7-Epiclusianone, a tetraprenylated benzophenone, relaxes airway smooth muscle through activation of the nitric oxide-cGMP pathway

Luciana Pontes Coelho, Magda Fráguas Serra, Ana Lúcia de Aguiar Pires, Renato Sérgio Balão Cordeiro, Patrícia Machado Rodrigues e Silva, Marcelo Henrique dos Santos and Marco Aurélio Martins.

Laboratory of Inflammation, Oswaldo Cruz Institute, Oswaldo Cruz Foundation (FIOCRUZ), Rio de Janeiro, RJ, Brazil (LPC, MFS, ALAP, SBC, PMRS, MAM).

Laboratory of Phytochemistry and Medicinal and Chemistry, Department of Pharmacy, Alfenas, Federal University of Alfenas, MG, Brazil (MHdS).
Running Title: 7-Epiclusianone relaxes airway smooth muscle.

Corresponding Autor:

Marco Aurélio Martins, PhD
Laboratory of Inflammation, Oswaldo Cruz Institute, Oswaldo Cruz Foundation (FIOCRUZ), Av. Brasil,4365, Manguinhos, CEP. 21045-900, Rio de Janeiro, RJ, Brazil.
E-Mail: mmartins@ioc.fiocruz.br Tel. + 55 21 25730844 FAX: + 55 21 25587382

Abbreviations: dimethylsulfoxide (DMSO), ethyleneglycol-bis-(β-aminoethyl)-N,N,N',N'-tetraacetic acid (EGTA), 5-hydroxytryptamine (5-HT), Nω-nitro-Larginine methyl ester (L-NAME), tetraethylammonium (TEA), iberitoxin (IbTX) and 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), nitric oxide synthase (NOS), soluble guanylate cyclase (sGC), adenosine 3’, 5’-cylcis monophosphate (cAMP), guanosine 3’, 5’-cyclic monophosphate (cGMP), hematoxilin-eosine (HE), trichloroacetic acid (TCA)

Recommended section assignment: Inflammation, Immunopharmacology and Asthma
Abstract

This study was undertaken in order to investigate the putative mechanism(s) underlying the antispasmodic effect of 7-epiclusianone, a naturally occurring compound isolated from the plant *Garcinia brasiliensis*. Guinea-pig tracheal rings were mounted in tissue baths filled with Krebs solution, and the contractile response to distinct stimuli measured in presence or absence of 7-epiclusianone. We also tested the effect 7-epiclusianone on methacholine-evoked airways obstruction in Balb/c mice using barometric plethysmography. 10 µM 7-epiclusianone inhibited epithelium-intact tracheal ring contraction induced by allergen, histamine, 5-HT or carbachol challenge. The relaxation effect was abrogated by epithelium removal, the presence of NOS inhibitor L-NAME (100 µM) or sGC inhibitor ODQ (10 µM). 7-epiclusianone (1-100 µM) induced a dose-dependent increase in the intracellular cGMP levels of cultured tracheal rings. The relaxation effect of 7-epiclusianone was also inhibited by K⁺ channel blockers TEA (10 µM), glibenclamide (1 µM) or apamin (1 µM), but not by SQ22.536 (100 µM), an adenylate cyclase inhibitor. In epithelium-intact tracheal rings, 7-epiclusianone also inhibited Ca²⁺-induced contractions in K⁺ (60 mM)-depolarized preparations, but appeared ineffective in assays in which epithelium-denuded tracheal ring preparations were employed. Oral administration of 7-epiclusinone (25-100 mg/Kg) dose-dependently inhibited airway obstruction triggered by aerosolized methacholine (6-25 mg/ml), in a mechanism sensitive to L-NAME (20 mg/Kg). In conclusion, the relaxation effect of 7-epiclusinone seems to be mediated by epithelium-, nitric oxide- and cGMP-dependent mechanisms. Furthermore, oral administration of 7-epiclusianone reduces episodes of bronchial obstruction, warranting further research on this compound regarding a putative application in asthma therapy.
Introduction

Ginkgo biloba, Artocarpus heterophyllus, Allium sativum, Glycyrrhiza glabra and Panax ginseng Ginseng are examples of plant species that have been employed in the so-called complementary and alternative medicine as herbal formulations, and for which a mechanism of action has been identified. The use of herb preparations for medicinal purposes has increased in popularity in recent years, and is already responsible for a 5 billion dollar market in the United States (Kaplan et al., 2007). The premise is that plants contain natural substances with marked health-promoting properties, though overall limited scientific data supporting it are available (Phillipson, 2001).

