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Research Paper

Gnaphaliin A and B relax smooth muscle of guinea-pig trachea and rat aorta via phosphodiesterase inhibition

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Abstract

Objectives To explore the relaxant mechanism of action of gnaphaliin A and gnaphaliin B in guinea-pig trachea and rat aorta, and to investigate the theoretical and experimental phosphodiesterase (PDE) inhibitory activity of these flavones.

Methods The relaxant effect and the inhibition of calcium chloride induced contractions of both flavones were evaluated on guinea-pig trachea and rat aorta rings. The PDE inhibitory activity was evaluated using a cyclic nucleotide PDE colorimetric assay kit with cAMP and cGMP as substrates. The docking analysis was carried out with AutoDock4 software and X-ray structure of PDE type 5. The activity of both gnaphaliins was compared with the activity of sildenafil, rolipram, aminophylline, IBMX and enoximone.

Key findings Gnaphaliin A and B were more actives as relaxants on rat aorta than guinea-pig trachea. They were less potent in the relaxation of guinea-pig trachea and rat aorta than sildenafil, but they were equal or more potent than the other PDE inhibitors tested. The relaxant effect of these flavones was potentiated by nitroprusside and forskolin, and blocked by 1H-[1,2,4]-oxadiazolo[4,3-a]quinoxalin-1-one but not by 2′,5′-dideoxyadenosine in guinea-pig trachea. L-NAME did not modify the relaxant effect of gnaphaliins. Gnaphaliins were more potent as PDE inhibitors when cGMP was used as substrate. Docking analysis revealed that gnaphaiins bind to the same binding site of sildenafil at PDE type 5.

Conclusions The results suggest that the main relaxant mechanism of action of gnaphaliin A and B is inhibition of PDEs with a preference to inhibit the degradation of cGMP. The docking study suggested that these flavones bind with high specificity to the same binding site of sildenafil at PDE type 5.

Keywords broncorelaxation; docking analysis; gnaphaliins; phosphodiesterases; vasodilatation

Introduction

The flavones gnaphaliin A and B (Figure 1) were identified as tracheal smooth muscle relaxant active principles from the *n*-hexane extract of *Gnaphalium liebmannii* Sch. Bp ex Klatt (family *Asteraceae*).[1] This medicinal plant is widely used in Mexican traditional medicine for the treatment of several respiratory diseases such as gripe, fever, asthma, cough, cold, bronchitis, expectorating and bronchial infections.[2–4] In a previous work, we reported that the relaxant effect of the *n*-hexane extract of *G. liebmannii* does not occur by activation of β-adrenergic receptors either by the blockade of ATP-sensitive potassium channels or by the antagonism of muscarinic or histamine receptors.[5] The relaxant effect of the *n*-hexane extract of *G. liebmannii* is potentiated by forskolin, nitroprusside and aminophylline in the smooth muscle of guinea-pig trachea.[5] It was also suggested that the mechanism of action of this extract is related to maintaining or increasing cAMP or cGMP levels.[5] On the other hand, some flavonoids have shown important pharmacological activity as relaxant agents of vascular smooth muscle[6,7] and airway smooth muscle.[8] These pharmacological effects are important in the treatment of diseases that involve vascular smooth muscle contraction such as hypertension[9] and airway smooth muscle contraction such as asthma and chronic obstructive pulmonary disease.[8] Inhibition of phosphodiesterases (PDE) has been demonstrated for several flavonoids, however not all flavonoids show this activity and not all of them are able to inhibit all types of PDE isoenzymes.[8]

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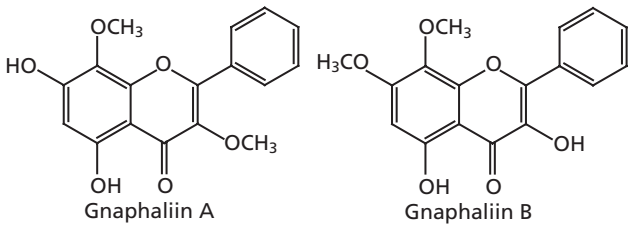

Figure 1 Chemical structure of gnaphaliins A and B.

In this study we have shown that gnaphaliin A and gnaphaliin B produce their relaxant effect on guinea-pig trachea and rat aorta smooth muscle mainly by inhibition of PDE, and for first time the docking analysis predicted that both gnaphaliins bind to the same catalytic site as sildenafil at PDE type 5 with a good correlation between theoretical and experimental in-vitro data.

Materials and Methods

Chemicals and drugs

Acetylcholine chloride (ACh), carbamylcholine chloride (carbachol, CCh) (–)norepinephrine, N^G-nitro-L-arginine methyl ester (L-NAME), 1H-[1,2,4]-oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), nitroprusside, 2′,5′-dideoxyadenosine, forskolin, rolipram, enoximone and aminophylline were purchased from Sigma Chemical Co. (St Louis, MO, USA). Sildenafil citrate was used as USP reference standard. Gnaphaliin A and gnaphaliin B were obtained from a previous study in our laboratory[1] and had a purity of 97% as determined by high-performance liquid chromatography. The drugs and flavones were suspended in 0.05% Tween 80 in distilled water. The final concentration of Tween 80 was a trace amount (less than 0.0005%) and did not affect the tracheal or vascular response. The drug solution or suspensions were freshly prepared each time a few minutes before experimentation. For the PDE assay, the drugs and flavones were dissolved in a binary mixture (1 : 1) of acetonitrile (Burdick & Jackson, Musquegon, MI, USA) and water (Direct-Q Millipore water purification system). PDE enzyme from bovine brain, 5′-nucleotidase from *Crotalus atrox* venom, 3′,5′-c-AMP, 3′,5′-cGMP, buffer (10 mM Tris-HCl, pH 7.4), BIOMOL Green reagent and IBMX were purchased from BIOMOL International, Inc. (Plymouth Meeting, PA, USA).

