J. Pharm. Pharmacol. 1996, 48: 975–980
Received November 2, 1995
Accepted March 8, 1996

Effect of Bakuchiol on Leukocyte Functions and Some Inflammatory Responses in Mice

MARÍA LUISA FERRÁNDIZ, BLANCA GIL, MARÍA JESÚS SANZ, AMALIA UBEDA, SILVIA ERAZO* ERNESTO GONZÁLEZ* ROSA NEGRETE* SERGIO PACHECO†, MIGUEL PAYÁ AND MARÍA JOSÉ ALCARAZ

*Departamento de Farmacología, Universidad de Valencia, Facultad de Farmacia, Avda. Vicent Andrés Estellés s/n, 46100 Burjassot, Valencia, Spain, *Departamento de Química Farmacológica y Toxicológica, Universidad de Chile, Facultad de Ciencias Químicas y Farmacéuticas, Casilla 233, Santiago-1, Chile and †Departamento de Biología Geral, Universidade Federal de Viçosa, 36570 Viçosa, Brasil*

Abstract

The effects of bakuchiol, a meroterpenoid isolated from the leaves of *Psoralea glandulosa* L., on phospholipase A_2 (PLA_2) activity from different sources, human neutrophil responses, zymosan air pouch and topical inflammation in mice, were investigated.

This natural product was a weak inhibitor of secretory and intracellular PLA_2 but dose-dependently reduced the formation of LTB_4 and TXB_2 by human neutrophils and platelet microsomes, respectively. In addition, bakuchiol inhibited degranulation in human neutrophils, whereas superoxide generation was not affected. In mice, bakuchiol decreased cell migration, myeloperoxidase activity and eicosanoid levels in the air pouch inflammation induced by zymosan. After topical administration, this compound was effective as an inhibitor of oedema and myeloperoxidase activity in the 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-induced ear oedema and significantly reduced the PGE_2 content and ear oedema in the arachidonic acid-induced response.

Bakuchiol is a natural anti-inflammatory agent able to control leukocytic functions such as eicosanoid production, migration and degranulation in the inflammatory site.

Some extracts of *Psoralea glandulosa* L. (Fabaceae) leaves have antipyretic and anti-inflammatory activities (Backhouse et al 1995). A major component of this plant is the meroterpenoid bakuchiol (Fig. 1), which exerts anti-inflammatory and anti-pyretic effects similar to those of sodium naproxen, with a lower toxicity (Zuñiga 1994). Nevertheless there are no data on the possible mechanisms of action of this novel anti-inflammatory compound.

PLA_2 activity releases arachidonic acid from membrane phospholipids as a first step in the synthesis of eicosanoids. The PLA_2 enzymes can be divided broadly into two major groups, the secretory PLA_2 (low molecular weight) and the intracellular PLA_2 (high molecular weight). The involvement of PLA_2 in inflammation is supported by the effects of secreted enzymes which cause acute inflammatory reactions when injected into animals and also by the anti-inflammatory activity of specific inhibitors. Indeed, several natural products, including a number of terpenoids, antagonize the actions of PLA_2 activity from different origins and exhibit potent anti-inflammatory activity which may result from their ability to interfere with a step in the PLA_2-induced inflammation.

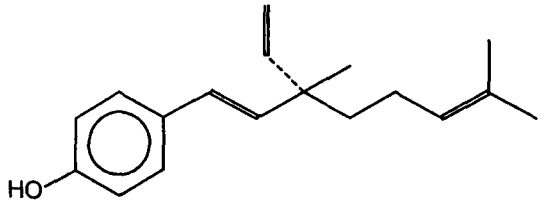

FIG. 1. Structure of bakuchiol.

Correspondence: M. J. Alcaraz, Departamento de Farmacología, Universidad de Valencia, Facultad de Farmacia, Avda. Vicent Andrés Estellés s/n, 46100 Burjassot, Valencia, Spain.

The objective of this study was to characterize the mode of action of bakuchiol in enzymic, cellular and in-vivo models relevant to the inflammatory process. To this end, we investigated the influence of bakuchiol on PLA_2 activity and human neutrophil responses as well as on zymosan air pouch and topical inflammation in mice.

Methods

Preparation of human neutrophils

Human neutrophils were separated from peripheral blood of healthy volunteers. The citrated blood was centrifuged at 200 *g* for 15 min at room temperature (21°C). The platelet-rich plasma was removed and the neutrophils which are contained in the residual blood were isolated by sedimentation with 2·0% dextran in 0·9% NaCl (saline). The supernatant was centrifuged at 1200 *g* for 10 min at 4°C. Contaminating erythrocytes were lysed by hypotonic treatment and neutrophils were purified by ficoll-hypaque sedimentation and resuspended in phosphate-buffered saline (PBS) containing 1·26 mM Ca^{2+} and 0·9 mM Mg^{2+}. Viability was greater than 95% by the trypan blue exclusion test.