Garcinia brasiliensis is a native plant of the Amazon region, typically tropical in distribution, and found widespread in the Brazilian territory. The fruit of this species has been used in the alternative medicine for the treatment of peptic ulcer, urinary and tumor diseases (Corrêa, 1926). Garcinia species are a rich source of oxygenated and prenylated phenol derivatives (Bennett and Lee, 1988) and some of them has been demonstrated to exhibit antifungal, anti-inflammatory, antitumoral, anti-oxidant and antilipidemic properties (Gopalakrishnan et al., 1997; Diaz-Carballo et al., 2003; Hay et al., 2004).

The pharmacological study of polyisoprenylated benzophenones has been shown to be of interest due to the wide spectrum of biological activities they provide and their putative applications in clinical conditions. Garcinol, isolated from the Garcinia indica fruit rind, is so far the best studied compound of this class. It is a potent inhibitor of histone acetyltransferases, and thereby gene expression (Balasubramanyam et al., 2004), being considered an interesting prototype in drug development for challenging diseases including cancer, atherosclerosis and HIV.

Prior studies reveal that 7-epiclusianone, a tetraisoprenylated benzophenone, first isolated from Rheedia gardneriana Miers ex Planch & Triana (Santos et al., 1998),
exhibits potent endothelium-dependent vasodilator effect on rat aortic rings (Cruz et al., 2006). We have further demonstrated that 7-epicusianone, isolated from the fruit pericarp of *Garcinia brasiliensis*, is also a potent inhibitor of allergen-induced tissue histamine release and ileum contraction triggered by either allergen, histamine or acetylcholine (Neves et al., 2007), giving support to the interpretation that 7-epicusianone should be further investigated as a putative lead compound in the context of allergic diseases and asthma therapy.

The present study was conducted to examine the putative mechanism(s) underlying the antispasmodic effect of 7-epicusianone on isolated guinea-pig trachea ring preparations. The potential effect of the oral treatment of 7-epicusianone in the bronchospasm triggered by aerosolized methacholine in Balb/c mice was also investigated.
Methods

Animals

Male guinea-pigs (300–400 g) and both male and female Balb/c mice (18-20 g) were provided by the Oswaldo Cruz Foundation breeding (Rio de Janeiro, Brazil). They were housed under conditions of constant temperature and controlled illumination. Food and water were available ad libitum. All the protocols and experimental procedures involving animal in this study were examined and approved by the Committee on Use of Laboratory Animals of the Oswaldo Cruz Foundation (License number - CEUA-FIOCRUZ, Prot. 00085-02).

Drugs

Sodium chloride, potassium chloride, potassium dihydrogen phosphate, sodium hydrogen carbonate, magnesium sulfate heptahydrate, calcium chloride dehydrate and dimethylsulfoxide (DMSO) were purchased from Merck, Darmstadt, Germany. Glucose, ethyleneglycol-bis-(β-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), histamine, 5-hydroxytryptamine (5-HT), ovalbumin, carbachol, methacholine, theophylline, Nω-nitro-Larginine methyl ester (L-NAME), tetraethylammonium (TEA), glibenclamide, apamin, iberitoxin (IbTX) and 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 7-epiclusianone was isolated from the *Garcinia brasiliensis* fruit pericarp, as reported (Neves et al., 2007), and provided by the Laboratory of Phytochemistry and Medicinal and Chemistry, Department of Pharmacy, Alfenas, Federal University of Alfenas, MG, Brazil.