Animals

Male guinea-pigs (Hartley), 400–450 g, were obtained from Biosupply S.A de C.V. (Mexico). Male Wistar rats, 200–300 g, were obtained from Harlan México (Mexico City, Mexico). Both species were maintained at a constant room temperature (22 ± 2°C) and submitted to a 12-h light/dark cycle with free access to food and water. Procedures involving animals were conducted in accordance with the Mexican Official Norm for Animal Care and Handing (NOM-062-ZOO-1999; Especificaciones Técnicas para la Producción, Cuidado y Uso de Animales de Laboratorio) and in compliance with international rules on the care and use of laboratory animals. Furthermore, clearance for conducting the studies was obtained from the Ethics Committee for the Use of Animals in Pharmacological and Toxicological Testing, Facultad de Química, Universidad Nacional Autónoma de México.

Guinea-pig trachea preparation

Guinea-pigs were killed by intraperitoneal injection of an overdose of sodium pentobarbital (95 mg/kg). The chest was opened to obtain the trachea, which was transferred to a dish containing warm Krebs solution of the following composition (mM): NaCl 118, KCl 4.7, NaH_2PO_4 1.2, $MgSO_4{\cdot}7H_2O$ 1.2, $CaCl_2{\cdot}2H_2O$ 2.5, $NaHCO_3$ 25, glucose 11.1. After removal of excess connective tissue and fat, the trachea was divided into eight small rings of about 2 mm in length containing 2–3 cartilaginous segments. Each tracheal ring was hung between two nichrome hooks inserted into the lumen and placed in a 10-ml organ bath containing Krebs solution. This solution was maintained at 37 ± 0.5°C and bubbled constantly with 5% CO_2/95% O_2. Isometric tension was recorded through an eight-channel Biopack System polygraph MP100 via a Grass FT 03E force transducer (Biopac System Inc., Goleta, CA, USA). The data were digitalized and analysed by means of software for data acquisition (Acknowledge 3.9.0; Biopac System Inc., Goleta, CA, USA). Tissues were placed under a resting tension of 1.5 g and allowed to stabilize for 60 min and they were washed with fresh Krebs solution at 15-min intervals before starting the experiments. After the stabilization period, the tracheal rings were subjected to one of the follow protocols.

Relaxant effect

Rings were contracted with ACh (30 μM) twice at 30-min intervals and then washed with fresh Krebs solution. After 30 min, the tissues were contracted with CCh (3 μM), and then cumulative concentrations of gnaphaliin A, gnaphaliin B, rolipram (PDE-4 inhibitor[10]), sildenafil citrate (PDE-5 inhibitor[11]), enoximone (PDE-3 inhibitor[8]), or aminophylline (unspecific PDE inhibitor[12]) (0.001–1000 μM) were added. The relaxant potencies were expressed as EC50.

Role of nitric oxide synthase

Rings were contracted with ACh (30 μM) twice at 30-min intervals and then washed with fresh Krebs solution. The rings were incubated with L-NAME (nitric oxide synthase inhibitor, 100 μM) at 20 min before the contraction of the tissue with CCh (3 μM) and then cumulative concentrations of gnaphaliin A or gnaphaliin B (0.0001–100 μM) were added.

Role of guanylate cyclase and adenylate cyclase

Rings were contracted with ACh (30 μM) twice at 30-min intervals and then washed with fresh Krebs solution. After 30 min, the tissues were contracted with CCh (3 μM). ODQ (guanylate cyclase soluble inhibitor, 10 μM) or 2′,5′-dideoxyadenosine (adenylate cyclase inhibitor, 10 μM) were added 5 min before the addition of cumulative concentrations of gnaphaliin A or gnaphaliin B, whereas nitroprusside (guanylate cyclase activator, 10 μM) or forskolin (adenylate cyclase activator, 10 μM) were added and immediately cumulative concentrations of gnaphaliin A or gnaphaliin B were added.

Inhibition of contraction induced by calcium

After the stabilization period, the trachea rings were incubated in a Krebs solution free of calcium. Each ring was depolarized

with a solution of potassium (80 mM) and immediately washed. This procedure was repeated every 15 min during 1 h. At the last addition of potassium solution the tissue was not washed and cumulative concentrations of calcium (0.1, 0.31, 1, 3.1, 10 mM) were added to contract the tissue in the presence or absence of gnaphaliin A or gnaphaliin B (0.1, 1.0, 10, 100 μM).

Rat aorta preparation

Male Wistar rats were killed in a CO_2 chamber and the thoracic aorta was removed and immediately immersed in a Krebs solution at 37°C. After removal of excess connective tissue and fat, the aorta was divided into eight small rings of about 2 mm in length. Each aorta ring was hung between two nichrome hooks inserted into the lumen and placed in a 10-ml organ bath containing Krebs solution at 37°C and bubbled constantly with 5% CO_2/95% O_2. The tissue stabilization period and data recording were carried out as described for guinea-pig trachea preparation. After the stabilization period the rings were contracted with norepinephrine (0.1 μM) twice at 30-min intervals. The integrity of the endothelium was verified by the relaxant response to ACh (10 μM); intact endothelium showed relaxation over 84%, whereas the endothelium denuded aorta did not relax with ACh. After the stabilization period the aorta rings were subjected to one of three protocols.

Relaxant effect

Intact endothelium rings or denuded aorta were contracted with norepinephrine (0.1 μM) and then cumulative concentrations of gnaphaliin A, gnaphaliin B, rolipram, sildenafil, aminophylline or enoximone (0.0001–100 μM) were added. The effective concentration to relax 50% of the norepinephrine induced contraction was expressed as the EC50.

Relaxant effect in the presence of L-NAME

The rings were incubated with L-NAME (100 μM) 20 min before the contraction of the tissue with norepinephrine (0.1 μM) and then cumulative concentrations of gnaphaliin A and gnaphaliin B (0.0001–100 μM) were added.

Inhibition of contraction induced by calcium

For this evaluation the same procedure described above for the guinea-pig trachea model it was followed.