Superoxide generation

A neutrophil suspension (0·5 mL) containing $2{\cdot}5 \times 10^6$ cells mL^{-1} was preincubated for 5 min at 37°C with test compounds or vehicle (ethanol, 5 μL) and 12-*O*-tetradecanoylphorbol 13-acetate (TPA, 1 μM) was added to induce superoxide generation, which was estimated as the superoxide dismutase inhibitable reduction of cytochrome C at 550 nm (Payá et al 1993).

Cytotoxicity studies
Lactate dehydrogenase was determined by measuring the rate of oxidation of NADH (Bergmeyer & Bernt 1974). Tubes containing 0·5% Triton X-100 were used for measurement of the total cellular content of LDH.

Elastase release
Neutrophils ($2·5 \times 10^6$ mL^{-1}) were preincubated with test compound or vehicle for 5 min and then stimulated with cytochalasin B (10 μM) and *N*-formyl-L-methionil-L-leucyl-L-phenylalanine (10 μM) for 10 min at 37°C. After centrifugation at 1200 *g* at 4°C, supernatants were incubated with *N-tert*-butoxy-carbonyl-L-alanine *p*-nitrophenyl ester (200 μM) for 10 min at 37°C (Barrett 1981). The extent of *p*-nitrophenol release was measured at 414 nm in a microtitre plate reader.

Elastase and myeloperoxidase activities from human neutrophils
Effects on elastase and myeloperoxidase were assessed using supernatants of human neutrophils. Neutrophils (10^8 mL^{-1}) were lysed by sonication and the mixture was centrifuged at 100 000 *g* for 1 h at 4°C. The supernatant was diluted in PBS at a protein concentration of 0·21 mg mL^{-1}. Samples of the diluted supernatant were used for the above reaction (elastase) or for myeloperoxidase determination following published procedures (Suzuki et al 1983; De Young et al 1989).

Synthesis and release of LTB_4 by human neutrophils
A suspension of human neutrophils (5×10^6 mL^{-1}) in PBS was preincubated with test compounds or vehicle and then stimulated with 1 μM A23187 for 10 min at 37°C. After centrifugation at 1200 *g* for 10 min at 4°C, the supernatants were frozen at − 80°C until the radioimmunoassay for LTB_4 was performed in supernatant samples (Moroney et al 1988).

Synthesis of TXB_2 by human platelet microsomes
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 haematin and 1 mM L-tryptophan. The reaction was terminated by boiling the samples for 5 min and TXB_2 levels were determined by radioimmunoassay (Moroney et al 1988).

Synthesis of PGE_2 by human leukocyte microsomes
After platelet isolation, the leukocyte fraction of human blood was resuspended in RPMI1640 culture medium containining aspirin (30 μM) and incubated at 37°C for 15 min. The cells were washed twice, resuspended in RPMI1640 with 10% foetal bovine serum and incubated with *Escherichia coli* lipopolysaccharide (10 μg mL^{-1}) at 37°C for 4 h. 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. PGE_2 synthesis was determined by radioimmunoassay (Moroney et al 1988).

Synthesis of LTB_4 by high-speed supernatants from human neutrophils
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). LTB_4 levels were measured by radioimmunoassay (Moroney et al 1988).

Assay of secretory PLA_2
Secretory PLA_2 was assayed by using [^{3}H]oleate-labelled autoclaved *Escherichia coli* following a modification of the method of Franson et al (1974). *E. coli* strain CECT 101 were seeded in medium containing 1% tryptone, 0·5% NaCl and 0·6% sodium dihydrogen orthophosphate pH 5·0 and grown for 6–8 h at 37°C in the presence of 5 μCi mL^{-1} [^{3}H]oleic acid (sp. act. 10 Ci mmol^{-1}). After centrifugation at 2500 *g* for 10 min, the cells were washed in buffer (0·7 M Tris-HCl, 10 mM $CaCl_2$, 0·1% bovine serum albumin, pH 8·0), resuspended in saline and autoclaved for 30–45 min. At least 95% of the radioactivity was incorporated into phospholipids. Three secretory enzymes were assayed, *Naja naja* venom enzyme, bee venom enzyme and human recombinant synovial enzyme, which were diluted in 10 μL 100 mM Tris-HCl, 1 mM $CaCl_2$ buffer pH 7·5 and preincubated at 37°C for 15 min with 2·5 μL test compound solution or its vehicle (methanol). Incubation proceeded for 15 min in the presence of 10 μL autoclaved oleate-labelled membranes and was terminated by addition of 100 μL ice-cold 0·25% bovine serum albumin in saline to a final concentration of 0·07%. After centrifugation at 2500 *g* for 10 min at 4°C, the radioactivity in the supernatants was determined by liquid scintillation counting.