All solutions were freshly prepared in distillated water, dimethylsulfoxide (DMSO, final concentration of 0.1%) or Tween 80 (final concentration of 0.2%) and protected from the light.
Guinea-pigs used for the anaphylactic contraction assay were previously sensitized with a subcutaneous injection of a saline suspension containing 50 µg ovalbumin and 5 mg of Al(OH)₃ in a final volume of 0.2 mL. Animals were killed in a CO₂ atmosphere 14 days after sensitization for tracheal removal, which was quickly immersed in nutritional solution of Krebs (mM: NaCl 118, KCl 4.8, CaCl₂ 2.5, MgSO₄ 1.2, KH₂PO₄ 1.2, NaHCO₃ 24 and glucose 11). Tracheas were dissected free of adhering fat and connective tissue, and cut into rings. These tracheal rings were mounted in isolated organ baths filled with 10 ml of Krebs solution, maintained at 37°C and aerated with 95% O₂ and 5% CO₂. To achieve a steady spontaneous tone level, an initial tension of 1 g was applied. Contractions were measured isometrically with a force-displacement transducer (Ugo Basile, Italy) and recorded by an Isolated Organs Data Acquisition program (Proto5, Letica Scientific Instruments).

**Experimental protocols**

Tissues were allowed to stabilize for 60 min, while the bathing solution was exchanged at 10 min intervals. At the end of the equilibration period, the response to carbachol (2.5 µM) was recorded. After washout of carbachol and re-establishment of stable baseline tone, tissues were exposed to either carbachol (10⁻⁸-10⁻⁴ M), histamine (10⁻⁷-10⁻³ M), 5-HT (10⁻⁸ - 3 x 10⁻⁵ M) or antigen (ovalbumin, 0,001-100 µg/mL) in the presence or absence of 10 µM 7-epiclusianone. The preparations were pre-incubated with 7-epiclusianone 15 min before addition of the spasmodic agents. All responses were expressed as percentage of response to 2.5 µM carbachol.

In some experiments, the epithelial cells were removed mechanically by rubbing the internal tracheal surface with a fine silver wire (200 µm diameter) as previously described (Wu et al., 2004). The removal of the epithelial layer was confirmed by
histological examination. Briefly, segments of trachea were fixed in Millong-formalin (Carson et al., 1973) and embedded with Paraplast-Plus paraffin. Sections (5 µm) were stained with hematoxilin-eosine (HE) and examined under light microscopy. During the experiment, the contractile response to 100 µg/mL ovalbumin or 2.5 µM carbachol was measured before and after a 15 min exposure to 10 µM 7-epiclusianone of intact or denuded epithelium tracheal rings.

In order to evaluate the putative interference of 7-epiclusianone with calcium influx, Ca\(^{2+}\) concentration–response curves were established according to Foster et al. (1984). Briefly, the responses to 2.5 µM carbachol of tracheal ring segments from naive guinea pig were recorded. After washout of carbachol and re-establishment of stable baseline tone, tissues were exposed to successive cycles of 60 mM KCl stimulations/washouts in Ca\(^{2+}\)-free Krebs solution containing 2 mM ethyleneglycol-bis-(β-aminoethylether)-N,N,N',N'-tetraacetic acid until complete desensitization to the 60 mM KCl-evoked contractile response. Next, tracheal rings were immersed in Ca\(^{2+}\)-free Krebs solution containing 60 mM KCl, and the extracellular Ca\(^{2+}\) concentration was stepwise increased by the cumulative addition of CaCl\(_2\) (0.03-30 mM) in the presence or absence of 10 µM 7-epiclusianone or vehicle (0.1% DMSO). All responses were expressed as the percentage of response to 2.5 µM carbachol.

To further investigate the mechanisms of action of 7-epiclusianone, the tracheal rings were pretreated 10 min before its application with 10 µM ODQ, an inhibitor of guanylate cyclase; 100 µM N\(^\omega\)-nitro-L-arginine methyl ester (L-NAME), an inhibitor of nitric oxide synthase; 100 µM SQ22.536, an inhibitor of adenylate cyclase; 10 mM tetraethylammonium (TEA), a non-selective K\(^+\) channel blocker; 1 µM glibenclamide, a K\(_{ATP}\) channel blocker; 1 µM apamin or 0.1 µM iberitoxin (IbTX), a Ca\(^{2+}\)-dependent K\(^+\)
channel blockers of small and large conductance, respectively. All spasmolitics effects were expressed as a percentage of carbachol-induced maximal contractile responses.

