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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Functional relaxant effect of 6,7-dipropoxy-2*H*-chromen-2-one is mainly by calcium channel blockade in ex vivo assay of tracheal rings

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Abstract

Asthma is a prevalent disease characterized by airway chronic inflammation, airway hyperresponsiveness, edema and excess of mucus production. Molecules with coumarin scaffold have shown multiple biological effects, including anti-asthmatic. This study describes relaxant effect and mechanism of action of novel semisynthetic 6,7-dipropoxy-2*H*-chromen-2-one (**6,7-DPC**) on smooth muscle airways and in silico approaches. **6,7-DPC** induced significant relaxant effect on tracheal rings pre-contracted with carbachol ($E_{max} = 100\%$; $EC_{50} = 89\ \mu M$). Concentration-response curve of **6,7-DPC** showed a significant shift to the left with respect to theophylline (positive control). Moreover, pre-treatment with **6,7-DPC** inhibited the carbachol-induced contraction in a concentration-dependent manner with suppression of the maximum response. Also, KCl [80 mM]-induced contraction was partially abolished by **6,7-DPC** compared with nifedipine; however, $CaCl_2$ contraction curve only reached 30% of maximal response in the tissues pre-incubated with the test sample. **6,7-DPC** 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: IIIS5.M26, IIIP.T45, IIIP.F49, IIIP.G51, IIIP.P53, IVP.C46, IVP.G49, IVP.E50, IVP.A51, IVP.W52, IVP.Q53, IVP.D54, IVS6.I4, IVS6.F7, IVS6.I8, IVS6.F10, and IVS6.F11. Also, nifedipine made unique interactions with IIIP.E50, IIIP.W52, IIIP.L55. Meanwhile, **6,7-DPC** interacted with IIIS5.A22, IIIS2.Q27, and IVS6.C14, exclusively. In conclusion, **6,7-DPC** showed a relaxant effect due to combined mechanism of action on rat tracheal rings, through non-competitive muscarinic receptor functional antagonism and preventing the calcium influx. This provides pharmacological basis for the use of **6,7-DPC** in airways disorders, such as asthma or chronic obstructive pulmonary disease (COPD).

Keywords 6,7-dipropoxy-2*H*-chromen-2-one · Asthma · Coumarin · Calcium channel blockade · Tracheal ring

Introduction

Coumarins are aromatic compounds with common structure of 2*H*-chromen-2-one, which have been isolated from natural sources, especially green plants. Different authors reported several important pharmacological properties, such as: anticoagulant and photosensitization agent, edema and lymphedema reduction, antithrombotic, anti-HIV and anti-inflammatory effect, analgesic and immunomodulatory activity, proliferative response of human peripheral mononuclear cells inhibition, smooth muscle relaxant effect, total cholesterol reducer, hypotensive agent, prostaglandin synthesis induction and smooth muscle contraction inhibitor, among others (Gouda 2013; Ramesh and Pugalendi 2005; Venugopala et al. 2013; Wu et al. 2009). Airway obstructive diseases, for example asthma and chronic

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obstructive pulmonary disease (COPD) are non-communicable ailments that affect the airways with major impact in human health. Asthma is a prevalent sickness; it is estimated that 235 million people are suffering it around the world and is a common disease in children (GINA 2002). It is characterized by chronic inflammatory changes of the airway and distinguished by airway hyperresponsiveness, edema and excess of mucus production. Management of asthma has been focused on reversing the early airway changes and limiting the late effects of airway remodeling, such as bronchoconstriction (GINA 2002). In this context, coumarins have chemical scaffold that can be used to develop more effective anti-asthmatic molecules. **6,7-DPC** is a semisynthetic coumarin obtained from 6,7-dihydroxy-2*H*-chromen-2-one (**6,7-DHC**), which showed weak relaxing activity (Sánchez-Recillas et al. 2014). In this manner, the aim of current work was to identify the ex vivo tracheal relaxant effect of **6,7-DPC** and elucidate the functional mechanism of action in isolated rat tracheal rings pre-contracted with carbachol (an acetylcholine analog).