PDE activity

The enzyme inhibition assay was performed using an adaptation of the BIOMOL cyclic nucleotide PDE assay kit, consisting of a colorimetric non-radioactive assay designed in a microplate format. The basis for this assay is the cleavage of cAMP or cGMP by a cyclic nucleotide PDE. The 5′-nucleotide released is further cleaved into the nucleoside and phosphate by the enzyme 5′-nucleotidase. The phosphate released due to enzymatic cleavage was quantified using BIOMOL Green reagent in a modified malachite green assay.[13] It may be used to screen inhibitors and modulators of cyclic nucleotide PDE activity. The compounds and drugs were tested at different concentrations: 1.0–300 μM gnaphaliins A or B, 3.0–300 μM rolipram, 0.001–300 μM sildenafil, 0.03–3.0 mM aminophylline, 0.03–3.0 mM enoximone or 5–300 μM IBMX.

Molecular modelling (Docking)

Blind docking was carried out with AutoDock4 software (http://autodock.scripps.edu/)[14,15] using the default parameters, except for the number of GA runs (100) and the Lamarkian genetic algorithm with local search and 25 million energy evaluations per run. A parallel supercomputer of distributed memory was used; it contains 1368 processors AMD Opteron, around 3 terabytes of memory and 160 terabytes of storage (http://www.super.unam.mx/). The target in each docking run was the X-ray structure of PDE 5 (2H42.pdb) obtained from the Protein Data Bank (http://www.pdb.org). To generate more accurate and physically realistic models of the target, after several iterations of rebuilding and refinement, a final all-atom refinement with the idealization application of the Rosetta 3.1 release was performed (http://www.rosettacommons.org). This application rebuilds molecules using ideal bond lengths, bond angles, and torsion angles. The starting conformation of the ligand was an energy minimized form with geometric optimization using the program HyperChem 8 release (Hypercube Inc., Gainesville, FL, USA). Sildenafil, gnaphaliin A, gnaphaliin B, IBMX, rolipram, aminophylline, enoximone and PDE 5 protein were first prepared by AutoDockTools 1.5.4 (http://mgltools.scripps.edu/), adding polar hydrogen atoms, as well as Kollman and computing Gasteiger charges to PDE5. The protein was held rigid during the docking process. The grid box Å size was 126 Å × 126 Å × 126 Å in the x, y and z dimensions, with the centre of the grid corresponding to the Å protein, and later was 40 Å × 40 Å × 40 Å in the x, y and z dimensions, with the centre of the grid corresponding to the ligand.

Statistical analysis

The EC50 values for relaxant effect were calculated by linear regression.[16] All values are shown as mean ± SEM of at least six experiments. The differences among obtained values were statistically calculated by one-way analysis of variance, and then determined by Dunnett's *t*-test.[17] $P < 0.05$ was considered statistically significant. The inhibitory activities of PDE by gnaphaliins or reference drugs are reported as IC50 ± SEM of at least three experiments.

Results

Relaxant effect of gnaphaliins on guinea-pig trachea and rat aorta

Gnaphaliin A and gnaphaliin B relaxed carbachol precontracted (3 μM) guinea-pig tracheal rings (EC50 values of 181.58 ± 2.22 and 128.36 ± 1.88 μM, respectively; Figure 2) and noradrenaline precontracted (0.1 μM) rat aortic rings in a concentration-dependent manner (EC50 values of 1.53 ± 0.26 and 5.36 ± 0.61 μM, respectively; Figure 3). The rat aorta tissue was more sensitive than guinea-pig trachea in its response to the relaxant effect induced by gnaphaliins and PDE inhibitors (Table 1). Gnaphaliin A and B were more

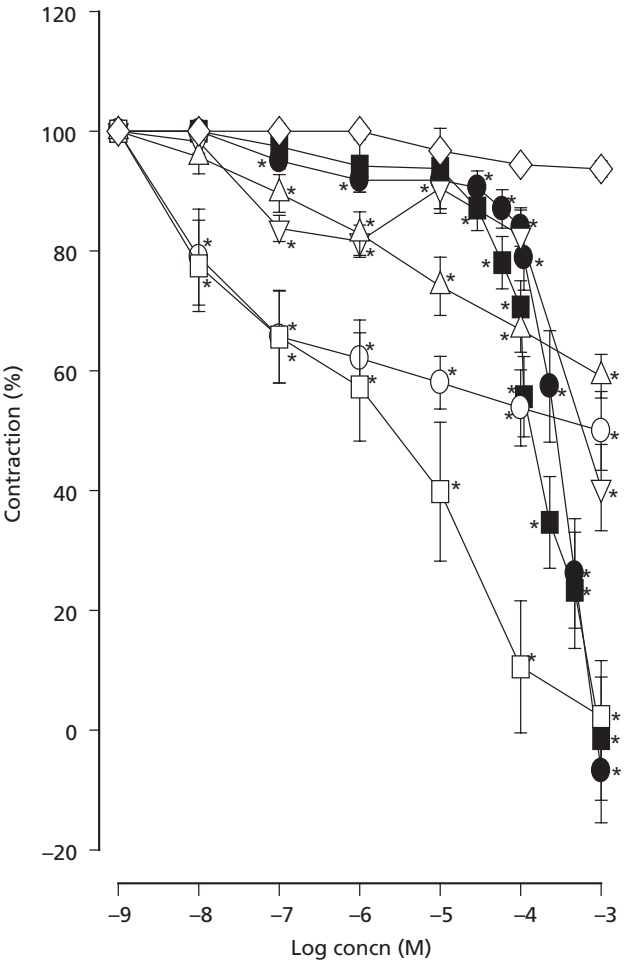

Figure 2 Relaxant effects of gnaphaliins on guinea-pig trachea. Relaxant effects of sildenafil (□), rolipram (○), enoximone (△), gnaphaliin B (■), gnaphaliin A (●), aminophylline (▽) and control (◇) on carbachol (3 μM) induced precontraction in guinea-pig trachea. Each point represents the mean ± SEM, $n = 6$. *$P < 0.05$, significantly different compared with the respective control at the same time (Dunnett's *t*-test after analysis of variance).

