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Inhibition of inflammatory responses by a series of novel dolabrane derivatives

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Abstract

Four dolabrane derivatives isolated from *Endospermum diadenum* have been studied for their inhibitory effects on murine models of inflammation and human neutrophil functions in vitro. After topical application, akendo 1, akendo 2 and akendo 3 potently inhibited the mouse ear oedema induced by 12-*O*-tetradecanoylphorbol acetate (TPA) with a striking effect on myeloperoxidase levels. After oral administration, akendo 2 and akendo 3 inhibited mouse paw oedema induced by carrageenan, with a significant reduction in myeloperoxidase levels. In contrast to indomethacin, they did not modify the prostaglandin E_2 content of the inflammed paw. None of the compounds affected superoxide generation by human neutrophils. On the contrary, they inhibited degranulation induced by different stimuli. The most effective compounds were akendo 2 and akendo 3, which also inhibited myeloperoxidase activity. All compounds were weak inhibitors of leukotriene B_4 synthesis and release by human neutrophils, whereas only akendo 3 decreased 5-lipoxygenase activity. Cyclo-oxygenase-1 from human platelets was inhibited mainly by akendo 2 and akendo 3, although with a low potency. The latter compound also reduced weakly the synthesis of prostaglandin E_2 by cyclo-oxygenase-2. The anti-inflammatory activity of these dolabrane derivatives was not related to arachidonic acid mobilization or metabolism.

Keywords: Dolabrane; Anti-inflammatory; Neutrophil, human; Myeloperoxidase; Elastase; Prostaglandin; Leukotriene

1. Introduction

Natural products represent a source of new chemical structures of pharmacological interest. The design of novel anti-inflammatory agents can provide alternatives to current therapeutic agents or probes that can be used to establish the mechanisms controlling inflammatory processes at the cellular and molecular levels. We have reported previously the anti-inflammatory activity of a number of diterpene derivatives (Alcaraz and Ríos, 1991). Recently, we have studied the effects on experimental inflammation of epitaondiol, a diterpene which modify some leukocyte functions like migration into the inflammatory site (Gil et al., 1995). Neutrophils are essential for host defense and are also involved in the pathology of inflammatory conditions since the activation of these cells leads to generation of reactive oxygen species and extracellular release of granule constituents which participate in the propagation and maintenance of acute and chronic inflammation (for review see Smith, 1994). Suppression of neutrophil functions could control the inflammatory response and has been implicated in the mechanisms of action of some non-steroidal anti-inflammatory agents since inhibition of constitutive and inducible cyclo-oxygenases does not seem to completely justify their anti-inflammatory effects (for review see Abramson and Weissmann, 1989 and Kankaanranta et al., 1994).

Endospermum diadenum Airy Shaw (Euphorbiaceae) is the only representative of this genus found in Thailand. This plant has been used in folk medicine for the relief of

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fever and skin infections. Little is known on the chemical constituents of this species although a series of diterpene derivatives have been isolated recently from the wood. They include ent-5α,2,15-dioxodolabr-3-ene-3,16-diol (akendo 1), ent-5α,16-chloro-2-oxodolabr-3-ene-3,15ξ-diol (akendo 2), ent-5α,3,15-dioxodolabr-1,4(18)-diene-2,16-diol (akendo 3) and ent-2-seco-3-nor-5α,4,15-dioxo-16-hydroxydolabran-2-oic acid (akendo 4), which are novel diterpenes possessing the dolabrane skeleton (Kijjoa et al., 1994).

We carried out the present work to find out their effects on murine models of inflammation as well as on human neutrophil functions and several enzymes relevant to the inflammatory process. This is the first report concerning the pharmacological properties of these natural products.

2. Materials and methods

2.1. Mouse ear oedema

12-*O*-Tetradecanoylphorbol acetate (TPA, 2.5 μg) or arachidonic acid (2.0 mg) dissolved in 20 μl acetone was applied in 10 μl volumes to both inner and outer surfaces of the right ear of Swiss mice (20–25 g). The protocol was approved by the institutional Animal Care and Use Commitee. Test compounds were applied topically in acetone before TPA administration or 20 min prior to arachidonic acid. The left ear (control) received only acetone. The animals were killed by cervical dislocation after 4 h (TPA) or 1 h (arachidonic acid) and equal sections of both ears were punched out and weighed. The increase in the weight of the right ear punch over that of the left indicated the oedema (Carlson et al., 1985). The ear sections were homogenized in 750 μl saline and after centrifugation at 10000 $\times g$ for 15 min at 4°C, the prostaglandin E_2 and leukotriene C_4 content in supernatants was determined by radioimmunoassay (arachidonic acid oedema) or myeloperoxidase activity was measured in aliquots of supernatants of TPA-treated ears by a modification of the method of Suzuki et al. (1983) (De Young et al., 1989). The reaction mixture contained 50 μl supernatant, 150 μl phosphate buffered saline, 20 μl 0.22 M NaH_2PO_4 (pH 5.4), 20 μl 0.026 (v/v) % H_2O_2 and 20 μl 18 mM tetramethylbenzidine in 8% (v/v) aqueous dimethylformamide. After 10 min reaction at 37°C, 30 μl 1.46 M sodium acetate, pH 3.0 was added and absorbance at 620 nm was read using a microtiter plate reader.