Materials and methods

Chemicals and drugs

1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), calcium chloride ($CaCl_2$), carbamoylcholine chloride (carbachol; CCh), glibenclamide, indomethacin, isoproterenol, N^G-nitro-L-arginine methyl ester (L-NAME), nifedipine, phenobarbital, potassium chloride (KCl), theophylline and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). All other reagents and solvents (analytical grade) were obtained from local suppliers. **6,7-DPC** was previously obtained by semi-synthesis from **6,7-DHC** (Sánchez-Recillas et al. 2014). Stock solution of **6,7-DPC** was prepared with distilled water and DMSO (1%) at the same day of experimentation.

Ex vivo pharmacological evaluation

Animals

Healthy male Wistar rats (250–300 g body weight) were obtained from the Animal House of Facultad de Estudios Superiores Iztacala (UNAM), Mexico. Animals were housed in polycarbonate cages and maintained under standard laboratory conditions (12 h light/dark cycle, 25 ± 2 °C temperature and 45–65% humidity) and were fed with standard rodent diet and water *ad libitum*. All animal procedures were conducted in accordance to our Federal Regulations for Animal Experimentation and Care (NOM-062-ZOO-1999, SAGARPA, Mexico) (SAGARPA 2001), 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 were euthanized by cervical dislocation after deep anesthesia with phenobarbital (65 mg/Kg, intraperitoneal administration).

General procedures

Trachea was dissected and cleaned out of connective tissue and cut into 4–5 mm segments (two cartilage rings). Then, tissue segments were fixed using stainless steel hooks, under optimal tension of 2.5 g in organ baths containing 10 mL of warmed (37 °C) and oxygenated (O_2/CO_2, 95:5, v-v) Krebs-Henseleit solution (KHS; composition mM: NaCl 119, KCl 4.6, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 1.5, $NaHCO_3$ 20, glucose 11.4, EDTA 0.027, pH = 7.4, in distilled water). Tension variations were recorded by Grass-FT03 force transducers (Astro Med, West Warwick, RI, USA) connected to MP100 analyzer (Biopac Instruments, Santa Barbara, CA, USA) as described in Estrada-Soto et al. (2012). After the stabilization period (20 min), sensitization was carried out. The tissues were stimulated with carbachol (CCh [1 μM]) during 15 min, then washed with fresh KHS, and allowed to stabilize for 15 min. This procedure was repeated twice.

Airway smooth muscle relaxant activity of 6,7-DPC on contraction induced by CCh

After sensitization, tissues were allowed to stabilize for 20 min and then contracted with CCh [1 μM]. **6,7-DPC** [1.16 to 382 μM], vehicle (DMSO [1% final concentration]) or positive control (theophylline [1.68 to 555 μM]) were added to the chamber in cumulative concentrations (Concentration-Response Curves, CRC). The relaxant effect of the samples was determined by their ability to reduce the maximal tracheal contraction induced by CCh, comparing tissue tension before and after their addition.

Determination of the relaxant mode of action of 6,7-DPC

In order to establish the underlying mode of action of **6,7-DPC** the following ex vivo experiments were carried out:

a. For the interaction with β_2-adrenergic receptors or inhibition of phosphodiesterase (PDE) and cAMP increase, independent tissues were pre-incubated for 15 min with isoproterenol [10 μM], a β-adrenergic agonist, or theophylline [10 μM], a PDE inhibitor; then the maximal relaxing effect of **6,7-DPC** was compared in absence and presence of isoproterenol or theophylline.