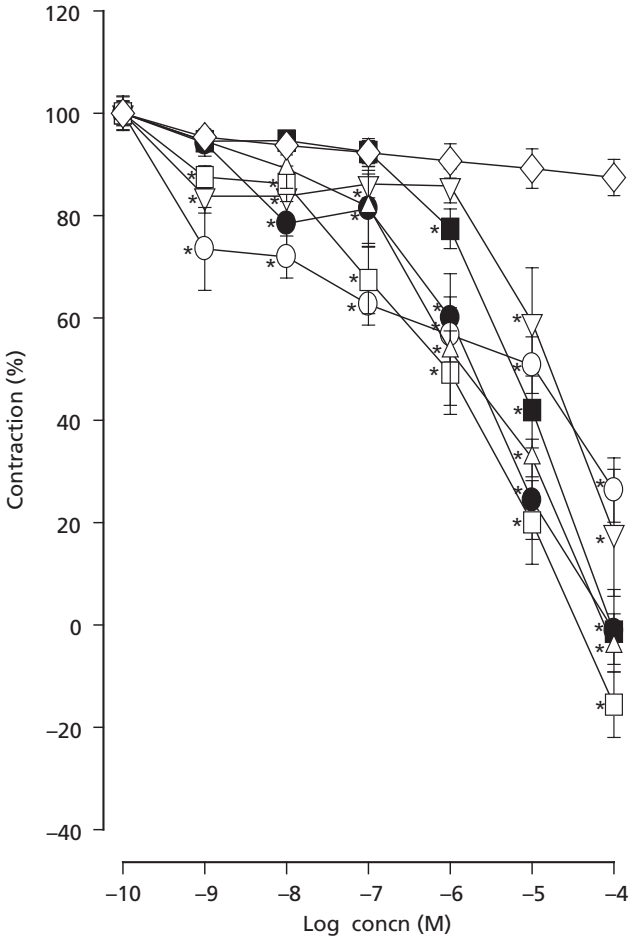

Figure 3 Relaxant effects of gnaphaliins on rat aorta. Relaxant effects of sildenafil (□), rolipram (○), enoximone (△), gnaphaliin B (■), gnaphaliin A (●), aminophylline (▽) and control (◇) on norephinephrine (0.1 μM) induced precontraction in rat aorta. Each point represents the mean ± SEM, $n = 6$, *$P < 0.05$, significantly different compared with the respective control at the same time (Dunnett's *t*-test after analysis of variance).

potent in the relaxation of guinea-pig trachea than enoximone (PDE-3 inhibitor), rolipram (PDE-4 inhibitor) and aminophylline (unspecific PDE inhibitor), but they were less potent than sildenafil (PDE-5 inhibitor). Both flavones were slightly more potent than aminophylline in the relaxation of rat aorta, showed similar potency to enoximone and rolipram, but were less potent than sildenafil in this model (Table 1).

Effect of gnaphaliins on extracellular Ca^{2+} induced contraction activated by KCl

In the Ca^{2+}-free solution plus 80 mM KCl, cumulative addition of $CaCl_2$ (0.1–10 mM) induced a stepwise tension increase in tracheal and aortic rings. Gnaphaliin A (Figure 4a) and gnaphaliin B (Figure 4c) attenuated $CaCl_2$ induced contractions in the control group in a non-parallel manner and depressed its maximal response, suggesting that Ca^{2+} influx was reduced by these flavones. The flavones were more active at inhibiting the Ca^{2+} influx in guinea-pig trachea than the Ca^{2+} influx in rat aorta (Figure 4b and 4d). At 100 μM both flavones were able to completely inhibit the calcium induced contractions in guinea-pig trachea, whereas gnaphaliin A and B inhibited only 47 and 21% of the calcium induced contractions in rat aorta tissue, respectively (Figure 4b and 4d).

Role of nitric oxide synthase on relaxation induced by gnaphaliins

The presence or absence of endothelium did not alter the relaxant effect of gnaphaliin A (Figure 5a) or gnaphaliin B (Figure 5b) on rat aorta. Also, the pretreatment with L-NAME (a nitric oxide synthase inhibitor, 100 μM) did not inhibit the relaxant effect of both gnaphaliins in this tissue (Figure 5a and 5b). Similarly, L-NAME did not inhibit the relaxant effect of gnaphaliins in guinea-pig tracheal rings (Table 2).

Table 1 Relaxant effects of gnaphaliin A, gnaphaliin B and phosphodiesterase inhibitors on smooth muscle

	Guinea-pig trachea	Rat aorta
Gnaphaliin A	181.58 ± 2.22 ($r^2 = 0.972$)	1.53 ± 0.26 ($r^2 = 0.991$)
Gnaphaliin B	128.36 ± 1.88 ($r^2 = 0.977$)	5.36 ± 0.61 ($r^2 = 0.996$)
Enoximone	>1000	1.49 ± 0.30 ($r^2 = 0.901$)
Rolipram	>1000	2.26 ± 0.72 ($r^2 = 0.914$)
Sildenafil	1.02 ± 0.48 ($r^2 = 0.940$)	0.28 ± 0.15 ($r^2 = 0.931$)
Aminophylline	363.15 ± 18.04 ($r^2 = 0.923$)	13.42 ± 4.17 ($r^2 = 0.984$)

EC50 (μM) values are presented as the means ± SEM, $n = 6$.