Mouse air pouch
Male Swiss mice, 25–30 g, were anaesthetized with ethyl ether and injected with 10 mL sterile air into the subcutaneous tissue of the back and three days later 5 mL sterile air was injected into the same cavity. After three days mice received via the air pouch 1 mL 1% zymosan in saline + vehicle (10 μL ethanol : control group) or 1 mL 1% zymosan in saline + test drug (dissolved in 10 μL ethanol). Another group received only 1 mL saline + vehicle (saline group). Four hours after administration animals were killed by cervical dislocation and the exudate in the pouch was collected with 1 mL saline (Edwards et al 1981). Samples of exudate were used to measure the leukocyte number using a Coulter counter and to determine superoxide anion release by the reduction of cytochrome C (Payá et al 1993). After centrifugation of the exudate at 1200 *g* at 4°C for 10 min, the supernatants were used to measure myeloperoxidase activity (Suzuki et al 1983; De Young et al 1989) as well as LTB_4 and PGE_2 levels by radioimmunoassay (Moroney et al 1988).

Preparation of intracellular PLA_2 (mouse air pouch)
Intracellular PLA_2 was prepared from the mouse air pouches obtained in the above experiment which were homogenized with a Polytron homogenizer in 25 mM Tris-HCl buffer, pH 7·4, containing 0·25 M sucrose, 2 mM EDTA, 2·5 mM dithiothreitol, 140 mM NaCl, 5 mM KCl, 1 mM phe-

nylmethylsulphonyl fluoride, 0·1 mM leupeptin, 0·32 mM benzamidine and 3·2 units mL^{-1} bacitracin. The homogenate was centrifuged at 2000 *g* for 10 min at 4°C and the resulting supernatant was further centrifuged at 100 000 *g* for 60 min at 4°C to obtain the cytosolic fraction. Protein concentration was determined (Bradford 1976) using bovine serum albumin (BSA) as standard.

Assay of intracellular PLA_2 (mouse air pouch)

Intracellular PLA_2 activity was measured as the release of radiolabeled arachidonic acid according to the method of Clark et al (1990). 1-Palmitoyl-2-[^{14}C]-arachidonoyl phosphatidylcholine (100 μM, 1 × 10^5 counts min^{-1}) was dried under nitrogen, then suspended in 0·1 mL 100 mM glycine buffer pH 9·0 containing 200 μM Triton X-100, 10 mM $CaCl_2$, 0·25 mg mL^{-1} BSA and 40% glycerol. The suspension was then sonicated to form mixed micelles of phospholipid and Triton X-100. The reaction was started by adding the enzyme solution (approximately 20 μg protein of cytosolic fraction from mouse air pouch) to 100 μL Tris-HCl buffer, pH 7·4 containing 150 mM NaCl, 1 mM $CaCl_2$, 1 mg mL^{-1} BSA, 2·5 mM dithiothreitol, 2 mM 2-mercaptoethanol, 40% glycerol and 10 μL micelles (1 × 10 counts min^{-1}). The reaction was stopped after a 60-min incubation period at 37°C by mixing with 0·5 mL isopropyl alcohol : heptane : 0·5 M H_2SO_4 (10 : 5 : 1). Heptane (0·65 mL) and water (0·2 mL) were then added, and the solution was vigorously mixed for 15 s. The heptane phase was mixed with silica (E. Merck, 100 mg) and centrifuged, and the radioactivity in each supernatant was measured (Zhang et al 1991). The percentage of enzyme inhibition was obtained by comparison with the zymosan control group at the same protein concentration.

Mouse ear oedema

TPA (2·5 μg) or arachidonic acid (2·0 mg) in acetone was applied to both surfaces of the right ear of Swiss mice. Test compounds were administered topically in acetone before TPA or 20 min before arachidonic acid. After 4 h (TPA) or 1 h (arachidonic acid) the animals were killed by cervical dislocation and equal sections of both ears were punched out and weighed (Carlson et al 1985). The ear sections were homogenized and the PGE_2 and LTC_4 content in supernatants was determined by RIA (arachidonic acid-induced oedema), or myeloperoxidase activity was measured in supernatants (TPA-induced oedema).

Materials

Bakuchiol was isolated from *Psoralea glandulosa* L. (Papilionaceae), as previously described (Erazo et al 1990). Human recombinant synovial PLA_2, the 5-lipoxygenase inhibitor ZM230,487 and antibody against LTB_4 were gifts from Dr R. M. McMillan, Zeneca Pharmaceuticals, Macclesfield, Cheshire, UK. [9,10-^{3}H]Oleic acid and L-3-phosphatidylcholine 1-palmitoyl-2-arachidonyl [arachidonyl-1-^{14}C] were purchased from Du Pont, (Itisa, Madrid, Spain). The radioactive eicosanoids [5,6,8,9,11,12,14,15(n)-^{3}H]TXB_2, [5,6,8,11,12,14,15(n)-^{3}H]PGE_2, [5,6,8,9,11,12,14,15(n)-^{3}H]LTB_4 and [14,15(n)-^{3}H]LTC_4 were from Amersham Iberica (Madrid, Spain). NS398 was purchased from Universal Biologicals Ltd (London, UK). All other reagents were from Sigma Chemicals (MO, USA).