2.2. Mouse paw oedema

Swelling was induced following a modification of the technique of Sugishita et al. (1981). Female Swiss mice (20–25 g) were fasted for 12 h with free access to water. Drugs or vehicle (ethanol, Tween 80, distilled water: 5/5/90, v/v/v) was administered orally (0.5 ml) 1 h before injection of carrageenan (0.05 ml; 3% w/v in saline) into the subplantar area of the right hind paws of groups of 6 animals. The volumes of injected and contralateral paws were measured at 1, 3 and 5 h after induction of oedema by using a plethysmometer (Ugo Basile, Comerio, Italy). The volume of oedema was expressed for each animal as the difference between the carrageenan-injected and contralateral paws. A control saline group was injected with 0.05 ml of saline instead of carrageenan suspension. After the last determination (5 h), animals were killed by cervical dislocation and right hind paws were homogenized in 2 ml saline. Aliquots of supernatants were used to determine prostaglandin E_2 levels by radioimmunoassay (Moroney et al., 1988) and myeloperoxidase activity as above.

2.3. Preparation of human neutrophils

Human neutrophils were isolated from the peripheral blood of healthy volunteers. The citrated blood was centrifuged at 200 $\times g$ for 15 min at room temperature. The platelet-rich plasma was removed and the neutrophils which are contained in the residual blood were isolated by sedimentation with 2.0% (w/v) dextran in 0.9% NaCl at room temperature. The supernatant was centrifuged at 1200 $\times g$ for 10 min at 4°C. Contaminating erythrocytes were lysed by hypotonic treatment. The pellet was resuspended in phosphate-buffered saline solution (PBS), and neutrophils were purified by Ficoll-hypaque sedimentation. The cells were resuspended in PBS containing 1.26 mM Ca^{2+} and 0.9 mM Mg^{2+} (Bustos et al., 1995). Viability was greater than 95% by the trypan blue exclusion test. To assess possible cytotoxic effects of test drugs, the cytoplasmic marker enzyme lactate dehydrogenase (LDH) was determined by measuring the rate of oxidation of NADH. The absorbance change was followed at 340 nm (Bergmeyer and Bernt, 1974). Tubes containing 0.5% Triton X-100 were used for measurement of total cellular content of LDH.

2.4. Superoxide generation by human neutrophils

Aliquots of 0.5 ml neutrophils (2.5×10^6 cells/ml) were preincubated for 5 min at 37°C with test compounds or the vehicle (methanol, 5 μl). TPA (1 μM) was used to stimulate neutrophils for 10 min and superoxide generation was measured by the reduction of cytochrome *c* at 550 nm (Payá et al., 1993).

2.5. Elastase release by human neutrophils

The cells (2.5×10^6/ml) were preincubated with test compound or vehicle for 5 min at 37°C and then stimulated with different agents and incubated 10 min at 37°C.

The stimulus used were: cytochalasin B (10 μM) and *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP, 10 μM), platelet activating factor (PAF, 0.5 μM), leukotriene B_4 (1 μM) or calcium ionophore A23187 (1 μM). After centrifugation at 1200 × *g* for 10 min at 4°C, supernatants were incubated with *N-tert*-butoxy-carbonyl-L-alanine *p*-nitrophenyl ester (200 μM) for 20 min at 37°C and absorbances were determined at 414 nm in a microplate reader (Bustos et al., 1995).

2.6. Elastase and myeloperoxidase activities from human neutrophils

Effects on elastase and myeloperoxidase were assessed using supernatants of human neutrophils stimulated with cytochalasin B (10 μM) + fMLP (10 μM), which were incubated with test compounds for 10 min at 37°C. Aliquots of these reactions were used for elastase determination as above, or myeloperoxidase determination following published procedures (Suzuki et al., 1983; De Young et al., 1989).

2.7. Synthesis and release of leukotriene B_4 by human neutrophils

Leukocytes (5×10^6/ml) in a volume of 500 μl, were preincubated with test compound or vehicle (5 μl) for 10 min at 37°C. Calcium ionophore A23187 (final concentration 1 μM) was added in a volume of 5 μl and the mixture was incubated for 10 min. After centrifugation at 1200 × *g* for 10 min at 4°C, the radioimmunoassay for leukotriene B_4 was performed in supernatants (Moroney et al., 1988).