b. To identify the participation of prostaglandins, tissues were pre-incubated for 15 min with indomethacin [10 μM], a cyclooxygenases inhibitor. Maximal relaxing effect of **6,7-DPC** was compared in absence and presence of indomethacin.
c. For interaction with sGC–cGMP-pathway, independent tissues were pre-incubated for 15 min with ODQ [10 μM], a soluble guanylyl cyclase inhibitor or L-NAME [10 μM], a nonselective NOS inhibitor. Maximal relaxing effect of **6,7-DPC** was compared in absence and presence of ODQ or L-NAME.
d. In order to identify the opening of K^+ channels on **6,7-DPC**-induced relaxation, tracheal rings were pre-incubated with K^+ channel blocker, glibenclamide [10 μM], for 15 min before CCh [1 μM] was added, then **6,7-DPC** was incorporated cumulatively.
e. To establish a possible blockade of **6,7-DPC** on L-type calcium channel, the tracheal rings were pre-contracted with KCl [80 mM]. Once a plateau was attained, CRCs of **6,7-DPC**-induced relaxation were obtained by adding cumulative concentrations of compound to the bath. In this experiment, nifedipine (calcium channel blocker) was used as positive control [1.15×10^{-5} to 10 μM].
f. To determine whether the inhibition of extracellular Ca^{2+} influx was involved in **6,7-DPC**-induced relaxation, the experiments were carried out in Ca^{2+}-free Krebs-Henseleit solution. Tracheal rings were washed with Ca^{2+}-free Krebs solution containing KCl [80 mM], and the cumulative concentration-response curve for $CaCl_2$ [0.06 to 20 μM] were obtained in the absence of **6,7-DPC** (control group) or after 15 min incubation with **6,7-DPC** [89 μM]. Finally, the contractile effect induced by $CaCl_2$ was compared in absence and presence of **6,7-DPC**. In this experiment nifedipine [10 μM] was used as positive control [10 μM].
g. For the interaction with the cholinergic receptors (AChR), CRCs were obtained with carbachol [0.006–540 μM]. Later, tissue was washed and incubated with **6,7-DPC** [44, 89, 140, 255, 455 μM]. Carbachol-contractile effect was determined comparing the contraction induced by carbachol in absence and presence of increasing concentrations of **6,7-DPC**.

In silico docking studies

Human L-type calcium channel previously modeled was used as reference target (Lipkind and Fozzard 2003). Nifedipine was used as reference ligand for human L-type calcium channel. Nifedipine and **6,7-DPC** were constructed using MarvinSketch by ChemAxon (2018). Docking experiments were directed to the known binding site of the protein. L-type calcium channel grid center was at (0,0,0) (previously orienting the pore to the Z-axis) with a size of $30.0 \times 30.0 \times 22.50$ Å^3. Grid box had a 1 Å of grid spacing. L-type calcium channel and ligands were prepared using Pymol 2.2.0 (Schrödinger 2010) and the Autodock Vina plugin for Pymol (Seeliger and de Groot 2010). Autodock Vina (Trott and Olson 2010) software was used to conduct all the docking studies. 1000 independent docking runs for each ligand were conducted. All images and protein-ligand interaction maps were created with Maestro by Schrödinger (2018).

Data analysis

Results are expressed as the mean ($n = 6$) ± standard error of the mean (S.E.M). Concentration-response curves (CRC) were plotted, and the experimental data from the CRC were adjusted by the nonlinear DoseResp equation with the curve-fitting program ORIGIN 8.0. Pharmacological parameters efficacy (E_{max}) and half maximal effective concentration (EC_{50}) values were calculated. The statistical significance of differences between means was assessed by a one-way analysis of variance (ANOVA) followed by the Tukey post-hoc test; p-values < 0.05 ($*p < 0.05$) were considered statistically significant (Bailey 1995; Daniel 2002).

