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Pharmacological Antagonism of *Anchietia salutaris* Extracts on the Contraction Induced by Prostaglandin D_2 and U46619 in Guinea-pig Lung Parenchymal Strips

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Abstract

Anchietia salutaris tea is traditionally used in Brazil to treat allergies, suggesting it contains compounds with antagonistic activity on the allergic mediators. We have evaluated extracts and semi-purified fractions of *Anchietia salutaris* as a source of compounds having this type of antagonism on the contraction induced in guinea-pig lung parenchymal strips and on platelet aggregation and shape change.

After 10 min pre-incubation dichloromethane extracts containing 30 or 100 $\mu g\ mL^{-1}$ inhibited the contraction induced by prostaglandin D_2 (PGD_2) in guinea-pig lung parenchymal strips with dose ratios (DR) of $0{\cdot}76 \pm 0{\cdot}14$ and $0{\cdot}93 \pm 0{\cdot}19$, respectively; the amount of inhibition depended both on the concentration and on the time of pre-incubation (DR after 30 min pre-incubation was $1{\cdot}21 \pm 0{\cdot}51$). The dichloromethane extract and its semi-purified fractions also inhibited the contractions induced by U46619, a more potent, stable, synthetic agonist of thromboxane A_2 (TxA_2) prostanoid (TP) receptors, the receptors acted upon by PGD_2 to produce lung contractions. The dichloromethane extract did not inhibit the lung parenchymal contractions induced by histamine, leukotriene D_4 (LTD_4) or platelet-activating factor (PAF). Platelet aggregation induced by U46619, adenosine 5′-diphosphate (ADP) or PAF was not inhibited by the dichloromethane extract. Indeed, the extract potentiated platelet aggregation induced by low concentrations of these agonists and also potentiated the shape change induced by U46619.

These results imply that the dichloromethane extract of *Anchietia salutaris* and its semi-purified fractions contain an active principle that competitively inhibits TxA_2 TP receptors, the stimulation of which causes lung parenchymal contraction. The inhibition seems to be selective for this receptor subtype, because the extract fails to inhibit platelet aggregation or shape change. This provides additional support of earlier reports suggesting the occurrence of TP receptor subtypes.

Inflammatory mediators considered to play a major role in the pathophysiology of asthma include preformed mediators, such as histamine, those newly synthesized by basophils or mast cells after antigen stimulation, such as prostaglandins and leukotrienes, and mediators generated as a result of primary mediator release. Several prostaglandins, leukotrienes, platelet-activating factor (PAF), bradykinin, histamine and other mediators cause bronchoconstriction in addition to plasma exudation, activation of neural mechanisms and mucus secretion that are all symptoms characteristic of asthma (Bochner et al 1994). Many of these mediators are also involved in the pathology of other allergies, for example rhinitis and urticaria (Amon et al 1994; Yamasaki et al 1997).

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The treatment of allergies is problematic not only because of the number of mediators and receptors involved and the variety of the actions they cause but also because there are insufficient selective antagonists to these mediators that can be used to treat man (Bochner et al 1994). This situation highlights the importance of the search for compounds that antagonize allergic mediators.

Anchietia salutaris St. Hil is a Brazilian plant traditionally used in the treatment of allergic diseases. Because plants used in this type of folk medicine could contain compounds with antagonistic activity on allergic mediators we have studied extracts and

semi-purified fractions from *A. salutaris* as a possible source of compounds with antagonist activity to histamine, PGD_2, LTD_4 and PAF.

Materials and Methods

Drugs and reagents

Prostaglandin D_2 (PGD_2), histamine diphosphate salt, leukotriene D_4 (LTD_4), DL-α-phosphatidylcholine β-acetyl-γ-*O*-hexadecyl (platelet activating factor, PAF), adenosine 5′-diphosphate (ADP) and 9,11-dideoxy-11α,9α-epoxymethano-prostaglandin $F_{2\alpha}$ (U46619) were purchased from Sigma (St Louis, MO). Reagents used were all of analytical grade.