2.8. Assay of phospholipase A_2

Secretory phospholipase A_2 was assayed by using [^{3}H]oleate labelled autoclaved *Escherichia coli* following a modification of the method of Franson et al. (1974) described previously (Payá et al., 1996). Human recombinant synovial enzyme was diluted in 10 μl (0.03 μg protein) of 100 mM Tris-HCl, 1 mM $CaCl_2$ buffer pH 7.5.

2.9. 5-Lipoxygenase assay

High speed (100 000 × *g*) supernatants from human neutrophils were obtained and incubated with 10 μM arachidonic acid as previously described (Tateson et al., 1988). Leukotriene B_4 levels were measured by radioimmunoassay (Moroney et al., 1988).

2.10. Cyclo-oxygenase-1 assay

Platelet-rich plasma was obtained as above and centrifuged at 1500 × *g* for 10 min. The pellet was resuspended in 50 mM Tris HCl + 1 mM EDTA, pH 7,4 and sonicated at 4°C in an ultrasonicator at maximum potency. Platelet microsomes were prepared by centrifugation at 15 000 × *g* for 20 min at 4°C followed by centrifugation of the supernatant at 100 000 × *g* for 100 min at 4°C. Microsomes (20 μg protein/tube) were incubated for 30 min at 37°C in 50 mM Tris HCl, pH 7.4 with 5 μM arachidonic acid and test compound or vehicle in the presence of 2 μM hematin and 1 mM L-tryptophan. The reaction was terminated boiling the samples for 5 min and thromboxane B_2 levels were determined by radioimmunoassay (Moroney et al., 1988).

2.11. Cyclo-oxygenase-2 assay

The leukocyte fraction of human blood was resuspended in RPMI1640 culture medium containing aspirin (30 μM) and incubated at 37°C for 15 min. The cells were washed twice, resuspended in RPMI1640 with 10% fetal bovine serum and incubated with *E. coli* lipopolysaccharide (10 μg/ml) at 37°C for 4 h (Grossman et al., 1995). After centrifugation the cells were sonicated at 4°C in an ultrasonicator at maximum potency and microsomes were prepared by centrifugation at 100 000 × *g* for 100 min at 4°C. Microsomes (40 μg protein/tube) were used as a source of cyclo-oxygenase-2 and reactions were carried out in the same conditions as above. Prostaglandin E_2 synthesis was determined by radioimmunoassay (Moroney et al., 1988).

2.12. Materials

Akendo 1-4 were purified as previously reported (Kijjoa et al., 1994). The structures of these compounds are shown

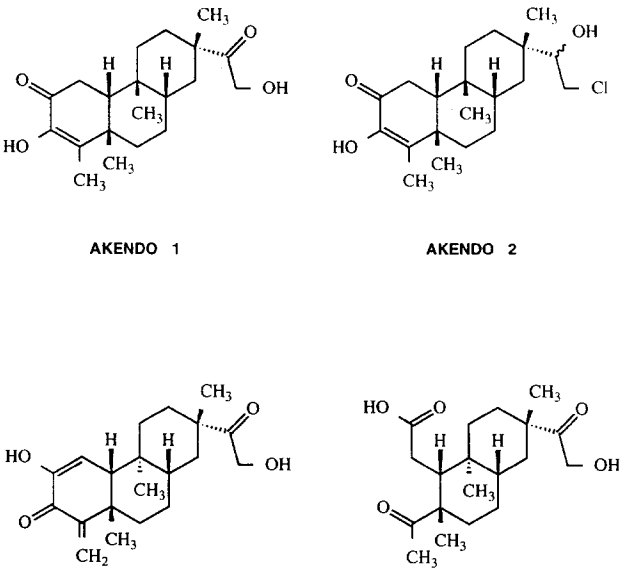

Fig. 1. Chemical structures of akendo 1–4.

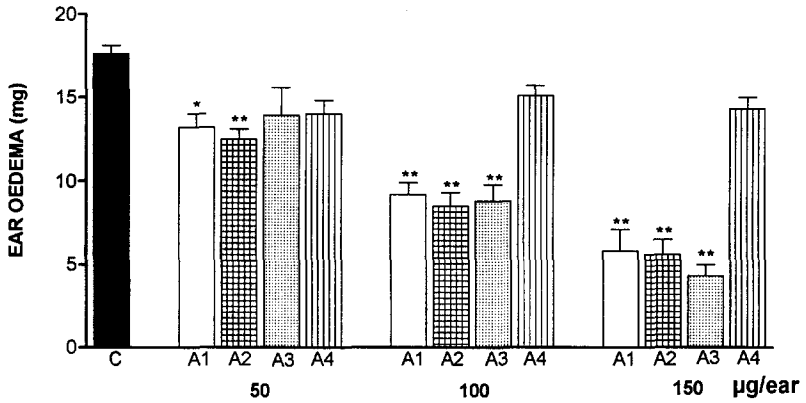

Fig. 2. Effect of akendo 1–4 on the mouse ear oedema induced by TPA. Data represent means ± S.E.M., $n = 6–12$. * $P < 0.05$, ** $P < 0.01$. Compounds were applied topically at the same time as the irritant. A1 = akendo 1, A2 = akendo 2, A3 = akendo 3 and A4 = akendo 4.