**Measurement of cyclic GMP**

Intracellular cyclic GMP concentrations in guinea-pig tracheal rings were assayed as previously described by Wu et al. (2004) with several modifications. Isolated trachea was cut into rings cells, quickly immersed in nutritional solution of Krebs and incubated with 7-epiclusianone (1–100 µM), L-NAME (100 µM) or ODQ (10 µM) in the presence of 100 µM IBMX for 20 min. Some tracheal rings were pre-treated with 100 µM L-NAME or 10 µM ODQ 10 min before addition of 100 µM 7-epiclusianone. Tissues sections were rapidly frozen by immersion in liquid nitrogen. The frozen tracheal rings were homogenized in cold 6% trichloroacetic acid (TCA). The homogenate was centrifuged at 2000 x g for 15 min at 4°C. To remove TCA, the supernatants were washed four times with 5 volumes of water-saturated diethyl ether. The upper ether layer was discarded after each wash. Then, the supernatants were lyophilized and the cyclic GMP of each sample was determined by using commercially available enzymeimmunoassay kits (Amersham, GE Healthcare, Buckinghamshire, England).

**Assessment of the effect 7-epiclusianone on airway obstruction in vivo**

Using barometric whole body plethysmography (Buxco Research System, USA) and increases in enhanced pause (Penh) as an index of airway obstruction, we measured responses to inhaled methacholine (6, 12 and 25 mg/mL, during 2.5 min, in 5 min intervals) in conscious, unrestrained naïve Balb/c mice, as reported (Hamelmann, 1997). Penh measurements were performed within 1 h and 4 h following treatment with 7-epiclusianone (25-100 mg/Kg) or vehicle (1% polysorbate 80, diluted in 0.9% NaCl) administered via the oral route (gavage). The effect of oral treatment with theophylline (60 mg/Kg) was also assessed for comparison.
Statistical analysis

The results were expressed as mean ± standard error of the mean (S.E.M). Statistical differences were determined by the analysis of variance (ANOVA), followed by the Newman-Keuls Student test. *P* values of 0.05 or less were considered significant.
Results

Effect of 7-epicusianone on guinea pig tracheal contractions induced by either allergen, histamine, 5-HT or carbachol

As illustrated in figure 1A, pretreatment with 10 and 100 µM 7-epicusianone but not its vehicle (0.1% DMSO) clearly inhibited the tracheal contraction triggered by increasing concentrations of ovalbumin (0.001-100 µg/mL). No changes in allergen-evoked contractile response were noted between untreated and vehicle-treated groups (data not shown). Our findings indicated that responses triggered by either histamine (10^{-7}-10^{-3} M) (Figure 1B), carbachol (10^{-8}-10^{-4} M) (Figure 1C) or 5-HT (10^{-8} - 3 x 10^{-5} M) (Figure 1D) were also significantly reduced by the 7-epicusianone treatment.

Role of epithelium in the antispasmodic effect of 7-epicusianone

We studied whether the epithelium would be implicated in the effect of 7-epicusianone by mechanically removing the epithelial cells of the internal tracheal surface with a fine silver wire as reported (Wu et al., 2004). Histological examination confirmed the epithelial layer removal as shown in Figure 2B (epithelium denuded), as compared to figure 2A (epithelium intact). The results showed that in the absence of tracheal epithelium, 10 µM 7-Epiclusianone failed to inhibit contraction triggered by either ovalbumin (100 µg/mL) or carbacol (2.5 µM), as shown in figures 2C and 2D, respectively.

Role of nitric oxide in the antispasmodic effect of 7-epicusianone

The putative role of nitric oxide in the spasmolitic activity of 7-epicusianone was assessed by pretreating tracheal rings with 100 µM L-NAME 10 min before exposure to the benzophenone. As shown in figure 3A, L-NAME clearly prevented the 7-epicusianone-relaxating effect under condition of carbachol-induced tracheal contraction. Such an effect was also inhibited by pretreatment with sGC inhibitor 10 µM ODQ (Figure
3B), but remained unchanged after pretreatment with the adenylate cyclase inhibitor 100 µM SQ 22536 (Figure 3C).