Statistical analysis

Statistical analysis was performed using Dunnett's test for multiple comparisons.

Results

Superoxide generation and elastase release

After stimulation with TPA, human neutrophils generated superoxide anion and released elastase, which represents a marker of the degranulation process. The first response was not modified by bakuchiol at concentrations up to 100 μM (data not shown). Nevertheless this compound inhibited elastase release in a concentration-dependent manner (Fig. 2) with an inhibitory concentration 50% (IC50) value and 95% confidence limits (CL) of 11·8 (9·3–13·3) μM. As expected, indomethacin did not inhibit superoxide generation and exerted a weak effect on degranulation, with 29·2 ± 2·1% inhibition at 100 μM (means ± s.e.m, n = 6, $P < 0.01$). Cytotoxic effects of bakuchiol on human neutrophils were discounted as lactate dehydrogenase release was not increased after incubation with this compound at concentrations up to 100 μM (data not shown).

Elastase and myeloperoxidase activities from human neutrophils

Bakuchiol did not exert a direct inhibition of elastase activity since this compound failed to modify enzyme activity in experiments using supernatants of sonicated human neutrophils (data not shown). On the contrary, myeloperoxidase activity was potently inhibited by bakuchiol, with an IC50 value of 0·1 (0·05–0·2) μM.

Synthesis and release of LTB_4 by human neutrophils

After stimulation with ionophore A23187, control incubations released 49·8 ± 2·8 ng/5 × 10^6 neutrophils/mL LTB_4, (n = 30), while non-stimulated samples released 0·5 ± 0·1 ng/5 × 10^6 neutrophils/mL LTB_4 (n = 30). Bakuchiol decreased this response in a concentration-dependent manner, with an IC50 value of 23·5 (21·8–24·5) μM. The specific 5-lipoxygenase inhibitor ZM 230,487 inhibited potently LTB_4 production and showed an IC50 value of 60·3 (35·5–95·5) nM.

Synthesis of TXB_2 by human platelet microsomes

Bakuchiol exerted a weak effect on cyclo-oxygenase 1 activity from human platelet microsomes, with 30·9 ± 1·4% inhibition

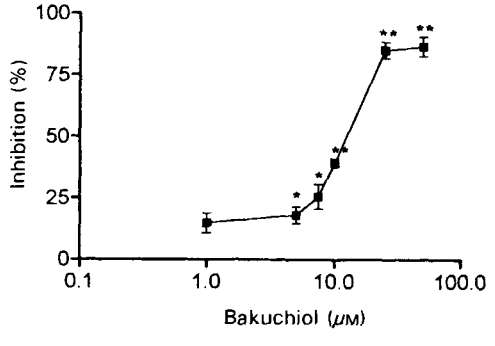

FIG. 2. Effect of bakuchiol on elastase release by human neutrophils. Means ± s.e.m., n = 6–20. *$P < 0.05$, **$P < 0.01$.

of TXB_2 synthesis at 100 μM (n = 6, $P < 0.05$). In contrast, indomethacin was very potent in inhibiting this response (IC50 = 19·4 (4·4–55·7) nM).

Synthesis of PGE_2 by human leukocyte microsomes

The selective inhibitor of cyclo-oxygenase 2 activity, NS398 decreased PGE_2 synthesis in a concentration-dependent manner, with an IC50 value of 2·0 (0·8–4·7) μM. In this system, bakuchiol showed 34·8 ± 6·0% inhibition at 100 μM (n = 6, $P < 0.05$).

Synthesis of LTB_4 by high-speed supernatants from human neutrophils

Bakuchiol showed a higher effect on LTB_4 synthesis, with a concentration-dependent inhibition and the calculated IC50 value was 16·0 (9·6–35·6) μM. The potency of bakuchiol in this system was lower than that of the reference inhibitor of 5-lipoxygenase activity, ZM 230,487 which showed an IC50 value of 6·9 (3·9–17·4) μM.

Secretory PLA_2

Bakuchiol exerted weak inhibitory effects on *Naja naja* venom and human recombinant synovial PLA_2 with percentages of inhibition at 100 μM of 28·0 ± 5·6 and 27·5 ± 2·5, respectively (n = 6, $P < 0.05$). Nevertheless this compound did not affect bee venom enzyme activity (data not shown). The reference inhibitor mepacrine at 1 mM gave 67·3 ± 4·3, 48·9 ± 2·6 and 58·5 ± 2·4% inhibition (n = 6, $P < 0.01$) for *Naja naja* venom, human recombinant synovial and bee venom PLA_2, respectively.