in Fig. 1. [5,6,8,11,12,14,15(n)-^{3}H]Prostaglandin E_2, [5,6,8,9,11,12,14,15(n)-^{3}H]thromboxane B_2, [5,6,8,9,11,12,14,15(n)-^{3}H]leukotriene B_4 and leukotriene C_4 radioimmunoassay kit were from Amersham Iberica (Madrid, Spain). [9,10-^{3}H]Oleic acid was purchased from Du Pont (Itisa, Madrid, Spain) and NS 398 was purchased from Universal Biologicals (London, UK). Other materials were from Sigma Chemical Co. (St. Louis, MO, USA). 6-([3-Fluoro-5-(4-methoxy-3,4,5,6-tetrahydro-2H-pyran-4-yl)phenoxy]methyl)-ethyl-2-quinolone (ZM 230,487), human recombinant synovial phospholipase A_2 and antibody against leukotriene B_4 were a gift from Dr R.M. McMillan and Dr S.J. Foster, Zeneca Pharmaceuticals, Macclesfield, Cheshire, UK.

2.13. Statistical analysis

Data are presented as the means ± S.E.M. Statistical analysis was performed by using one-way analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons.

3. Results

3.1. Mouse ear oedema

Fig. 2 shows the effect on ear oedema induced by TPA of akendo 1–4 at the doses of 50, 100 and 150 µg/ear. Akendo 1, akendo 2 and akendo 3 dose dependently decreased ear oedema and exhibited the same potency since their approximated inhibitory dose 50% (ID_{50}) were 106, 99 and 99 µg/ear, respectively. These natural products were more potent than indomethacin, which in this test showed an ID_{50} value of 168 µg/ear. In contrast, akendo 4 slightly reduced oedema without reaching statistical significance. The myeloperoxidase content of inflammed ears is taken as an index of leukocyte migration and was significantly decreased by all the compounds tested (Fig. 3). Akendo 4 was less effective than the rest of compounds and also failed to show a dose-effect relationship. In contrast, akendo 1, akendo 2 and akendo 3 decreased myeloperoxidase levels by more of 50% even at the lowest dose tested (50 µg/ear). Indomethacin was less

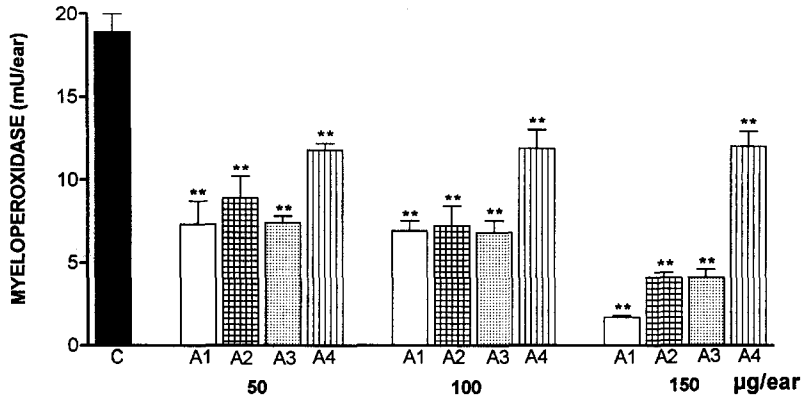

Fig. 3. Effect of akendo 1–4 on myeloperoxidase levels in the mouse ear oedema induced by TPA. Data represent means ± S.E.M., $n = 6–12$. * $P < 0.05$, ** $P < 0.01$. Compounds were applied topically at the same time as the irritant. A1 = akendo 1, A2 = akendo 2, A3 = akendo 3 and A4 = akendo 4.