Effect of 7-epiclusianone on cGMP levels in cultured tracheal rings

As illustrated in Figure 4, 7-epiclusianone (1-100 µM) dose-dependently increased the tissue content of cGMP in cultured tracheal rings. The phenomenon was entirely abolished by pretreatment with either the NOS inhibitor L-NAME (100 µM) or the sGC inhibitor ODQ (10 µM).

Role of K+ channels in the antispasmodic effect of 7-epiclusianone

TEA (non-selective K+ channels blocker) (10 µM), glibenclamide (K_{ATP} channel blocker) (1 µM), apamin (small conductance K^{+}_{Ca^{2+}}) (1 µM) and IbTX (a large conductance K^{+}_{Ca^{2+}} blocker) (0.01 µM) were used to examine the putative involvement of K+ channels in the antispasmodic activity of 7-epiclusianone. Note that application of TEA (Figure 5A), glibenclamide (Figure 5B) or apamin (Figure 5C) clearly impaired the protective effect of 7-epiclusianone upon carbachol-induced tracheal contraction, whereas IbTX (Figure 5D) was inactive.

Effects of 7-epiclusianone on voltage-dependent calcium channels

In experiments with preparations maintained in Ca^{2+}-free medium and depolarized with 60 mM KCl, cumulative Ca^{2+} addition (0.1–20 mM) produced a concentration-dependent contraction, which was sensitive to pretreatment with 10 µM 7-epiclusianone (Figure 6A), confirming previous data (Neves et al., 2007). As illustrated in figure 6B, mechanically removal of the tracheal epithelial barrier abrogated such protective effect.

Role of nitric oxide on the effects of 7-epiclusianone on methacholine-evoked airways spasm in vivo

In naive Balb/c mice treated orally with 100 mg/Kg 7-epiclusianone, we found a marked attenuation in the elevation of bronchial spasm induced by 12 or 25 mg/mL aerosolized methacholine 1 and 4 h after the treatment (Figure 7 A and B, respectively).
Used here as reference treatment, oral administration of theophylline (60 mg/Kg) clearly inhibited methacholine-induced increases in Penh at 1 h, but in this case the protective effect disappeared 4 h post-treatment (Figure 7 C and D, respectively).

Thus, it was interesting to determine whether the blockade of nitric oxide synthesis in vivo would modify the protective effect of 7-epiclusianone as noted in the ex-vivo setting (tracheal system). As illustrated in Figure 8, the intraperitoneal pretreatment of Balb/c mice with L-NAME (20 mg/Kg), 30 min before 7-epiclusianone pretreatment (100 mg/Kg, oral), entirely reversed its effect upon methacholine-evoked elevation in the Penh values.
Discussion

These studies showed that a tetraprenylated benzophenone, 7 epclusianone, can act as an antispasmodic agent on both *ex-vivo* and in *in vivo* settings of airway smooth muscle contraction. What are the possible mechanisms by which this occurs? Activation of nitric oxide production is generally accepted to play an important role in the regulation of airway function under physiological and pathological conditions. Our findings demonstrated that L-NAME prevented the 7-epclusianone effect upon methacholine-evoked airways bronchoconstriction in Balb/c mice. In the *ex-vivo* settings, the epithelial cells were shown to be essential in the relaxation effect of 7-epclusianone upon allergen- and carbachol-induced tracheal ring contractions. In addition, 7-epclusianone induced a dose-dependent augmentation in the tissue cGMP levels of cultured tracheal rings. These effects were also sensitive to the blockade of nitric oxide synthase and sGC enzymes, strongly suggesting that 7-epclusianone is probably acting through activation of the nitric oxide-cGMP pathway.