Results and discussion

6,7-DPC was previously obtained by semi-synthesis from **6,7-DHC** (Sánchez-Recillas et al. 2014). Figure 1a shows the CRC of **6,7-DPC** relaxing effect, which displayed significant effect on isolated rat tracheal rings pre-contracted with CCh [1 μM] (maximum effect: $E_{max} = 100\%$; half maximal effective concentration: $EC_{50} = 89$ μM). **6,7-DPC** curve was shifted to left respect to theophylline ($E_{max} = 100\%$ and $EC_{50} = 144$ μM) used as positive control (phosphodiesterase inhibitor), which indicates that **6,7-DPC** is more potent than positive control. Moreover, Fig. 1b shows the contractile effect of carbachol in the presence of increasing concentrations of **6,7-DPC**, which showed concentration-dependent inhibition at 89 μM ($E_{max} = 92\%$), 140 μM ($E_{max} = 56\%$), 255 μM ($E_{max} = 45\%$) and 455 μM ($E_{max} = 30\%$), respectively. Therefore, since carbachol-induced contraction (cholinergic agonist and acetylcholine stable bioisostere) was modified in the presence of **6,7-DPC**, followed by non-parallel shift with suppression of the maximum effect in a concentration-dependent manner, suggesting a non-competitive antagonism (Katzung 2004). The non-competitive antagonism has been observed when the maximum effect of agonist is modified due to increasing

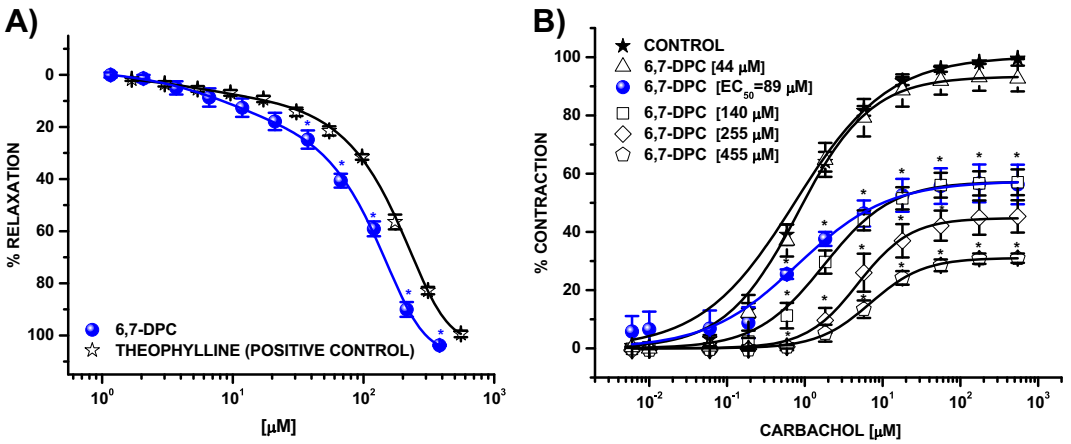

Fig. 1 **a** Concentration-response curves of the relaxant effect of **6,7-DPC** [1.16–382 μM] and theophylline (positive control) on tracheal rat rings pre-contracted with carbachol [1 μM] and **b** effect of **6,7-DPC** increasing concentration on carbachol-induced contraction [0.006–540 μM] in tracheal rings. All results are expressed as the mean ± S.E.M of six experiments. $*p < 0.05$ represent significant difference respect to control

concentrations of antagonist, without and/or slim modification of the EC_{50} values of agonist, as shown in Fig. 1b. This suggests that **6,7-DPC** could interact at the same site as carbachol, irreversibly or pseudo-irreversibly (with slow dissociation but not through covalent bond). It causes a shift of the CRC to the right with further depression of the maximal response. Another suggestion is an allosteric effect that may occur when the antagonist binds to a different site on the muscarinic receptor, inhibiting the maximum response. This effect is saturable and inhibition reaches a limiting value when the allosteric site is fully occupied. Finally, a third event might occur, i.e., a functional antagonism when it is inhibited a step downstream after carbachol binds with muscarinic receptor, such as calcium channel blockade, an increasing of intracellular cGMP and/or cAMP or K^+ channels opening (Sánchez-Recillas et al. 2014; Fylaktakidou et al. 2004; Katzung 2004; Estrada-Soto et al. 2012; Black and Barnes 1990; Buxton 2006; Susuki et al. 1985), among others.