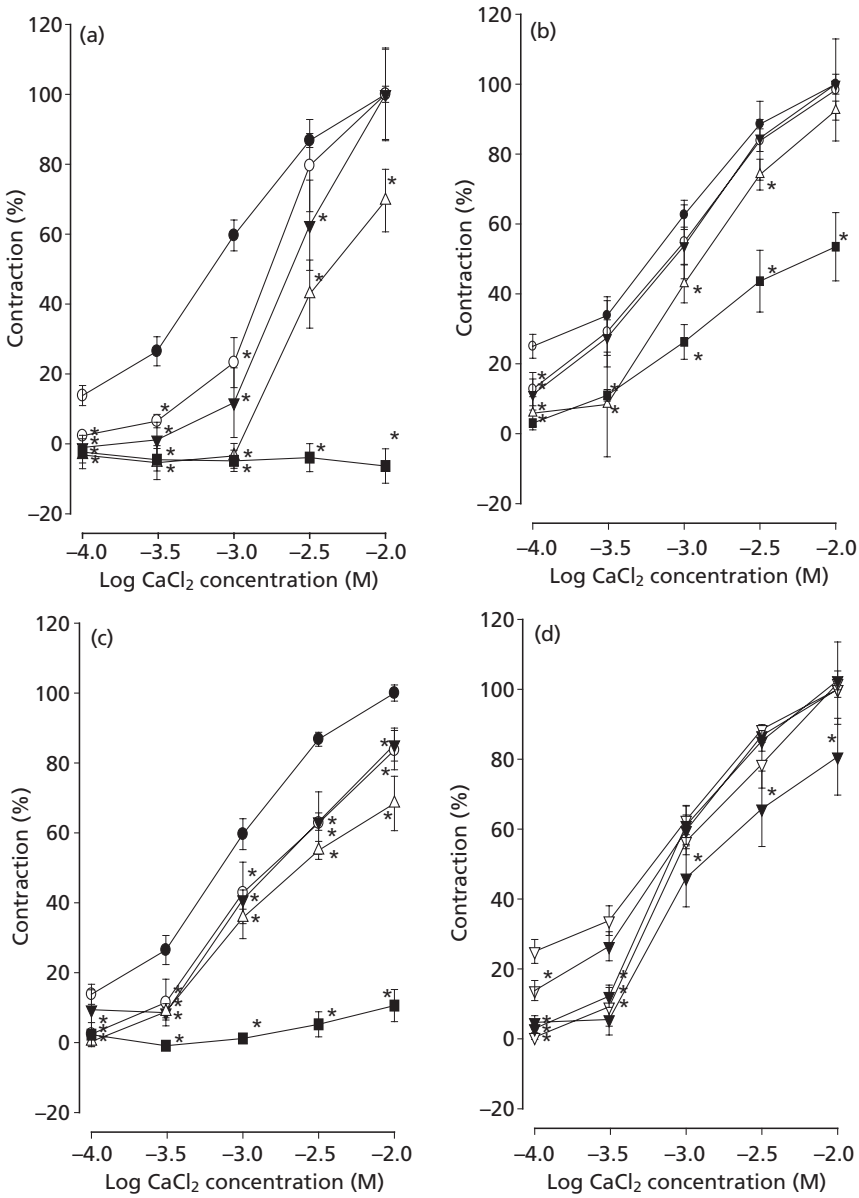

Figure 4 Effects of gnaphaliins on extracellular Ca^{2+} induced contraction activated by KCl. Inhibition of the contraction induced by calcium chloride (0.1, 0.31, 1, 3.1 and 10 mM) in the presence of gnaphaliin A (a and b) or gnaphaliin B (c and d) at 0.1 (○),1 (▼),10 (▲) and 100 (■) μM in guinea-pig trachea (a and c) and rat aorta (b and d). Each point represents the mean ± SEM, $n = 6$. *$P < 0.05$, significantly different compared with the contraction curve of calcium at the same concentration (Dunnett's t-test after analysis of variance).

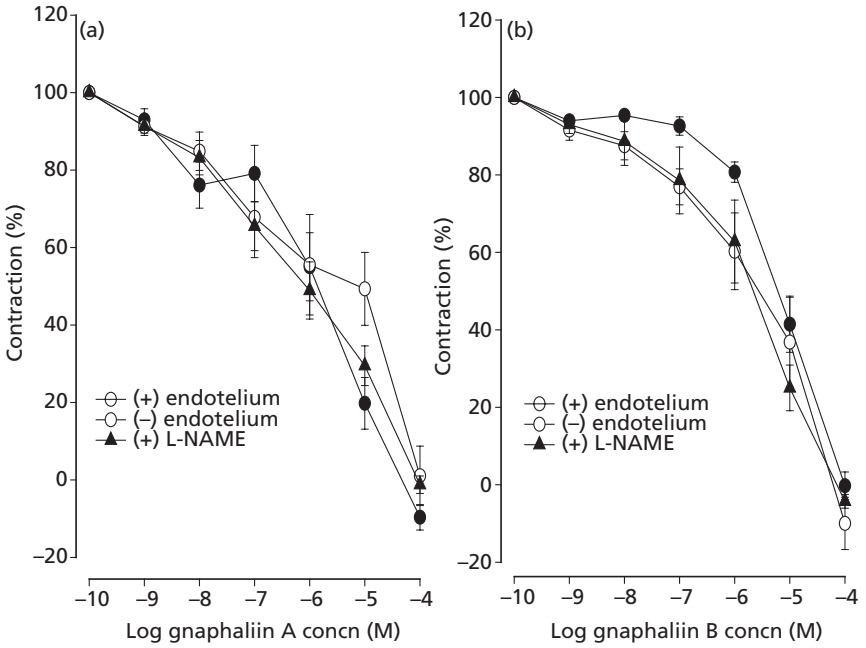

Figure 5 Role of nitric oxide synthase on the relaxation induced by gnaphaliins. Influence of the endothelium and L-NAME on the relaxant effects of gnaphaliin A (a) and gnaphaliin B (b) in rat aorta precontracted with norepinephrine (0.1 μM). Each point represents the mean ± SEM, $n = 6$.