*Garcinia* is the most numerous genus of the Guttiferae (Clusiaceae) – vegetal family typically tropical in distribution (Ampofo et al., 1986). *Garcinia* species are rich in oxygenated and prenylated phenol derivatives (Bennett and Lee, 1988) and some of them exhibit interesting biological activities such as antifungal, anti-inflammatory, anti-tumoral, anti-oxidant, anti-lipidemic and vasodilator (Gopalakrishnan et al., 1997; Diaz-Carballo et. al., 2003; Hay et al., 2004; Cruz et al., 2006). In a recent study, we obtained a yellow crystalline solid from *Garcinia brasiliensis* fruit pericarp, which was identified as the tetraprenylated benzophenone 7-epclusianone (Neves et al., 2007), a substance previously isolated from *Rheedia gardneriana* Miers ex Planch & Triana (Santos et al., 1998). The current study was mainly motivated by the findings that 7-epclusianone inhibited the anaphylactic contraction of isolated guinea pig ileum and prevented allergen-evoked...
histamine release with potency comparable to that of the anti-allergic agent azelastine (Neves et al., 2007).

In this study, we found that 7-epiclusianone inhibited guinea pig tracheal contraction induced by allergen, histamine, 5-HT or carbachol. The fact that 7-epiclusianone impairs contraction by allergen as well as those triggered by histamine, 5-HT and carbachol suggests that the manner by which 7-epiclusianone exerts its effect is by interfering with the mechanism of smooth muscle contraction. The next step was to investigate whether the effect of 7-epiclusianone could be dependent of the production of smooth muscle relaxation factors by epithelial cells. In fact, it was observed that the antispasmodic activity of 7-epiclusianone upon allergen- and carbachol-evoked contraction was unapparent following tracheal epithelium removal, clearly showing the crucial role of epithelial cells in this protective effect. We also studied the possibility that 7-epiclusianone would be acting via activation of nitric oxide production by epithelial cells, which is a mechanism implicated in the regulation of the airway tone, under physiological and pathological conditions in guinea pigs and humans (Folkerts and Nijkamp, 2006). In fact, our findings revealed that the 7-epiclusianone’s protective effect upon carbachol-evoked contraction was abolished by prior exposure of tracheal rings to the nitric oxide synthase inhibitor, L-NAME.

It is well known that epithelium-derived nitric oxide activates guanylate cyclase and increases cGMP intracellular concentration, leading to relaxation of airway smooth muscles (Katsuki and Murad, 1977). The effect of 7-epiclusianone under conditions of intact epithelium was then shown to be inhibited by pretreatment with the sGC inhibitor ODQ. Notably, the response to 7-epiclusianone was not modified by pretreatment with the adenylate cyclase inhibitor, SQ 22536. In another set of experiments, we demonstrated that 7-epiclusinone was also able to augment the trachea ring content of cGMP, in a mechanism
clearly sensitive to blockade of either nitric oxide synthase or sGC, strongly supporting the interpretation that the activity of this compound depends on the activation of nitric oxide and sGC/cGMP, but not cAMP pathway.