Anticholinergic therapy of asthma or COPD is mainly aimed on the inhibition of bronchoconstriction by antagonizing muscarinic M_3 receptor. Currently, a few anti-asthmatic drugs are recognized as anticholinergic. Moreover, some coumarin derivatives with inhibitory action of calcium influx on smooth muscle cells were previously described (Susuki et al. 1985). Chen and Duan (1990) reported the anti-asthmatic effect of coumarin due to activation of β_2-adrenergic receptors in guinea pig, likewise (Xiong et al. 2012) described the anti-allergic and bronchial hyper-attenuation of coumarin potential due to suppression of inflammation induced by ovalbumin in mice. Thus, previous results suggest that coumarins, as **6,7-DPC**, could have dual therapeutic effect as bronchodilator and anti-inflammatory. All of these are applicable in development of new drugs for the treatment of obstructive diseases of the airways as asthma.

As described above, it seems that **6,7-DPC** induces its airway smooth muscle (ASM) relaxation due to a functional antagonism mechanism. Thus, in order to corroborate this hypothesis, we decided to explore the relaxant effect related to Ca^{2+} channel blockade. Asthma is characterized by excessive ASM contraction and contraction is dependent on the phosphorylation of the regulatory myosin light-chain, mediated by myosin light-chain kinase that is activated through the calmodulin-Ca^{2+} complex (Chen and Sanderson 2017; Pfitzer 2001). Therefore, an increase of intracellular calcium concentration ($[Ca^{2+}]_i$) induces the contraction of ASM. In this context, **6,7-DPC** was able to slightly relax the KCl [80 mM]-induced contraction (E_{max} = 52%) and Ca^{2+}-induced contraction was 70% inhibited in the presence of **6,7-DPC** [89 μM] compared with nifedipine (L-type calcium channel blocker used as positive control), which inhibited 100% of the $CaCl_2$ cumulative-contraction curve (Fig. 2a, b) suggesting it as a potential Ca^{2+} channel blocker.

Previous works mentioned that contraction of smooth muscle, induced by high K^+, is dependent upon the entry of Ca^{2+} into the cells through the voltage-operated channels (VOC) and cell membrane depolarization; while inhibition of high K^+-induced contraction is due to blockade of Ca^{2+} entry through those channels (Godfraind 2017; Ghayur et al. 2006; Yagi et al. 2002). Moreover, a variety of Ca^{2+} entry pathways are proposed to contribute to Ca^{2+} homeostasis in ASM contraction, including VOC, receptor-operated Ca^{2+} channels (ROCC) and reverse-mode Na^+/Ca^{2+} exchangers (Chen and Sanderson 2017). Consequently, as **6,7-DPC** was capable to induce relaxation against high K^+ and $CaCl_2$ contractions, we propose that