Table 1
Effect of akendo 1–4 on arachidonic acid-induced mouse ear oedema

	Oedema (mg)	Prostaglandin E_2 (ng/ear)	Leukotriene C_4 (ng/ear)
Control	15.3 ± 1.1	44.1 ± 10.7	41.6 ± 2.8
Akendo 1			
250 μg/ear	15.6 ± 0.9	39.2 ± 8.6	46.4 ± 7.0
500 μg/ear	14.3 ± 0.8	41.5 ± 11.1	57.3 ± 5.8
Akendo 2			
250 μg/ear	11.9 ± 1.3	34.0 ± 6.2	56.0 ± 11.0
500 μg/ear	11.8 ± 0.2	33.5 ± 12.3	49.9 ± 8.7
Akendo 3			
250 μg/ear	15.7 ± 0.6	29.4 ± 5.3	36.0 ± 3.3
500 μg/ear	13.0 ± 1.1	22.7 ± 6.9	43.2 ± 4.5
Akendo 4			
250 μg/ear	14.0 ± 1.2	44.6 ± 5.6	57.2 ± 5.8
500 μg/ear	12.3 ± 1.4	43.8 ± 9.4	38.4 ± 3.1

Data represent means ± S.E.M., $n = 6–8$.

Table 2
Effect of akendo 1–4 on myeloperoxidase activity and prostaglandin E_2 levels in the carrageenan mouse paw oedema

	Myeloperoxidase (mU/paw)	Prostaglandin E_2 (ng/paw)
Control	78.4 ± 12.0	90.2 ± 9.7
Akendo 1 (30 mg/kg p.o.)	55.2 ± 4.0	114.0 ± 12.8
Akendo 2 (30 mg/kg p.o.)	33.6 ± 5.2 [b]	81.4 ± 7.7
Akendo 3 (30 mg/kg p.o.)	37.6 ± 0.8 [a]	135.6 ± 18.7
Akendo 4 (30 mg/kg p.o.)	68.0 ± 8.0	135.2 ± 10.2
Indomethacin (10 mg/kg p.o.)	33.2 ± 4.0 [b]	6.0 ± 1.0

Data represent means ± S.E.M., $n = 6–10$. [a] $P < 0.05$, [b] $P < 0.01$.

effective than these dolabrane derivatives on myeloperoxidase levels and showed an approximated ID_{50} value of 59 μg/ear. When the ear inflammation was induced by arachidonic acid, none of the compounds at doses up to 500 μg/ear were able to significantly reduce swelling or modify eicosanoid levels (Table 1). Indomethacin at 500 μg/ear abolished prostaglandin E_2 levels but only inhibited oedema by 27.5 ± 2.0% ($n = 6$, $P < 0.05$).

3.2. Mouse paw oedema

As depicted in Fig. 4, when administered orally at a dose of 30 mg/kg, only akendo 2 and akendo 3 significantly inhibited mouse paw oedema induced by carrageenan. Akendo 2 was effective at the three determinations (1, 3 and 5 h), whereas akendo 3 acted at 3 and 5 h after carrageenan administration. At these two time points the inhibition of oedema achieved by both compounds was similar, with values of 41% and 31% of inhibition at 3 h for akendo 2 and akendo 3, respectively, while indomethacin at 10 mg/kg p.o. inhibited oedema by 46%. In the same test, the group treated with indomethacin at 10 mg/kg p.o. showed oedema values of 71.3 ± 9.2 μl (1 h), 69.3 ± 7.8 μl (3 h, $P < 0.01$) and 59.4 ± 7.9 μl (5 h, $P < 0.01$) ($n = 6$). Akendo 2 and akendo 3 also inhibited myeloperoxidase levels in inflammed paws measured 5 h after induction of inflammation (Table 2) and this parameter was affected to a greater extent than oedema. The administration of carrageenan increased the paw content of prostaglandin E_2 from 26.8 ± 1.8 ng/paw ($n = 6$) to 90.2 ± 9.7 ng/paw ($P < 0.05$, $n = 10$) measured at 5 h after the induction of the inflammatory response. As expected, indomethacin-treated animals showed prostaglandin E_2