It has been reported that nitric oxide is capable of stimulating Ca\(^{2+}\)-activated K\(^+\) channels in smooth muscles (Bolotina et al., 1994), including those in the airways (Abderrahmane et al., 1998), causing membrane hyperpolarization, decrease in calcium influx, and smooth muscle relaxation (Lincoln and Cornwell, 1991). The opening of several K\(^+\) channels, such small conductance K\(^+\)\(_{Ca^{2+}}\) and the K\(^+\)\(_{ATP}\) channels, produce relaxation of the smooth muscle (Archer et al., 1998; Kume et al, 1989 and Kotlikoff, 1993). We then examined the effectiveness of distinct K\(^+\) channel blockers to interfere with the antispasmodic activity of 7-epicusianone. We noted that tracheal relaxations were no more observed when 7-epicusianone was preceded by the non-selective K\(^+\) channel blocker TEA, indicating the involvement of K\(^+\) channels in this process. The 7-epicusianone relaxation response was also sensitive to the K\(^+\)\(_{ATP}\) channel blocker glibenclamide and to apamin, specific inhibitor of the small-conductance K\(_{Ca^{2+}}\) channel, but remained unchanged following pretreatment with the specific inhibitor of high-conductance K\(_{Ca^{2+}}\) channel iberiotoxin. These findings are consistent with the interpretation that the 7-epicusianone tracheal effects do involve activation of the cGMP/PKG signaling cascade, leading to the phosphorylation-dependent opening of both K\(^+\)\(_{ATP}\) and small conductance K\(_{Ca^{2+}}\) channels, blockade of Ca\(^{2+}\) entrance and eventually tracheal relaxation. They also suggest, in line with a prior study (Wu et al., 2004), that the high-conductance K\(_{Ca^{2+}}\) channel is probably not the predominant K channel expressed by the guinea pig tracheal tissue, despite the fact that this channel is highly implicated in nitric oxide/sGC/cGMP pathway-dependent relaxation of a number of other smooth muscle cell systems (Kaczorowski et al., 1996).
There is a significant body of evidence for the existence of voltage-dependent Ca\(^{2+}\) channels in airway smooth muscle (Marthan et al., 1989; Hisada et al., 1990; Worley and Kotlikoff, 1990). In guinea-pig airway smooth muscle, contraction evoked by KCl is due to membrane depolarization with influx of Ca\(^{2+}\) through voltage-dependent Ca\(^{2+}\) channels (Knox and Tattersfield, 1994). It is noteworthy that in epithelium-intact trachea rings, 7-epiclusianone dose-dependently inhibited Ca\(^{2+}\)-induced contractions in K\(^+\) (60 mM)-depolarized preparations, suggesting that 7-epiclusianone could inhibit Ca\(^{2+}\) influx via blockade of voltage-dependent Ca\(^{2+}\) channels (Neves et al., 2007). Nevertheless, this interpretation does not find support in similar experiments carried out in epithelium-denuded tracheal rings. Under this condition, 7-epiclusianone failed to alter contractile response induced by changes in extracellular Ca\(^{2+}\) concentration during high K\(^+\) depolarization, discarding the involvement of voltage-dependent Ca\(^{2+}\) channels, while reinforcing the crucial role of the epithelial layer in the antispasmodic effect of 7-epiclusianone.

Finally, while trying to get an insight in the putative therapeutic application of this benzophenone derivative, we have used non invasive barometric plethysmography to assess the effectiveness of 7-epiclusianone in animals. Essentially, we measured methacholine-evoked airway obstruction in Balb/c mice subjected to oral treatment with 7-epiclusianone, theophylline or vehicle. Both compounds significantly attenuated methacholine-induced increases in Penh at 1 h as compared to the vehicle-treated group, but only 7-epiclusianone remained active 4 h post-treatment, showing for the first time that this natural product was active orally and displayed a longer-lasting effect in comparison to theophylline. Moreover, it was interesting to find that the blockade of nitric oxide synthesis in the animal model also reversed the antispasmodic effect of 7-epiclusianone, adding support to the interpretation that nitric oxide is indeed mediating the phenomenon. On the
other hand, the perspective of having some systemic cardiovascular repercussions for the oral 7-epiclusianone treatment should not be ruled out. This possibility is also supported by in vitro studies in which 7-epiclusianone exhibited an endothelium-dependent vasodilator effect in rat aortic rings (Cruz et al., 2006). A better characterization of the clinical value of 7-epiclusianone for the treatment of allergic and inflammatory diseases will require additional investigations.

In conclusion, these studies suggest that the airways relaxation effect of 7-epiclusinone is accounted for by epithelium-, nitric oxide- and sGC/cGMP-dependent mechanisms. The fact that oral administration of 7-epiclusianone can inhibit airways spasm in animal model emphasizes the possibility that this compound may be beneficial for the treatment of airflow limitation triggered by allergen challenge in humans.
Acknowledgements

We are indebted to Fernanda Maria da Silva Alves and Francisco Alves Faria Filho for the technical support. Luciana P Coelho thanks FAPERJ for her Fellowship.
References


Footnotes:

These investigations were financially supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação Oswaldo Cruz (PAPES IV), Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ), IM-INOFAR(BR, #420015/05-1) and PRONEX 2006.
Legends for Figures:

**Figure 1** - Antispasmodic effects of 1 µM (●), 10 µM (■) or 100 µM (▲) 7-epiclusianone on the guinea-pig tracheal contraction induced by (A) ovalbumin (10^{-9} – 10^{-4} g/mL); (B) histamine (10^{-7} – 10^{-2} M); (C) carbachol (10^{-8} – 10^{-4} M) or (D) 5-HT (10^{-9} – 3 x 10^{-5} M). Each point represents the mean ± S.E.M. of eight experiments. All results were expressed as a percentage of contractile responses induced by 2.5 µM carbachol. *, p< 0.05 as compared with tracheal responses from vehicle treated preparations (o).