A. salutaris *extracts and fractions*

Herbarium sheets were deposited in the Herbarium BOTU of the Biosciences Institute, Unesp, Botucatu, S.P., Brazil. They were collected, dehydrated at 50°C, powdered, extracted with methanol for 24 h, concentrated to 30% of the initial volume and subjected to chlorophyll extraction. Chlorophyll-free methanol extract (crude extract) was used in the biological assays and for the extraction with hexane and dichloromethane. The dichloromethane extract was separated by flash chromatography with dichloromethane, dichloromethane–ethyl acetate (9 : 1, 7 : 3, 1 : 1), ethyl acetate and methanol as mobile phases. After complete evaporation of the methanol, hexane, dichloromethane and dichloromethane–ethyl acetate the resulting fractions were diluted first with dimethylsulphoxide (DMSO) and then with H_2O or saline (platelet aggregation experiments). The final concentration of DMSO in the assays (0·2% v/v) had no effect on the biological preparation and did not alter the effects of PGD_2 or U46619.

Lung parenchymal strips

Dunkin–Hartley guinea-pigs of both sexes, 300–500 g, were killed by cervical dislocation, exsanguinated, and the lungs were removed. Two parenchymal strips were carefully cut from the distal edges of the lower lobe of the right lung (approximately 1·5 cm × 0·4 cm × 0·3 cm) and suspended in 5-mL organ baths containing Krebs solution (mM: NaCl 118, KCl 4·7, $CaCl_2.2H_2O$ 2·8, $MgCl_2.6H_2O$ 2·5, $NaHCO_3$ 25, $NaH_2PO_4.H_2O$ 1·0, glucose 11) at 37°C and oxygenated with 95% O_2–5% CO_2. A resting tension of 1 g was applied and the preparations were equilibrated for 45 min, during which time they were washed with the physiological solution every 15 min. The extracts or fractions were then added to one of the organ baths (the other being kept as control) and left for 10 min before addition of agonist. Isometric contractions were recorded with a Gold model 2200S recorder. Cumulative concentration–effect curves for the agonists were constructed according to the method of Van Rossum (1963). The maximum response was considered as the contraction induced by addition of 3×10^{-2} M $BaCl_2$. The contractions induced by the mediators were expressed as a percentage of the contraction induced by $BaCl_2$. The concentration–effect curves were used to calculate dose ratios (DR), i.e. the difference between the logarithm of the EC25 (the concentration of agonist inducing 25% of the $BaCl_2$ response) from the curves obtained with and without addition of antagonist.

Platelet aggregation and shape change

Guinea-pigs of both sexes were anaesthetized with sodium pentobarbital (45 mg kg^{-1}, i.p.) and blood was collected by heart puncture into plastic tubes containing 3·8% sodium citrate. Platelet-rich plasma was prepared by centrifuging the citrated blood at 200 *g* and room temperature for 10 min. Platelet aggregation was determined by turbidimetry (Born 1962) with a Zenite Z-1000 aggregometer. Samples (400 μL) of platelet-rich plasma were incubated for 5 min at room temperature with saline or extract before addition of aggregating agent causing irreversible control aggregation. The shape-change signal, defined as the initial increase in optical density of the platelet-rich plasma (prepared in an identical manner) was monitored with a Helena Laboratories Packs-4 aggregometer.

Statistical analysis

Results are expressed as means ± s.e.m. Student's *t*-test was used to evaluate the significance of differences between paired and unpaired samples as indicated in the results. The level of significance was set at $P < 0.05$.

Results

Effects of the extracts on the parenchymal strips

Chlorophyll-free methanol and hexane extracts were themselves active on guinea-pig parenchymal lung strips. Contractions of 18·5 ± 3·9% (n = 6) and 15·7 ± 2·7% (n = 5) were induced by chlorophyll-free methanol extracts containing 30 and 100 μg mL^{-1}, respectively, and the hexane extract (100 μg mL^{-1}) induced a relaxation of 10·3 ± 2·2% (n = 4). The activity of these extracts lasted longer than 10 min. Because of these problems we used only the hexane (30 μg mL^{-1}) and the dichloromethane extracts, which did not affect the preparations.