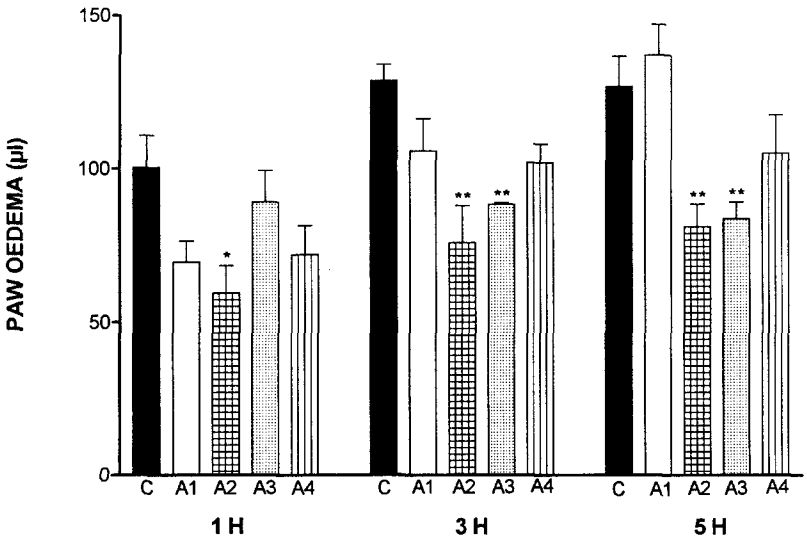

Fig. 4. Effect of akendo 1–4 on mouse paw oedema induced by carrageenan. Data represent means ± S.E.M., $n = 6–10$. * $P < 0.05$, ** $P < 0.01$. Compounds were administered orally at the dose of 30 mg/kg, 1 h before the injection of carrageenan. A1 = akendo 1, A2 = akendo 2, A3 = akendo 3 and A4 = akendo 4.

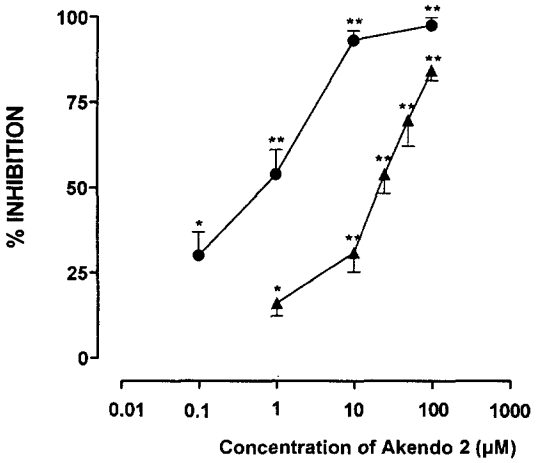

Fig. 5. Effect of akendo 2 on elastase release by human neutrophils. Data represent means ± S.E.M., $n = 6–10$. * $P < 0.05$, ** $P < 0.01$. (▲) cytochalasin B + PAF; (●) cytochalasin B + leukotriene B_4.

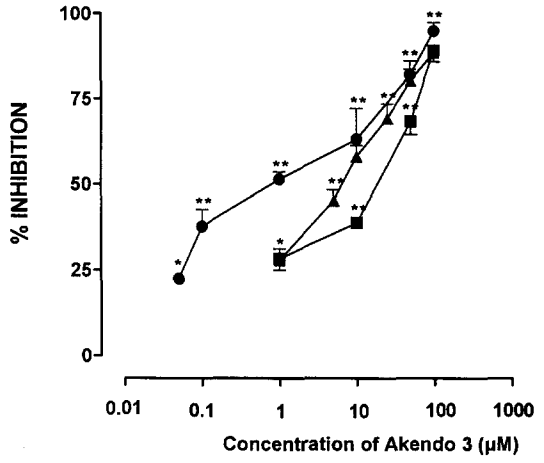

Fig. 6. Effect of akendo 3 on elastase release by human neutrophils. Data represent means ± S.E.M., $n = 6–10$. * $P < 0.05$, ** $P < 0.01$. (▲) cytochalasin B + PAF; (■) cytochalasin + fMLP; (●) cytochalasin B + leukotriene B_4.

values lower than basal levels whereas the dolabrane derivatives did not modify in a significant way the paw content of this eicosanoid with respect to the control group (Table 2).

3.3. Superoxide generation and elastase release by human neutrophils

Initial experiments were conducted to determine if akendo 1–4 exerted cytotoxic effects on human neutrophils. Once excluded such effects, experiments were performed to assess the influence of these dolabrane derivatives on the production of superoxide by stimulated neutrophils. None of the compounds tested at concentrations up to 100 μM, had any significant effect on superoxide generation during a 10 min incubation with TPA. Nevertheless, preincubation of isolated human neutrophils with akendo 2 or akendo 3 elicited a concentration-dependent inhibition of leukotriene B_4- and PAF-induced degranulation measured as elastase release (Fig. 5 and Fig. 6). The inhibitory concentration 50%(IC_{50}) values and 95% confidence limits were 0.7 (0.1–2.2) μM and 1.1 (0.4–2.0) μM for akendo 2 and akendo 3, respectively, in the first response and 22.3 (15.1–30.2) μM and 6.9 (4.0–12.0) μM when PAF was the stimulus used. Akendo 1 decreased degranulation induced by leukotriene B_4 ($IC_{50} = 2.4$; 0.5–9.2 μM) but exerted weak effects if the cells were stimulated by PAF, with 40.7 ± 4.5% inhibition ($n = 10$, $P < 0.01$) at 100 μM. In contrast, akendo 4 inhibited PAF-induced elastase release ($IC_{50} = 29.5$; 11.9–79.4 μM) but decreased leukotriene B_4-induced degranulation only by 46.7 ± 9.0% ($n = 6$, $P < 0.05$) at 100 μM. In the presence of fMLP, only akendo 3 afforded significant inhibition with an IC_{50} value of 33.6 (23.3–40.0) μM, whereas none of the compounds at concentrations between 0.1 and 100 μM inhibited degranulation induced by the calcium ionophore A23187 (data not shown).