Mouse air pouch (Tables 1,2,3)

In an air pouch inflammation model in mice, the number of leukocytes that had infiltrated into the pouch fluid collected 4 h after zymosan challenge was significantly reduced by bakuchiol at the dose of 1000 nmol/mouse and ZM 230,487 at 100 nmol/mouse, while indomethacin was without effect at the three doses tested (1, 10 and 100 nmol/mouse). As expected, PGE_2 levels in the exudates were strongly reduced by indomethacin and ZM 230,487 exerted similar effects on LTB_4. Bakuchiol significantly inhibited PGE_2 and LTB_4 levels at 100 and 1000 nmol/mouse although a better dose–response relationship was observed for the first eicosanoid. As observed in human neutrophils in-vitro, none of the drugs modified the generation of superoxide anion by the leukocytes present in inflammatory exudates (data not shown). Nevertheless myeloperoxidase levels in exudates were dose-dependently inhibited by bakuchiol, whereas indomethacin and ZM 230,487 were ineffective (Table 4). Bakuchiol also inhibited intracellular PLA_2 activity, at the highest dose tested (1000 nmol/mouse).

Mouse ear oedema

After topical administration, bakuchiol reduced significantly oedema and myeloperoxidase levels (an index of leukocyte migration) in the TPA-induced inflammation model. No direct inhibitory effects on myeloperoxidase activity were observed after in-vitro incubation of bakuchiol with supernatants from TPA-treated ears (data not shown). Indomethacin and ZM 230,487 were more effective than bakuchiol on both parameters (Table 5). When inflammation was induced by arachidonic acid, bakuchiol gave significant results with inhibition of oedema and PGE_2 levels but failed to reduce LTC_4 levels at the doses administered, 250 and 500 μg/ear (Table 6). Its anti-oedematous effect was similar to that of ZM 230,487 and higher than that of indomethacin. The reference compounds strongly inhibited eicosanoid levels with a high selectivity for the cyclo-oxygenase (indomethacin) or lipoxygenase (ZM 230,487) pathway.

Table 1. Effect of bakuchiol on leukocyte accumulation in the mouse air pouch.

	Cell number × 10^6 (mL^{-1})
Saline without zymosan	6·1 ± 0·7**
Control	20·0 ± 1·9
Indomethacin	
1 nmol/mouse	25·2 ± 1·1
10 nmol/mouse	22·5 ± 2·4
100 nmol/mouse	15·2 ± 1·9
ZM 230,487	
1 nmol/mouse	24·8 ± 4·0
10 nmol/mouse	18·8 ± 1·9
100 nmol/mouse	12·0 ± 1·0**
Bakuchiol	
10 nmol/mouse	21·6 ± 3·1
100 nmol/mouse	20·3 ± 2·1
1000 nmol/mouse	13·2 ± 1·2**

Mean ± s.e.m., (n = 6). **$P < 0.01$ compared with control.

Table 2. Effect of bakuchiol on PGE_2 levels in the mouse air pouch.

	PGE_2 (ng mL^{-1})
Saline without zymosan	2·4 ± 0·7**
Control	19·8 ± 1·0
Indomethacin	
1 nmol/mouse	4·3 ± 0·8**
10 nmol/mouse	2·3 ± 0·4**
100 nmol/mouse	1·0 ± 0·2**
Bakuchiol	
10 nmol/mouse	18·2 ± 5·1
100 nmol/mouse	13·1 ± 3·2*
1000 nmol/mouse	4·2 ± 1·6**

Mean ± s.e.m., (n = 6). *$P < 0.05$, **$P < 0.01$ compared with control.

Table 3. Effect of bakuchiol on LTB_4 levels in the mouse air pouch.

	LTB_4 (ng mL^{-1})
Saline without zymosan	0·3 ± 0·01**
Control	35·5 ± 2·9
ZM 230,487	
1 nmol/mouse	9·8 ± 2·2**
10 nmol/mouse	9·5 ± 1·2**
100 nmol/mouse	2·1 ± 0·3**
Bakuchiol	
10 nmol/mouse	22·8 ± 6·0
100 nmol/mouse	22·0 ± 3·5*
1000 nmol/mouse	20·7 ± 5·2*

Mean ± s.e.m., (n = 6). *$P < 0.05$, **$P < 0.01$ compared with control.

Table 4. Effect of bakuchiol and reference compounds on myeloperoxidase and intracellular PLA_2 activities in the mouse air pouch

	Inhibition (%)	
	Myeloperoxidase	Intracellular PLA_2
Bakuchiol		
10 nmol/mouse	41·9 ± 17·5	0
100 nmol/mouse	52·0 ± 13·2*	8·1 ± 3·6
1000 nnol/mouse	78·3 ± 3·7**	32·9 ± 6·9**
Indomethacin		
1 nnol/mouse	3·7 ± 2·1	0
10 nmol/mouse	5·9 ± 5·8	0
100 nmol/mouse	25·7 ± 9·9	13·4 ± 4·9
ZM 230,487		
1 nmol/mouse	1·7 ± 1·7	0
10 nmol/mouse	17·9 ± 16·0	0
100 nmol/mouse	25·9 ± 10·8	9·4 ± 3·4

Data show means ± s.e.m. of 6 animals. *$P < 0.05$, **$P < 0.01$ compared with control. Drugs were injected into the air pouch at the same time as zymosan.