Table 2 Relaxant effect of gnaphaliins in carbachol (3 μM) precontracted guinea-pig tracheal rings in the presence or absence of L-NAME, ODQ, nitroprusside, 2′,5′-dideoxyadenosine or forskolin

Treatment	Gnaphaliin A	Gnaphaliin B
Gnaphaliin alone	191.45 ± 15.11	138.12 ± 7.12
Gnaphaliin + L-NAME (100 μM)	208.33 ± 12.48	145.83 ± 17.62
Gnaphaliin + ODQ (10 μM)	254.66 ± 10.49**	182.78 ± 19.04*
Gnaphaliin + nitroprusside (10 μM)	6.77 ± 1.57**	4.78 ± 1.38**
Gnaphaliin + 2′,5′-dideoxyadenosine (10 μM)	247.39 ± 26.88	158.44 ± 22.18
Gnaphaliin + forskolin (10 μM)	16.22 ± 2.07**	21.41 ± 7.49**

EC50 (μM) values are presented as the mean ± SEM of six experiments. *$P < 0.05$, **$P < 0.01$, significantly different compared with the group treated only with the respective gnaphaliin (Dunnett's *t*-test after analysis of variance).

Role of guanylate cyclase and adenylate cyclase in relaxation induced by gnaphaliins

ODQ (10 μM), a soluble guanylate cyclase inhibitor, significantly increased the EC50 value of gnaphaliin A and gnaphaliin B in guinea-pig tracheal rings (Table 2). 2′,5′-Dideoxyadenosine, an adenylate cyclase inhibitor, did not modify the EC50 values of either gnaphaliin (Table 2). However, sodium nitroprusside (10 μM), a guanylate cyclase activator and forskolin (10 μM), an adenylate cyclase activator, potentiated the relaxant effect of both gnaphaliins (Table 2).

PDE inhibitory effect of gnaphaliins

Gnaphaliin A and gnaphaliin B showed a concentration-dependant inhibitory effect on PDE enzymes. The IC50 values for these flavones and for rolipram, sildenafil, aminophylline, enoximone and IBMX are shown in Table 3. The gnaphaliins and the other PDE inhibitors evaluated were more potent as PDE inhibitors when cGMP was used as substrate than when cAMP was used. For cAMP as substrate, the PDE inhibitory activity order was: sildenafil > IBMX > gnaphaliin B > rolipram > gnaphaliin A > aminophylline and enoximone. For cGMP as substrate, the activity order was: sildenafil > rolipram > gnaphaliin A > gnaphaliin B > aminophylline > IBMX and enoximone (Table 3).

Molecular docking

Table 4 shows the results of the docking study for gnaphaliin A and B, and PDE inhibitors. All compounds bind to the same pocket, which corresponds to the binding site of sildenafil. Each docking run predicted a binding conformation with an associate value for estimated free energy of binding in kcal/mol and an estimated inhibition constant (K_i) in molar concentration. The K_i values for sildenafil, gnaphaliin A, gnaphaliin B, rolipram, aminophylline and enoximone were over the range 0.045–29.52 μM. The residues Typ612, Asn661, Asp764, Leu765, Ala767, Ile768, Ile778, Val782, Phe786, Gln817 and Phe820 interact with sildenafil and they

Table 3 Phosphodiesterase inhibitory effects of gnaphaliin A, gnaphaliin B and phosphodiesterase inhibitors

	cAMP	cGMP
Gnaphaliin A	120.798 ± 26.599 (r^2 = 0.980)	2.963 ± 0.350 (r^2 = 0.985)
Gnaphaliin B	72.153 ± 11.858 (r^2 = 0.993)	6.454 ± 2.293 (r^2 = 0.933)
Sildenafil	0.116 ± 0.016 (r^2 = 0.997)	0.0037 ± 0.0008 (r^2 = 0.991)
Aminophylline	>3000	182.770 ± 20.386 (r^2 = 0.989)
Rolipram	74.877 ± 9.190 (r^2 = 0.995)	2.866 ± 0.739 (r^2 = 0.986)
IMBX	21.347 ± 4.411 (r^2 = 0.899)	>300
Enoximone	>3000	>3000

IC50 (μM) values are presented as the means ± SEM, n = 3.

Table 4 Theoretical parameters of phosphodiesterase inhibitors

Compound	K_i (μM)	EFEB (kcal/mol)
Sildenafil	0.045	−10.02
Gnaphaliin A	0.908	−8.24
Gnaphaliin B	1.55	−7.93
IBMX	29.52	−6.18
Rolipram	0.288	−8.92
Aminophylline	340	−4.73
Enoximone	6.04	−7.12

EFEB, estimating free energy of binding. Sildenafil K_i theoretical value 14.7 nM (Kim *et al*.).[18]

correspond to binding sites in the PDE 5–sildenafil complex in the crystallographic structure.[19] The residues involved in the interaction with gnaphaliin A were Leu765, Ala767, Ile768, Gln775, Val782, Phe786, Met816, Gln817 and Phe820. This compound forms an H-bond with Leu765. For Gnaphaliin B the residues involved in the interaction were Ala767, Ala779, Val782, Phe786, Leu804, Ile813, Met816, Gln817 and Phe820. Figure 6 shows the theoretical binding model for sildenafil, gnaphaliin A and gnaphaliin B, superimposed on the X-ray structure of the protein with the classical inhibitor of PDE 5, sildenafil.

Discussion

This work demonstrated that the relaxant effect of gnaphaliin A and B is predominantly due to the inhibition of PDE activity (Table 3). When cAMP was used as substrate, gnaphaliin B was slightly more potent than gnaphaliin A, whereas when cGMP was used as substrate, gnaphaliin A was 2-times more potent than gnaphaliin B. These results suggest that small structural changes in these flavones have an effect on preference for one or other catalytic site of PDE enzymes. The enzymatic kit used in this work constituted a mixture of PDE isoforms as it is known that PDE-1 preferentially inactivates cGMP and cAMP, PDE-4 and PDE-3 preferentially inactivate cAMP, and PDE-5 preferentially inactivates cGMP.[20] Enoximone did not inhibit the degradation of cAMP or cGMP, suggesting that this enzymatic kit does not contain PDE isoform type 3 and indirectly suggests that neither gnaphaliin inhibited PDE-3. The preference to inhibit degradation of cGMP suggests that both gnaphaliins could inhibit PDE-1, PDE-5 or both isoforms, however an additional study using isolated PDEs of each type would be necessary to determine if these flavones are able to inhibit a specific type of PDE.