Effects of the dichloromethane and hexane extracts on the contraction of parenchymal strips induced by PGD_2, histamine, LTD_4 and PAF

The dichloromethane extract (30 and 100 μg mL^{-1}) inhibited the contraction of the guinea-pig parenchymal lung strips induced by PGD_2, producing a significant rightward shift on the concentration–effect curves (Figure 1). The inhibition increased with the concentration of the dichloromethane extract, as shown by the DR values in the legend to Figure 1, and also increased with the time of pre-incubation with the 100 μg mL^{-1} dichloromethane extract (DR after 10 and 30 min were

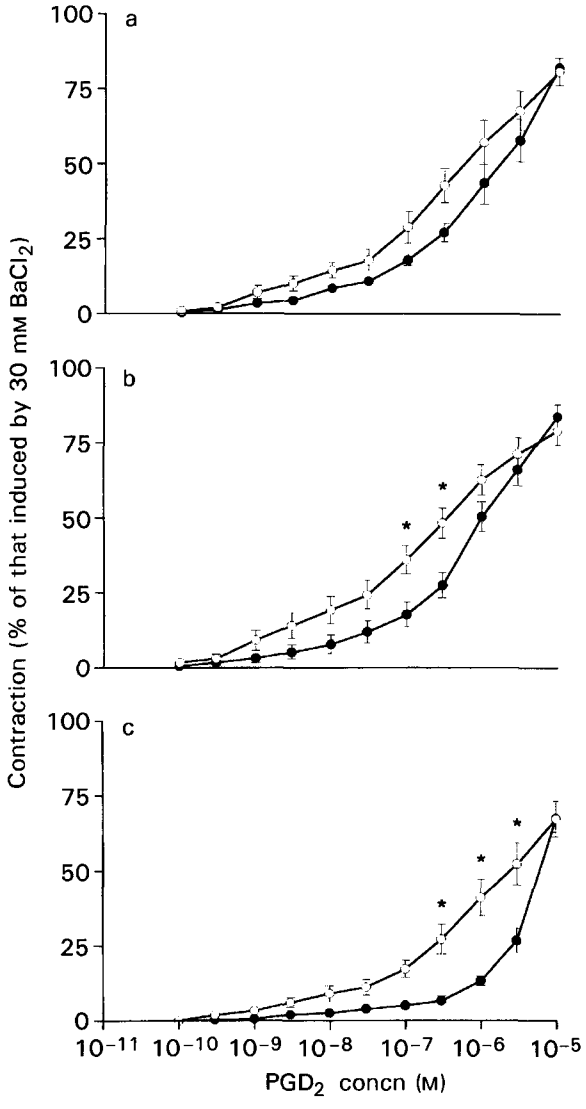

Figure 1. Inhibition by dichloromethane extracts of the contractions induced by PGD_2 in lung parenchymal strips: ○, control; ●, extract, 30 (a), 100 (b) or 300 μg mL^{-1} (c), dose ratios (DR) 0·50±0·26, 0·76±0·14 and 0·93±0·19 (mean ±s.e.m.), respectively. Symbols indicate means of results from 4–8 experiments and the bars show the s.e.m. *$P < 0.05$, significantly different from the result for the corresponding treated strip (Student's *t*-test for unpaired samples).