Table 5. Effect of bakuchiol and reference compounds on mouse ear inflammation induced by TPA.

	Inhibition (%)	
	Oedema	Myeloperoxidase
Bakuchiol		
250 μg/ear	34·5 ± 14·1*	48·1 ± 11·7*
500 μg/ear	52·6 ± 6·0**	56·3 ± 4·9**
Indomethacin		
250 μg/ear	65·5 ± 7·7**	59·2 ± 2·4**
500 μg/ear	88·9 ± 3·8**	71·9 ± 2·5**
ZM 230,487		
250 μg/ear	64·4 ± 6·9**	50·8 ± 3·4**
500 μg/ear	80·8 ± 2·4**	62·1 ± 1·7**

Data show means ± s.e.m. of 6 animals. *$P < 0.05$, **$P < 0.01$ compared with control. The oedema value in the control group was 16·8 ± 0·7 mg. Drugs were topically administered before the application of TPA.

Table 6. Effect of bakuchiol and reference compounds on mouse ear inflammation induced by arachidonic acid.

	Inhibition (%)		
	Oedema	PGE_2	LTC_4
Bakuchiol			
250 μg/ear	37·6 ± 7·9*	43·1 ± 5·1**	10·0 ± 3·0
500 μg/ear	61·9 ± 7·9**	53·5 ± 8·3*	15·2 ± 9·4
Indomethacin			
250 μg/ear	23·7 ± 3·7	100 ± 0·0**	0
500 μg/ear	34·4 ± 8·6*	100 ± 0·0**	7·7 ± 4·5
ZM 230,487			
250 μg/ear	49·2 ± 7·9**	13·6 ± 1·4	90·5 ± 1·6**
500 μg/ear	55·5 ± 7·3**	25·1 ± 3·8	93·9 ± 5·3**

Data show mean ± s.e.m. of 6 animals. *$P < 0.05$, **$P < 0.01$ compared with control. The oedema value in the control group was 15·1 ± 0·7 mg. Arachidonic acid-treated ears in the control group showed PGE_2 and LTC_4 levels of 208·3 ± 15·3 ng/ear and 37·6 ± 2·9 ng/ear, respectively. Drugs were topically administered before the application of arachidonic acid.

Discussion

Activation of neutrophils during the inflammatory process results in radical species generation and degranulation, effector functions which have been implicated in a variety of disease states. In fact, activated neutrophils are present in synovial fluids of patients with rheumatoid arthritis where they release reactive oxygen species besides lysosomal enzymes like elastase and myeloperoxidase which can have a pronounced role in the pathogenesis of inflammation and joint destruction (Weiss 1989; Smith 1994). Accordingly, inhibition of neutrophil functions can participate in the mechanism of action of a number of drugs, including some non-steroidal anti-inflammatory agents (Abramson & Weissmann 1989; Kankaanranta et al 1994).

Bakuchiol did not inhibit the respiratory burst of human neutrophils or activated cells present in the inflammatory exudates of the mouse air pouch. In contrast, this natural product inhibited degranulation in human neutrophils in-vitro and this effect was also confirmed in-vivo in zymosan-treated mice undergoing inflammation. In addition, this natural product inhibited potently myeloperoxidase activity, thus preventing the generation of oxidants such as hypochlorous acid. As a result, bakuchiol could decrease tissue damage caused by hydrolytic enzymes and besides by some oxidant species, in human leukocytes.

On the other hand, this compound also affected eicosanoid production by the cyclo-oxygenase and 5-lipoxygenase pathways in-vitro and in-vivo, with some differences according to the experimental model used. Cyclo-oxygenase 1 is constitutively expressed in mammalian cells, while cyclo-oxygenase 2 is induced by a variety of pro-inflammatory factors (Masferrer et al 1990, 1994). In our experiments, bakuchiol was a weak inhibitor of cyclo-oxygenases 1 and 2 in-vitro using human enzyme preparations. Nevertheless, in animal models of inflammation we observed that bakuchiol induced a significant decrease in the synthesis of PGE_2, probably derived from cyclo-oxygenase 2 activity. On the other hand, bakuchiol was about equipotent in inhibiting LTB_4 release in human neutrophils and LTB_4 production by high-speed supernatants from human neutrophils. Thus, the reduction of LTB_4 synthesis in human neutrophils could be a consequence of 5-lipoxygenase inhibition by bakuchiol.