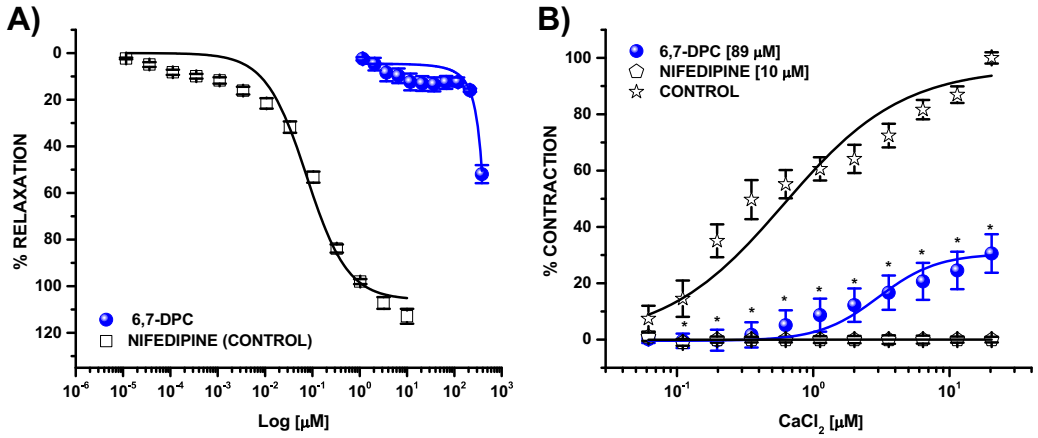

Fig. 2 **a** Relaxant effect of **6,7-DPC** on the contraction induced by KCl [80 mM] in rat tracheal rings and **b** Inhibitory effect of **6,7-DPC** on the cumulative-contraction curve dependent on extracellular Ca^{2+} influx induced by KCl [80 mM] in Ca^{2+}-free solution. Results are presented as mean ± S.E.M. of six experiments. *$p < 0.05$ compared with control

coumarin, being a VOC and ROCC blocker, uses this action as main ASM relaxant mechanism.

On the other hand, with the purpose to continue exploring about the functional mode of action of **6,7-DPC**, individual experiments were carried out in the presence of isoproterenol, indomethacin and theophylline to establish the participation of β_2-adrenergic receptors, relaxant prostanoids and/or phosphodiesterases inhibition, respectively. Likewise, relaxation-CRC's were constructed in the presence of ODQ, L-NAME and glibenclamide to identify activation of cGMP-NO pathway and opening of ATP-sensitive K^+ channels, respectively. The **6,7-DPC**-relaxant effect was not modified in the presence of those agonists, inhibitors and/or blockers (Fig. 3), which allowed us to discard a potential β_2-adrenergic agonism, an augment of second messenger such as cAMP or cGMP and/or opening K^+ channel (Sánchez-Recillas et al. 2014; Fylaktakidou et al. 2004; Katzung 2004).

Once the relaxant effect of **6,7-DPC** was demonstrated and related with the calcium channel blockade, we decided to investigate the *in silico* putative interactions of the active compound with L-type calcium channel (LTCC). For this, nifedipine (a well know L-type calcium channel blocker) and **6,7-DPC** were docked on human LTCC model. **6,7-DPC** and nifedipine were docked on 1000 independent runs. All discussion is based on the most populated cluster of conformations (2 Å RMSD separation between each cluster). Nifedipine most populated cluster [Site B as reported (Alemán-Pantitlán et al. 2016) with a smaller box] had 689 members with an affinity energy of −6.77 ± 0.06 kcal/mol. **6,7-DPC** cluster had 995 members with −7.01 ± 0.04 kcal/mol of affinity energy. Even more, when both ligands were found in the same pocket (Fig. 4) [IIIS5, IIIP, IVP and IVS6, nomenclature and numeration as reported (Pandey et al. 2012)], **6,7-DPC** was located on a deeper cavity formed by those helixes, this was due to one of the two aliphatic tails. Both ligands interacted with IIIS5. M26, IIIP.T45, IIIP.F49, IIIP.G51, IIIP.P53, IVP.C46, IVP. G49, IVP.E50, IVP.A51, IVP.W52, IVP.Q53, IVP.D54, IVS6.I4, IVS6.F7, IVS6.I8, IVS6.F10, and IVS6.F11. Nifedipine made unique interactions with IIIP.E50, IIIP. W52, IIIP.L55; meanwhile, **6,7-DPC** interacted IIIS5.A22, IIIS2.Q27 and IVS6.C14, exclusively. **6,7-DPC** appeared to be stabilized by a π–π interactions with IVS6.F7. The stacking between both **6,7-DPC** rings and the phenylalanine appears to be more energetically favorable than the same interaction with nifedipine. Docking results found in this work showed that **6,7-DPC** might be able to bind to the L-type calcium channel with a subtle affinity of −0.24 kcal/mol greater than nifedipine does.