**Figure 2** - Hematoxylin-Eosin stained histological sections of guinea-pig trachea displayed before (A) and after removal of the epithelial layer (B). Antispasmodic effects of 10 µM 7-epiclusianone on tracheal contractions induced by ovalbumin (100 µg/mL) (C) or carbachol (2.5 µM) (D) in the presence (closed columns) or absence (open columns) of epithelium. Values represent the mean ± S.E.M. of at least six experiments. *, p<0.05 as compared to the group of untreated tracheal rings.

**Figure 3** - Effects of 7-epiclusianone (10 µM) on carbachol-contracted guinea-pig trachea, performed in absence or presence of L-NAME (A), ODQ (B) or SQ22536 (C). Each point represents the mean ± S.E.M of five experiments.

**Figure 4** - Effect of 7-epiclusianone (1 – 100 µM) on cGMP levels in tracheal smooth muscle tissue in absence or presence of L-NAME (100 µM) and ODQ(10 µM). Each point represents the mean ± S.E.M of three tracheal rings. +Indicates P<0.01 as compared to the DMSO 0,1% group. *Indicates p<0.01 as compared to the 100 µM 7-epiclusianone group

**Figure 5** - Effects of 7-epiclusianone (10 µM) on carbachol-contracted guinea-pig trachea, performed in the absence or presence of TEA (A), Glibenclamide (B), Apamin (C) or Ibtx (D). Each point represents the mean ± S.E.M of at least five experiments.
Figure 6 - Ex-vivo effect of 7-epiclusianone (1 µM) (■) and (10 µM) (●) on the contractile response triggered after cumulative application of calcium in tracheal rings mounted in calcium free high K+ (60mM) Krebs solution. Pretreatment with 7-epiclusianone led to decrease in tension after calcium addition in the presence of epithelium (A), but not in the absence of epithelium (B). Each point represents the mean ± S.E.M. from at least four experiments. The magnitude of the contractile tension induced by calcium on trachea preparations was expressed as a percentage of contraction response evoked by 2.5 µM carbachol. *, p<0.05 as compared with vehicle pretreated preparations.

Figure 7 - Relaxant effect of 7-epiclusianone (50 and 100 mg/kg) (upper panels) and theophylline (60 mg/kg) (lower panels), orally administered into Balb/c mice challenged with methacholine aerosol (6, 12 e 25 mg/mL) at 1 h (A and C) and 4 h (B and D) after 7-epiclusianone treatment. Each value represents the mean ± S.E.M. from at least 6 mice. *Indicates P<0.01 as compared to the untreated group. †, p<0.01 as compared with the saline group without methacholine aerosol.

Figure 8 - Effect of L-NAME (20 mg/kg) administered intraperitoneally on the capacity of 7-epiclusianone (100 mg/kg, oral) to inhibit increase in Penh values caused by aerosol of methacholine in Balb/c mice. Values represent the mean ± S.E.M. from at least 6 mice.
Figure 1

(A) % Carbachol 2.5 μM vs. Log [Ovalbumin] g/ml

(B) % Carbachol 2.5 μM vs. Log [Histamine] M

(C) % Carbachol 2.5 μM vs. Log [Carbachol] M

(D) % Carbachol 2.5 μM vs. Log [5-HT] M
Figure 5

(A) 10 μM TEA

(B) 1 μM Glibenclamide

(C) 1 μM Apamin

(D) 0.01 μM IbTx

- Vehicle
- 7-EPICUSLUSIONE

% Carbachol 2.5 μM

P < 0.05
Figure 8

![Graph showing the effect of methacholine on Penh with different treatments.](image-url)