The levels of the nucleotides cAMP and cGMP are regulated by the presence of PDEs in airway smooth muscle[12] and vascular smooth muscle,[10] and the relaxation of these tissues is related to the increase or maintenance of the levels of these second messengers.[20] Gnaphaliins A and B were more potent in the relaxation of both vascular smooth muscle and airway smooth muscle than aminophylline, a drug used to treat asthma.[12] A more important difference between these flavones and aminophylline was observed in their ability to inhibit PDE when cGMP was used as substrate: gnaphaliin A and gnaphaliin B were 60- and 28-times more active than aminophylline, respectively (Table 3). Likewise, gnaphaliins A and B were more active than aminophylline when cAMP was used as substrate (aminophylline was not active). In relation to rolipram, Turner *et al*[21] reported that this drug is able to completely relax guinea-pig trachea, however in our experiments rolipram showed less than 50% relaxant effect. This discrepancy could be due to the concentrations of carbachol used to contract the guinea-pig trachea: whereas Turner *et al*[21] used 0.1 μM CCh, we used 3 μM. On the other hand, the activity of both gnaphaliins was over the same range as rolipram in the relaxation of rat aorta (Table 1) and the inhibition of PDEs (Table 3). While enoximone, a PDE-3 inhibitor, was unable to relax the guinea-pig trachea (Figure 2), it showed relaxant activity on rat aorta at a similar potency to gnaphaliin A and it was slightly more potent than gnaphaliin B (Figure 2). These results suggest that PDE 3 is possibly not involved in the relaxation of airway smooth muscle in guinea-pig trachea, but it is involved in vascular smooth muscle relaxation in rat aorta where it is well documented that the relaxant activity is related to PDE 1 and 3 activity.[10] Sildenafil showed the greatest relaxant activity on both guinea-pig trachea and rat aorta, and inhibition of PDE (Figure 2; Table 3). These results emphasize the importance of PDE type 5 and cGMP levels on the relaxation of guinea-pig trachea, despite the fact that only the presence of PDE type 3 and 4 has been reported in this tissue.[8] The presence of PDE type 5 has been well documented in vascular smooth muscle,[11] where gnaphaliins and PDE inhibitors were more active. The relaxant effect of sildenafil in vascular smooth muscle is accompanied by the production of nitric oxide.[11] In contrast, the pretreatment with L-NAME, an inhibitor of nitric oxide synthase, did not modify the relaxant effect of gnaphaliins A and B in vascular smooth muscle (Figure 5) and airway smooth muscle (Table 2), suggesting that these flavones did not