With the intention of anticipating latent off-targets affinity and toxicity issues of **6,7-DPC**, a virtual prediction of safety profiles was calculated (Table 1). The toxicity parameters of **6,7-DPC** and nifedipine (a well know L-type calcium channel blocker) were predicted using ACD/Tox-Suite software, v. 2.95 (Colín-Lozano et al. 2018).

The *in silico* calculation of inhibition for the three main isoforms of cytochrome P_{450} for **6,7-DPC** was comparable to nifedipine at relevant clinical concentration (<10 μM), showing very low probabilities of drug-drug interactions. Also, several lipophilic coumarins are associated with cardiovascular risks due to human ether-a-go-go related gene (hERG) channel blockade (Żołek and Maciejewska 2017). **6,7-DPC** showed very low prediction of hERG channel blockage at clinically relevant concentration ($K_i < 10$ μM), being considered as theoretically non-cardiotoxic compound. In the calculation of acute toxicity, **6,7-DPC** demonstrated similar or even high predicted LD_{50} than

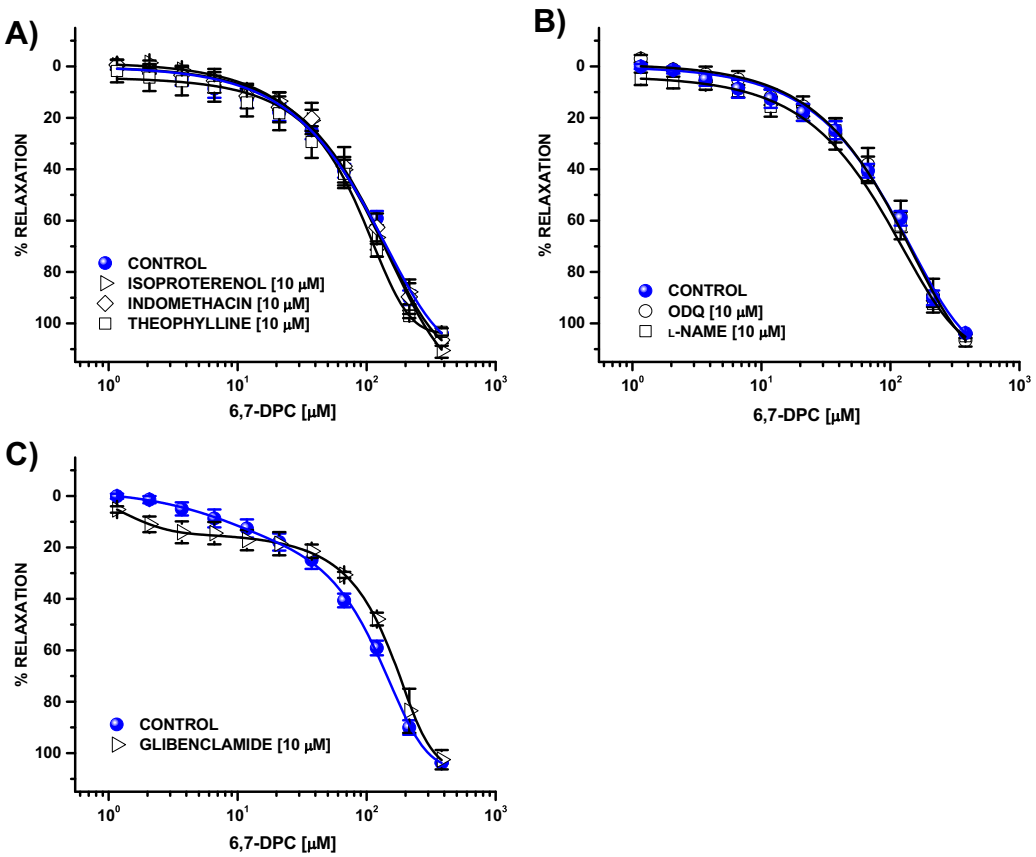

Fig. 3 Relaxant effect of **6,7-DPC** [1.16–382 μM] on isolated rat tracheal rings pre-contracted with carbachol [1 μM] **a** in presence of isoproterenol [10 μM], indomethacin [10 μM] and theophylline [10 μM], **b** in presence of ODQ [10 μM], and L-NAME [10 μM] and **c** in presence of glibenclamide [10 μM]. All results are expressed as the mean ± S.E.M of six experiments. *$p < 0.05$ compared with **6,7-DPC** curve without drugs (control)