3.4. Elastase and myeloperoxidase activities from human neutrophils

Direct inhibitory effects of akendo 1–4 on these enzyme activities released by human neutrophils stimulated by cytochalasin B + fMLP, were also assessed. Table 3 shows that only akendo 2 and akendo 3 decreased

Table 3
Effect of akendo 1–4 on several enzymatic activities

	Myeloperoxidase		5-Lipoxygenase		Cyclo-oxygenase-1	
	% inhibition	IC_{50} (μM)	% inhibition	IC_{50} (μM)	% inhibition	IC_{50} (μM)
Akendo 1	24.7 ± 2.0	N.D.	28.5 ± 7.0	N.D.	43.3 ± 5.6 [b]	N.D.
Akendo 2	59.5 ± 3.0 [b]	74.4 (65.7–83.1)	29.1 ± 8.8	N.D.	70.9 ± 3.5 [b]	49.7 (37.9–60.3)
Akendo 3	91.9 ± 1.4 [b]	4.8 (2.2– 7.9)	67.2 ± 0.9 [b]	43.6 (33.0–46.9)	73.1 ± 2.3 [b]	33.7 (13.7–112.4)
Akendo 4	16.6 ± 4.6	N.D.	9.0 ± 3.1	N.D.	30.4 ± 3.8 [b]	N.D.

Data represent means ± S.E.M., $n = 6–12$ of percentages of inhibition at 100 μM and IC_{50} (μM). [b] $P < 0.01$. N.D. = not determined. IC_{50} values are shown with their 95% confidence limits.

myeloperoxidase activity in a significant way, akendo 3 causing its effect at lower drug concentrations than akendo 2 ($IC_{50} = 4.8$; 2.2–7.9 μM and $IC_{50} = 74.4$; 65.7–83.1 μM, respectively). The four compounds at concentrations up to 100 μM were inactive on elastase (data not shown).

3.5. Arachidonic acid release and metabolism in vitro

These dolabrane derivatives at the concentration of 100 μM reduced the fatty acid release induced by recombinant human synovial phospholipase A_2 activity by less of 10% (data not shown). When human neutrophils were stimulated by A23187, leukotriene B_4 levels increased from 0.5 ± 0.01 to 115.2 ± 5.6 ng/ml ($n = 18$). Synthesis and release of leukotriene B_4 by A23187-stimulated human neutrophils was inhibited weakly, with percentages of inhibition at 100 μM of 16.4 ± 3.1 ($n = 5$, $P < 0.05$), 37.8 ± 5.6 ($n = 5$, $P < 0.01$), 56.1 ± 3.4 ($n = 8$, $P < 0.01$) and 41.9 ± 1.9 ($n = 9$, $P < 0.01$) for akendo 1–4, respectively. As seen in Table 3, akendo 3 yielded significant inhibition of leukotriene B_4 synthesized by 5-lipoxygenase activity present in $100\,000 \times g$ supernatants of human neutrophils. The selective inhibitor of 5-lipoxygenase activity, ZM230,487 potently inhibited leukotriene B_4 synthesis by whole cells or $100\,000 \times g$ supernatants with IC_{50} values of 0.06 (0.03–0.1) μM and 0.09 (0.06–0.13) μM. Synthesis of thromboxane B_2 by cyclo-oxygenase-1 present in microsomes from human platelets was inhibited mainly by akendo 2 and akendo 3 (Table 3), with a low potency compared to that of indomethacin, which showed an IC_{50} value of 0.01 (0.001–0.05) μM. On the other hand, synthesis of prostaglandin E_2 by cyclo-oxygenase-2 activity was inhibited only by akendo 3 at 100 μM ($43.1 \pm 6.1\%$, $n = 6$, $P < 0.01$). In this system, the selective inhibitor of cyclo-oxygenase-2, NS398 inhibited prostaglandin E_2 synthesis with an $IC_{50} = 2.0$ (0.8–4.7) μM.