Zymosan causes cell activation through a surface receptor coupled to a G protein leading to increased PLA_2 activity and arachidonic acid release from membrane phospholipids leading to an inflammatory response (Dawson et al 1991; Balsinde et al 1994b). The effects of reference cyclo-oxygenase and 5-lipoxygenase inhibitors in the mouse air pouch injected with zymosan, suggest that the observed reduction in leukocyte influx is more related to inhibition of 5-lipoxygenase than to inhibition of cyclo-oxygenase. Our results also indicate some species differences on eicosanoid generation since bakuchiol inhibited PGE_2 with preference to LTB_4 levels in mice.

Another in-vivo model in mice, the TPA-induced ear oedema model, can be dependent on generation of eicosanoid mediators, such as PGE_2 and LTB_4 (Carlson et al 1985; Rao et al 1993), although is not very specific and other mediators including histamine (Carlson et al 1985) or PAF (Merlos et al 1991) can be involved. In this inflammatory response, cyclo-oxygenase inhibitors usually reduce oedema with a lower

effect on cell influx, while 5-lipoxygenase or dual inhibitors are more effective on arachidonic acid-induced oedema (Rao et al 1993). After topical application, bakuchiol achieved significant anti-inflammatory effects in the responses induced either by TPA or arachidonic acid, with reduction of leukocyte influx into the ear (TPA-induced inflammation), whereas the results in arachidonic acid-induced inflammation confirmed a preferential inhibition by bakuchiol of the cyclo-oxygenase pathway product PGE_2 in mice.

Recently, the importance of intracellular PLA_2 enzymes in the release of arachidonic acid from phospholipids has been stressed (Reynolds et al 1993; Balsinde et al 1994a; Bartoli et al 1994; Mukherjee et al 1994). Apart from the fact that bakuchiol is a weak inhibitor of human recombinant synovial PLA_2, it is interesting to note the inhibitory effect exerted in-vivo on intracellular PLA_2 activity in the mouse air pouch. It remains to be determined if a modification of this enzyme activity may contribute to the anti-inflammatory activity of bakuchiol. Interestingly, the actions of this natural product present in plants used in folk medicine in Chile, go beyond those of an inhibitor of eicosanoid synthesis since this compound is able to control leukocytic functions such as migration and degranulation in the inflammatory site.

Acknowledgements

This work was supported by grant PB93-0357 from CICYT, Spanish Ministerio de Educación y Ciencia and Proyecto X-1, Subprograma X, CYTED. The authors thank Dr R. M. McMillan and Dr S. J. Foster, Zeneca Pharmaceuticals, Macclesfield, Cheshire, UK for the gifts of ZM230,487 and antibody against LTB_4.

References

Abramson, S. B., Weissmann, G. (1989) The mechanisms of action of nonsteroidal antiinflammatory drugs. Arthritis Rheum. 32: 1–9

Backhouse, N. , Delporte, C. , Negrete, R. , Salinas, P. , Pinto, A. , Aravena, S., Cassels, B. K. (1996) Anti-inflammatory and antipyretic activities of *Cuscuta chilensis*, *Cestrum parqui* and *Psoralea glandulosa*. Int. J. Pharmacognosy in press

Balsinde, J. , Barbour, S. E., Bianco, I. D., Dennis, E. A. (1994a) Arachidonic acid mobilization in P388D1 macrophages is controlled by two distinct Ca^{2+}-dependent phospholipase A_2 enzymes. Proc. Natl. Acad. Sci. USA 91: 11060–11064

Balsinde, J. , Fernández, B., Solis-Herruzo, J. A. (1994b) Ethanol inhibits zymosan-stimulated eicosanoid production in mouse peritoneal macrophages. Biochim. Biophys. Acta 1210: 195–201

Barrett, A. J. (1981) Leukocyte elastase. Methods Enzymol. 80: 581–588

Bartoli, F. , Lin, H.-K., Ghomashchi, F. , Gelb, M. H., Jain, M. K., Apitz-Castro, R. (1994) Tight binding inhibitors of 85 kDa phospholipase A_2 but not 14 kDa phospholipase A_2 inhibit release of free arachidonate in thrombin-stimulated human platelets. J. Biol. Chem. 269: 15625–15630

Bergmeyer, H. U., Bernt, E . (1974) Lactate dehydrogenase: UV assay with pyruvate and NADH. In: Bergmeyer, H. U. (ed.) Methods of Enzymatic Analysis. 2nd edn. Academic Press, New York, pp 574–577

Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248–254

Carlson, R. P., O'Neill-Davis, L. , Chang, J., Lewis, A. J. (1985) Modulation of mouse ear edema by cyclooxygenase and lipoxygenase inhibitors and other pharmacologic agents. Agents Actions 17: 197–204

Clark, J. D., Milona, N., Knopf, J. L. (1990) Purification of a 110 kilodalton cytosolic phospholipase A_2 from the human monocytic cell line U937. Proc. Natl. Acad. Sci. USA 87: 7708–7712