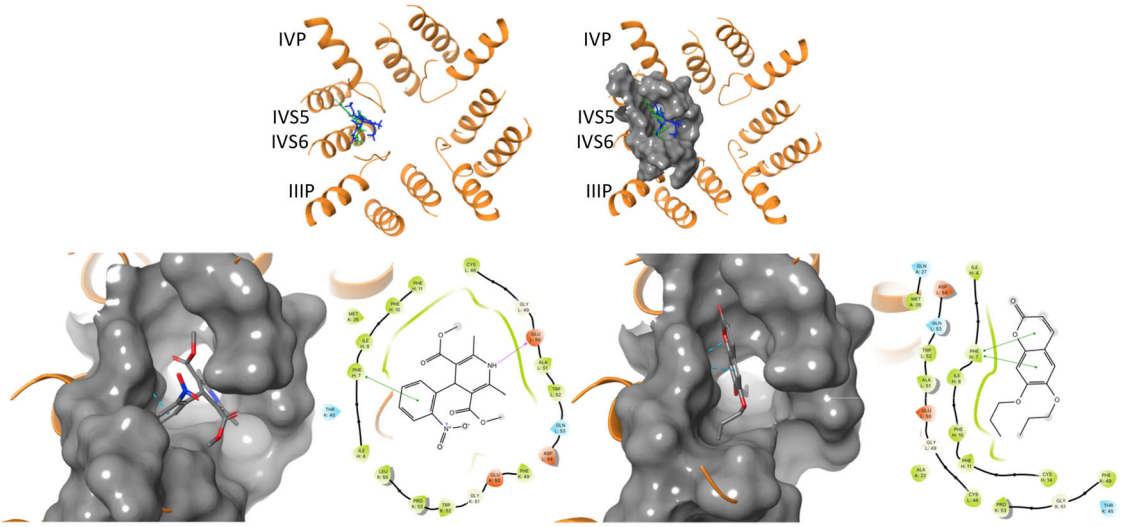

Fig. 4 Representative structures of the most populated clusters for nifedipine and **6,7-DPC** with human L-type calcium modeled. Upper images, location of both ligands on the calcium channel. Lower images, binding pocket and interaction map for nifedipine and **6,7-DPC** found in this work

Table 1 Toxicity profiles computed for **6,7-DPC** and nifedipine

Compound	LD_{50} (mg/kg)				Probability of inhibition/blockage (IC_{50} or $Ki < 10\ \mu M$)			
	Mouse		Rat		CYP_{450} isoform			hERG
	i.p.	p.o.	i.p.	p.o.	3A4	2D6	1A2	
6,7-DPC	330	500	490	1000	0.08	0.05	0.81	0.14
Nifedipine	185	202	230	1022	0.07	0.06	0.82	0.10

nifedipine by two different administration routes in two murine species, being predicted less toxic than this common L-type calcium channel blocker used as reference in this work.

Conclusion

6,7-DPC showed a relaxant effect due to combined mechanism of action on rat tracheal rings, through non-competitive muscarinic receptor functional antagonism and preventing the influx of extracellular calcium. This provides pharmacological basis for the use of **6,7-DPC** in airways disorders as asthma or chronic obstructive pulmonary disease.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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