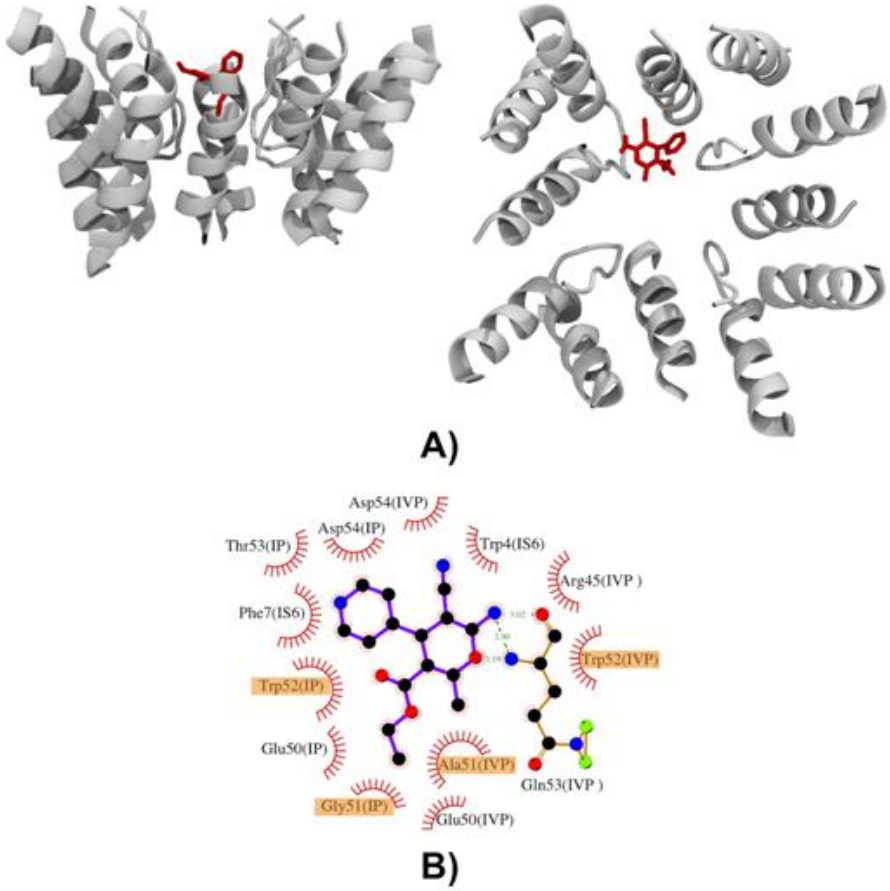


Fig. 10: Compound 1 binding site in the calcium channel model. A) Compound 1 binding site in the same orientation used in Figure 8. B) Compound 1 interactions found in current work. The notation is the same as Figure 9.

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Conflict of Interests: There are no conflicts of interest.

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