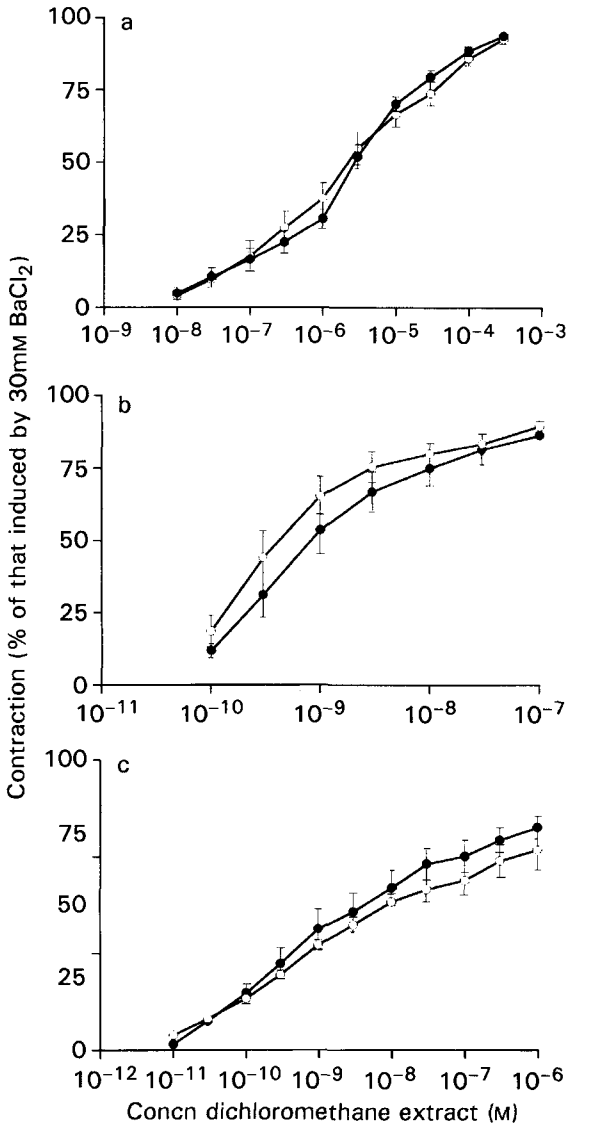

Figure 2. Concentration–effect curves for histamine (a), leukotriene D_4 (b) and platelet-activating factor (c) in lung parenchymal strips in the absence (○) or presence (●) of the 100 μg mL^{-1} dichloromethane extract. Symbols indicate the means of results from 6–8 experiments and the bars show the s.e.m.

0·76±0·14, n=8, and 1·21±0·51, n=4, respectively). Figure 2 shows that the dichloromethane extract did not inhibit the contractions induced by histamine, LTD_4 or PAF. The 30 μg mL^{-1} hexane extract did not change the cumulative concentration–effect curves obtained after treatment with histamine, LTD_4, PGD_2 or PAF (data not shown).

Effects of the dichloromethane extract and its semi-purified fractions on the contraction of parenchymal strips induced by U46619

The dichloromethane extract also inhibited the parenchymal contractions induced by another prostanoid agonist, U46619, a synthetic and stable

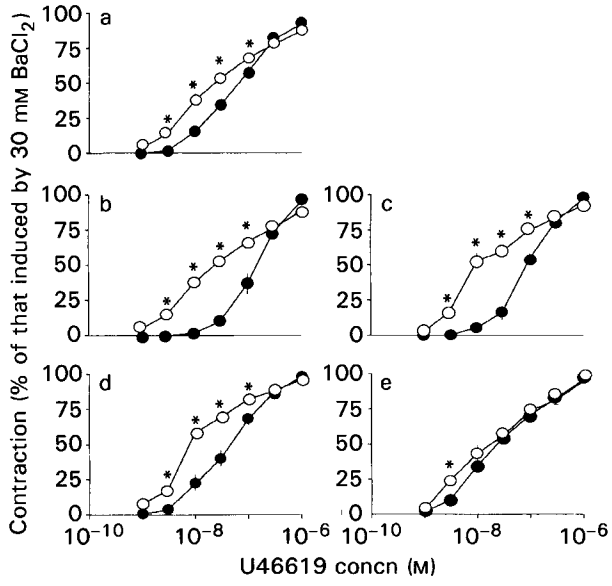

Figure 3. Concentration–effect curves for the contractions induced by U46619 in lung parenchymal strips in the absence (control, ○) or presence (●) of the 300 μg mL^{-1} dichloromethane extract (a, dose ratio (DR) 0·46 ± 0·09 (mean ± s.e.m.)) or its semi-purified fractions obtained by elution with: dichloromethane–ethyl acetate, 9 : 1 (b, fraction F2, dose ratio (DR) 1·09 ± 0·07), 7 : 3 (c, fraction F3, dose ratio (DR) 0·95 ± 0·07), 1 : 1 (d, fraction F4, dose ratio (DR) 0·54 ± 0·11), and ethyl acetate (e, fraction F5). Symbols indicate the means of results from 4–6 experiments and the bars show the s.e.m. *$P < 0·05$, significantly different from the result from the corresponding treated strip (Student's t-test for unpaired samples).