4. Discussion

Experiments were conducted to determine the effects of these dolabrane derivatives on animal models of inflammation. TPA application to mouse ear induces a marked increase in vascular permeability and oedema associated to cell influx and moderate eicosanoid synthesis. On the contrary, the response dependent on arachidonic acid shows lower oedema values and marked eicosanoid synthesis. The synthesis of leukotriene B_4 seems to play an important role in the TPA-induced oedema (Rao et al., 1993). In addition, prostaglandins or mediators such as histamine, bradykinin (Carlson et al., 1985) or PAF (Merlos et al., 1991) can also play a role in this response. On the other hand, leukotrienes and prostaglandins mediate the arachidonic acid-induced inflammatory response (Rao et al., 1993). Cyclo-oxygenase and lipoxygenase inhibitors are known to reduce TPA- and arachidonic acid-induced oedema, with some differences in potency (Rao et al., 1993; Bustos et al., 1995). In contrast, the inhibitory effect of akendo 1, akendo 2 and akendo 3 after topical application, on TPA-induced oedema was dose-dependent but they did not modify the inflammation induced by arachidonic acid and the levels of prostaglandin E_2 and leukotriene C_4 were not affected, suggesting that the anti-inflammatory effects of these natural products were not brought about through inhibition of arachidonic acid metabolism. On the contrary, they inhibited myeloperoxidase activity, indicating a reduction in leukocyte migration. Interestingly, the active dolabrane derivatives showed very potent topical anti-inflammatory effects on oedema and myeloperoxidase.

In the carrageenan mouse paw oedema the initial change in paw volume (approximately until the first hour) is unrelated to prostaglandin production, since it is not inhibited by indomethacin although the second phase of oedema (2–5 h) is very sensitive to cyclo-oxygenase inhibitors (Sugishita et al., 1981). In fact, the induction of cyclo-oxygenase-2 activity has been demonstrated in inflammed paws during the second phase of this response in rats (Seibert et al., 1994). In our experiments, indomethacin inhibited the second phase of carrageenan mouse paw oedema, myeloperoxidase levels and significantly decreased prostaglandin E_2 levels in the inflammed paw. Akendo 2 and akendo 3 inhibited paw oedema and myeloperoxidase levels when administered p.o. before challenge with carrageenan, although they did not modify prostaglandin E_2 levels, which confirm that they did not modify inflammatory responses in this study through the inhibition of cyclo-oxygenase activity.

During inflammation, activated neutrophils release reactive oxygen species and lysosomal enzymes which mediate tissue injury at the site of inflammation (Smith, 1994). Myeloperoxidase catalyzes the reaction of hydrogen peroxide and chloride to form HOCl, a strong oxidant. In addition, human neutrophils generate hydroxyl radical through a myeloperoxidase-dependent mechanism, by reaction of HOCl and superoxide (Ramos et al., 1992). The dolabrane derivatives tested may inhibit some aspects of the neutrophil function. They did not affect neutrophil-generated superoxide. Nevertheless, all the compounds inhibited the degranulation induced by the receptor agonists leukotriene B_4 and PAF with some differences in potency depending on the stimulus used. The most effective dolabrane derivatives on this response were akendo 2 and akendo 3. The last compound was also the only compound able to modify the response induced by cytochalasin B + fMLP. In contrast, all the dolabrane derivatives failed to decrease degranulation induced by A23187 and thus their inability to inhibit neutrophil degranulation under conditions where the requirement of cell surface receptors is bypassed, suggests the inhibition of the ligand-receptor

interaction or signal transduction by these compounds. An additional mechanism to control inflammatory responses could be the inhibition of myeloperoxidase activity by akendo 2 and akendo 3 and consequently, these compounds could prevent or slow the progression of neutrophil-mediated tissue injury.

Inhibition of secretory phospholipase A_2 activity has been related to neutrophil exocytosis (Barnette et al., 1994). Nevertheless, these compounds reduced human neutrophil degranulation but did not act as inhibitors of synovial phospholipase A_2 and exerted a weak inhibitory influence on leukotriene B_4 release in human neutrophils, which would indicate that these dolabrane derivatives do not modify arachidonic acid mobilization in membranes. On the other hand, these compounds had little effect on 5-lipoxygenase and cyclo-oxygenase activities in vitro since akendo 3 was the only compound which showed inhibition of cyclo-oxygenase-2 activity at 100 μM, whereas all of them inhibited weakly cyclo-oxygenase-1 and 5-lipoxygenase.

The comparison of the effects of this series of compounds on models of inflammation and in vitro inflammatory responses suggests certain structural features. The most active compounds are akendo 2 and akendo 3, which indicates that chloro substitution of the side chain or the presence of a conjugated 3-keto-4-methylene group, provides an increase in activity. According to this hypothesis, akendo 1 exhibits a lower anti-inflammatory potency. In contrast, the opening of the A ring results in a drastic reduction (akendo 4).

In this report we have described the activity of a series of dolabrane derivatives, a new class of natural anti-inflammatory agents which do no act by inhibiting the synthesis of arachidonic acid metabolites. Instead, they block recruitement of neutrophils into inflammatory lesions and also inhibit degranulation and myeloperoxidase activity in vitro.

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