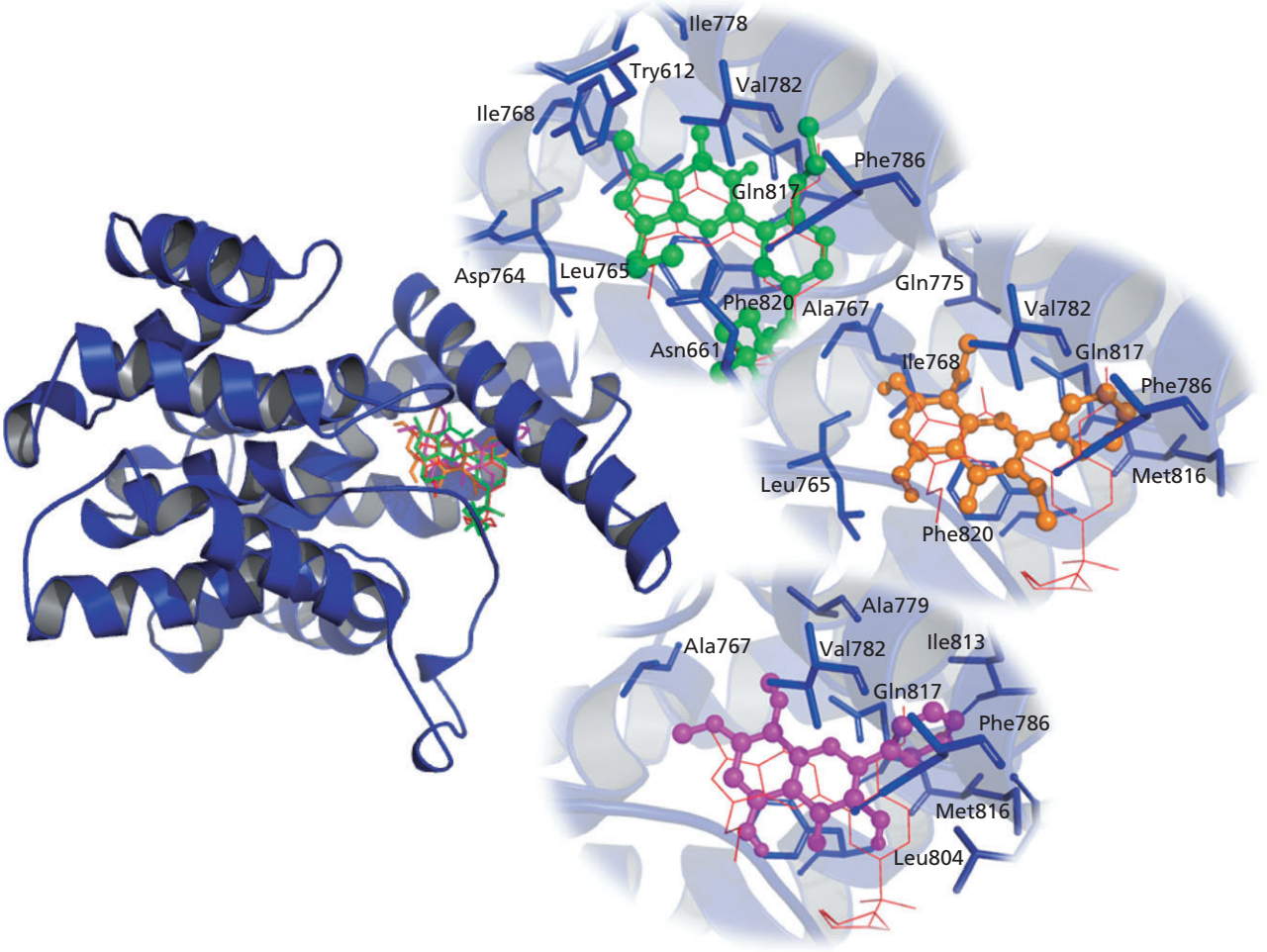

Figure 6 Docking of the phosphodiesterase 5 with sildenafil, gnaphaliin A and gnaphaliin B. The red line shows the crystallized sildenafil with phosphodiesterase 5, sildenafil modelling is depicted in green (positive control), gnaphaliin A and B are shown in orange and magenta, respectively. Residues involved in the interaction with ligands are showed in blue sticks.

induce their relaxant effect via nitric oxide. In addition the relaxant effect of gnaphaliins was not altered by the absence of endothelium in vascular smooth muscle, suggesting that the relaxant effect of these natural products is not dependant of the relaxation controlled by endothelium in this tissue.

It is well known that sodium nitroprusside and endogenous nitric oxide activate soluble guanylate cyclase which increases cGMP levels and relaxes airway smooth muscle.[22] Sodium nitroprusside potentiated the relaxant effect of both gnaphaliins, and ODQ, an inhibitor of soluble guanylate cyclase, blockaded this relaxant effect in airway smooth muscle (Table 2). These results are in agreement with the PDE inhibitory effect of the gnaphaliins (Table 3), because the activation of guanylate cyclase by nitroprusside increases the levels of cGMP and gnaphaliins inhibit its degradation, thus increasing the relaxant effect. Similarly, forskolin increases adenylate cyclase activity[23] and as a consequence cAMP levels increase and the relaxant activity of gnaphaliins is potentiated due to its PDE inhibitory activity (Table 2). The 2′,5′-dideoxyadenosine, an adenylate cyclase inhibitor, decreased the activity of gnaphaliins but this decrease was not significant (Table 2). These results reveal that cGMP is more important than cAMP in the relaxant effect of gnaphaliins and provide additional support to the importance of PDE type 5 and cGMP levels to relax guinea-pig trachea. The docking analysis provides additional support for this. The binding model for gnaphaliins A and B with PDE type 5 indicates that these compounds bind to the same binding site as the classical PDE 5 inhibitor sildenafil. To validate the docking simulation procedure, the docked conformation of sildenafil was compared with the crystalline structure of the PDE 5–sildenafil complex (2H42.pdb), and this conformation of sildenafil was overlapped in the same binding pocket of the crystal complex (Figure 6). This validation showed that the docking procedure was reasonable in identifying the binding conformation of the inhibitors. The affinity of compounds according to estimated K_i was: sildenafil > rolipram > gnaphaliin A > gnaphaliin B > enoximone > aminophylline > IBMX. The estimated K_i values for sildenafil, gnaphaliin A and gnaphaliin B (Table 4) showed a good

correlation ($r^2 = 0.982$) with the respective IC50 values to inhibit PDE when cGMP was used as substrate (Table 3). The correlation between theoretical and experimental data suggests that gnaphaliin A and B are PDE inhibitors with high specificity for PDE type 5. The residues Ala767, Val782, Phe786, Gln817 and Phe820 are common residues for the interaction of sildenafil, gnaphaliin A and gnaphaliin B with PDE 5. In addition, gnaphaliin A and sildenafil have two common additional residues, Leu765, Ile768. This can be related to the greater affinity of gnaphaliin A for PDE 5 compared with gnaphaliin B (Table 4), and with the greater PDE inhibitory activity of gnaphaliin A compared with gnaphaliin B when cGMP was used as substrate (Table 3).

In addition to PDE inhibition as a relaxant mechanism of gnaphaliins, these flavones also showed inhibition of calcium influx in guinea-pig trachea, although the inhibition was not significant in rat aorta (Figure 4). It is well known that calcium is the major promoter of contractions in airway smooth muscle and vascular smooth muscle.[24] The inhibition of calcium induced contractions is a mechanism described for several flavonoids in rat aorta and guinea-pig trachea.[8,10,25] In smooth muscle, the inhibition of calcium influx promotes relaxation that is initiated by decreasing intracellular calcium concentrations of $[Ca^{2+}]_i$ due to Ca^{2+} uptake by the sarcoplasmic reticulum and Ca^{2+} extrusion by the plasmalemmal Ca^{2+} pump and Na^{+}-Ca^{2+} exchanger. The decrease in $[Ca^{2+}]_i$ causes dissociation of the Ca^{2+}–CAM complex and the dephosphorylation of myosin light chain by MLC phosphatase.[26] A relationship between the PDE inhibitory effect of gnaphaliins and their effect to inhibit calcium influx can be recognized because it is known that cAMP activates protein kinase A and cGMP activates protein kinase G. These active protein kinases decrease the global influx of calcium with the resulting inhibition of the contraction.[22] To our knowledge, there are no studies of PDE inhibitor drugs that have demonstrated the inhibition of calcium influx as observed with gnaphaliins as PDE inhibitors. Therefore further studies are needed to determine if other PDE inhibitors also inhibit calcium influx in guinea-pig trachea.

Conclusions

Gnaphaliin A and gnaphaliin B showed relaxant effects in guinea-pig trachea and rat aorta through the inhibition of PDE with a preference to inhibit the degradation of cGMP and by inhibition of calcium influx. According to docking analysis, these flavones bind with high specificity to the same binding site as sildenafil at PDE type 5. Finally, the results highlight the potential use of these compounds and other flavonoids to treat diseases related to the contraction of vascular and tracheal smooth muscle.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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