analogue of TxA_2 with a potent activity on TP receptors. The fractions obtained with proportions of dichloromethane and ethyl acetate in the ratios 9 : 1 (fraction F2), 7 : 3 (fraction F3) and 1 : 1 (fraction F4) caused significant rightward shift of the U46619 concentration–effect curves. The ethyl acetate fraction (fraction F5) inhibited only the contraction induced by the 3×10^{-9} M concentration of U46619 (Figure 3). Fractions F1 and F6 (obtained with dichloromethane and methanol, respectively) induced sustained contraction of the parenchymal lung strips and, as for the chlorophyll-free methanol and 100 μg mL^{-1} hexane extracts mentioned above, were not used in the experiments.

Effects of the dichloromethane extracts on guinea-pig platelet aggregation and shape change

Although platelet aggregation induced by ADP (1 μM), PAF (1 μM) and U46619 (3 μM) were not inhibited by the dichloromethane extract, ten-times lower concentration of PAF and U46619 induced aggregation that was potentiated by the 100 and 300 μg mL^{-1} dichloromethane extract. The aggregation induced by ADP (0·1 μM) was potentiated only by the 100 μg mL^{-1} dichloromethane extract—the higher concentration (300 μg mL^{-1}) inhibited aggregation (Table 1). The use of a low concentration of U46619 (0·3 μM) enabled recording of the optical density (percentage increase) accompanying platelet-shape change. The control value (7·9 ± 3·0) was potentiated by the 30- and 100-μg mL^{-1} dichloromethane extracts (16·2 ± 3·4 and 18·8 ± 3·6, respectively), but not by the 300-μg mL^{-1} extract (5·7 ± 2·7) ($P < 0·05$, Student's t-test; n = 4). The dichloromethane extract alone did not cause platelet aggregation or shape change.

Discussion

Our results show that the dichloromethane extract from *Anchietia salutaris* contains a compound that inhibits the contractions induced by PGD_2 in guinea-pig parenchymal lung strips. This inhibitory effect depends on the time of pre-incubation and on the concentration in the organ bath. The active principle is certainly different from that found in the *Anchietia salutaris* hexane extract which inhibit histamine release (Gomes et al 1994) because the dichloromethane extract has higher polarity and also because the contraction induced by PGD_2 was not inhibited by the hexane extract. Cumulative concentration–effect curves obtained after treatment

Table 1. Percentage of maximum platelet aggregation induced by ADP, platelet-activating factor and U46619 in the presence of *Anchietia salutaris* dichloromethane extracts (0–300 μg mL^{-1}).

Extract concentration (μg mL^{-1})	Platelet aggregation (% of maximum)					
	ADP		Platelet-activating factor		U46619	
	0·1 μM	1 μM	0·1 μM	1 μM	0·3 μM	3 μM
0	71·0 ± 2·9	67·4 ± 3·7	77·2 ± 0·4	80·0 ± 2·5	88·4 ± 2·9	71·5 ± 2·5
30	75·2 ± 4·5	67·2 ± 2·7	78·7 ± 3·0	82·0 ± 3·9	92·7 ± 2·0	70·0 ± 2·6
100	78·5 ± 2·7*	67·2 ± 3·9	89·6 ± 2·6*	84·7 ± 3·0	94·4 ± 1·9*	74·0 ± 3·4
300	53·5 ± 6·5*	66·4 ± 4·4	91·0 ± 4·1*	81·3 ± 1·1	95·8 ± 3·2*	75·0 ± 1·7

Values are means ± s.e.m. of results from four experiments. *$P < 0·05$, significantly different from the respective control result (Student's t-test for unpaired samples).

with PGD_2 were shifted to the right without reduction of the maximum response, which suggests competitive antagonism (Kenakin 1993). The dichloromethane extract had no inhibitory activity on the cumulative concentration–effect curves obtained after treatment of guinea-pig parenchymal lung strips with histamine, LTD_4 or PAF, evidence of compound selectivity for PGD_2 receptors.