Dawson, J. , Sedgwick, A. D., Edwards, J. C., Lees, P. (1991) A comparative study of the cellular, exudative and histological responses to carrageenan, dextran and zymosan in the mouse. Int. J. Tissue React. 13: 171–185

De Young, L. M., Kheifets, J. B., Ballaron, S. J., Young, J. M. (1989) Edema and cell infiltration in the phorbol ester-treated mouse ear are temporally separate and can be differentially modulated by pharmacologic agents. Agents Actions 26: 335–341

Edwards, J. C. W., Sedgwick, A. D., Willoughby, D. A. (1981) The formation of a structure with the features of synovial lining by subcutaneous injection of air: an in vivo tissue culture system. J. Pathol. 134: 147–156

Erazo, S. , García, R., Delle Monache, F. (1990) Bakuchiol and other compounds from *Psoralea glandulosa*. Rev. Latinoamer. Quím. 21/2: 62

Franson, R. , Patriarca, P., Elsbach, P. (1974) Phospholipid metabolism by phagocytic cells. Phospholipases A_2 associated with rabbit polymorphonuclear leukocyte granules. J. Lipid Res. 15: 380–388

Kankaanranta, H., Moilanen, E., Vapaatalo, H. (1994) Effects of nonsteroidal anti-inflammatory drugs on polymorphonuclear leukocyte functions in vitro: focus on fenamates. Naunyn Schmiedebergs Arch. Pharmacol. 350: 685–691

Masferrer, J. L., Zwefel, B. S., Needleman, P. (1990) Selective regulation of cellular cyclooxygenase by dexamethasone and endotoxin in mice. J. Clin. Invest. 86: 1375–1379

Masferrer, J. L., Zweifel, B. S., Manning, P. T., Hauser, S. D., Leahy, K. M., Smith, W. G., Isakson, P. C., Seibert, K. (1994) Selective inhibition of inducible cyclooxygenase 2 in vivo is antiinflammatory and nonulcerogenic. Proc. Natl. Acad. Sci.USA 91: 3228–3232

Merlos, M. , Gómez, L. A., Giral, M. , Vericat, M. L., García-Rafanell, J., Forn, J. (1991) Effects of PAF-antagonists in mouse ear oedema induced by several inflammatory agents. Br. J. Pharmacol. 104: 990–994

Moroney, M. A., Alcaraz, M. J., Forder, R. A., Carey, F., Hoult, J. R. S. (1988) Selectivity of neutrophil 5-lipoxygenase and cyclo-oxygenase inhibition by an anti-inflammatory flavonoid glycoside and related aglycone flavonoids. J. Pharm. Pharmacol. 40: 787–792

Mukherjee, A. B., Miele, L., Pattabiraman, N. (1994) Phospholipase A_2 enzymes: regulation and physiological role. Biochem. Pharmacol. 48: 1–10

Payá, M. , Ferrándiz, M. L., Miralles, F., Montesinos, C., Ubeda, A., Alcaraz, M. J. (1993) Effects of coumarin derivatives on superoxide anion generation. Arzneim. Forsch. 43: 655–658

Rao, T. S., Currie, J. L., Shaffer, A. F., Isakson, P. C. (1993) Comparative evaluation of arachidonic acid (AA)- and tetradecanoylphorbol acetate (TPA)-induced dermal inflammation. Inflammation 17: 723–741

Reynolds, L. J., Hughes, L. L., Louis, A. I., Kramer, R. M., Dennis, E. A. (1993) Metal ion and salt effects on the phospholipase A_2, lysophospholipase, and transacylase activities of human cytosolic phospholipase A_2. Biochim. Biophys. Acta 1167: 272–280

Smith, J. A. (1994) Neutrophils, host defense, and inflammation: a double-edged sword. J. Leukoc. Biol. 56: 672–686

Suzuki, K., Ota, H. , Sasagawa, S., Sakatani, T., Fujikura, T. (1983) Assay method for myeloperoxidase in human polymorphonuclear leukocytes. Anal. Biochem. 132: 345–352

Tateson, J. E., Randall, R. W., Reynolds, C. H., Jackson, W. P., Bhattacherjee, P. , Salmon, J. A., Garland, L. G. (1988) Selective inhibition of arachidonate 5-lipoxygenase by novel acetohydroxamic acids: biochemical assessment in vitro and ex vivo. Br. J. Pharmacol. 94: 528–539

Weiss, S. J. (1989) Tissue destruction by neutrophils. N. Engl. J. Med. 320: 365–376

Zhang, Y. Y., Deems, R. A., Dennis, E. A. (1991) Lysophospholipases I and II from P388D1 macrophage-like cell line. Methods Enzymol. 197: 456–468

Zuñiga, A. (1994) Estudio de las actividades antiinflamatoria, antipirética y toxicidad aguda de bakuchiol, metabolito secundario de *Psoralea glandulosa* L. Thesis. Universidad de Chile