Characterization of the receptors that mediate the contraction by prostanoids of lung strips from guinea-pig and man shows that they are TxA_2 type receptors (Coleman & Sheldrick 1989). Specifically, PGD_2 is the second most potent agonist inducing contraction of guinea-pig lung by acting on the TP receptor, being only slightly less potent than TxA_2 (Coleman et al 1984). These data suggest that the inhibition caused by the dichloromethane extract is a result of action on TP receptors in the guinea-pig lung. This hypothesis is reinforced by the inhibition by the dichloromethane extract of the contraction induced, in guinea-pig parenchymal lung strips, by U46619, a stable synthetic agonist of TxA_2 TP receptors. This contraction is also inhibited by all the purified fractions obtained from the dichloromethane extract (i.e. F2, F3, F4 and F5) that did not themselves induce contraction; the activity was most concentrated in fraction F2 and almost absent from fraction F5. The separation process was quite effective because the DR calculated from the curves obtained with the fractions F2, F3 and F4 were higher than those obtained with the dichloromethane extract, indicating that the active principle is concentrated in these fractions.

TP receptors are widely distributed not only in lung parenchyma but in vascular smooth muscle and platelets (Coleman et al 1994). By acting on TP receptors U46619 elicits several biological effects, including platelet shape-change and aggregation (Morinelli et al 1987). ADP and PAF induce secondary platelet aggregation (second wave) that is promoted by formation of TxA_2 and its release from granules if the platelets are in a medium containing a low concentration of ionized calcium (Bretschneider et al 1994), a condition occurring in the citrated platelet-rich plasma used in this work. If the platelets' TP receptors are of the same type as exist in lung parenchyma it would be expected that the dichloromethane extract would inhibit platelet aggregation; it did not, however, alter guinea-pig platelet aggregation induced by normal concentrations of ADP (1 μM), PAF (1 μM) or U46619 (3 μM), implying that inhibition by the dichloromethane extract on guinea-pig lung parenchyma might be the result of action on a TP receptor subtype different from that which causes platelet aggregation. This hypothesis is supported by a preliminary report that U46619 affects a low-affinity site that mediates platelet aggregation, 5-hydroxytryptamine release, and fibrinogen receptor exposure, and a high-affinity site responsible for shape change and myosin light-chain phosphorylation (Morinelli et al 1987). Additional evidence was presented by Furci et al (1991) who showed that activation of TxA_2 receptors in rat vascular smooth muscle induces contraction by activation of a receptor subtype different from those that transduce platelet aggregation.

By use of a low concentration of agonist it is possible to monitor the platelet shape-change that precedes aggregation and secretion (Siess 1989). Under our conditions the TxA_2 mimetic U46619, ten times less concentrated, also induced an observable platelet shape-change. The change was not inhibited by the dichloromethane extract; in fact although significant shape-change potentiation resulted from doses of 30 and 100 $\mu g\ mL^{-1}$ the response to the 300-$\mu g\ mL^{-1}$ dose was no different from the control response. Platelet aggregation induced by PAF and ADP was also potentiated by the dichloromethane extract. Although we have no experimental evidence, a possible hypothesis for the failure of these dichloromethane extracts to induce inhibition is that the receptors that mediate the platelet shape-change and aggregation might be different from those that cause lung parenchyma contraction, whose stimulation in platelets would cause an inhibitory effect and, of course, its inhibition would potentiate the effect. The reversion of the dichloromethane extract potentiation of the platelet shape change (induced by U46619) and aggregation (induced by ADP, 0·1 $\mu g\ mL^{-1}$) could be because the dose of the dichloromethane extract was too high, perhaps having a non-specific effect in the platelets.

In total, our results reveal the presence in *Anchietia salutaris* of a compound that inhibits the contractions induced by PGD_2 and by the TxA_2 analogue U46619 in guinea-pig lung parenchymal strips. The characteristics of this inhibition suggest competitive antagonism quite selective to the lung TP receptors. We also provide additional support of earlier evidence that the TP receptor subtypes that mediate shape-change and platelet aggregation are different from those that cause lung parenchymal contraction.

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