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Modulation of acute and chronic inflammatory processes by cacospongionolide B, a novel inhibitor of human synovial phospholipase A_2

[1]Pablo García Pastor, [2]Salvatore De Rosa, [2]Alfonso De Giulio, [1]Miguel Payá & *,[1]M. José Alcaraz

[1]Departamento de Farmacología, Universidad de Valencia, Facultad de Farmacia, Avda. Vicent Andrés Estellés s/n, 46100 Burjassot, Valencia, Spain and [2]Istituto per la Chimica di Molecole di Interesse Biologico CNR, via Toiano 6, 80072 Arco Felice, Napoli, Italy

1 Cacospongionolide B is a novel marine metabolite isolated from the sponge *Fasciospongia cavernosa*. In *in vitro* studies, this compound inhibited phospholipase A_2 (PLA_2), showing selectivity for secretory PLA_2 ($sPLA_2$) versus cytosolic PLA_2 ($cPLA_2$), and its potency on the human synovial enzyme (group II) was similar to that of manoalide.

2 This activity was confirmed *in vivo* in the 8 h zymosan-injected rat air pouch, on the secretory enzyme accumulating in the pouch exudate. Cacospongionolide B, that is bioavailable when is given orally, reduced the elevated levels of $sPLA_2$ present in paw homogenates of rats with adjuvant arthritis.

3 This marine metabolite showed topical anti-inflammatory activity on the mouse ear oedema induced by 12-*O*-tetradecanoylphorbol acetate (TPA) and decreased carrageenin paw oedema in mice after oral administration of 5, 10 or 20 mg kg^{-1}.

4 In the mouse air pouch injected with zymosan, cacospongionolide B administered into the pouch, induced a dose-dependent reduction in the levels of eicosanoids and tumour necrosis factor α (TNFα) in the exudates 4 h after the stimulus. It also had a weak effect on cell migration.

5 The inflammatory response of adjuvant arthritis was reduced by cacospongionolide B, which did not significantly affect eicosanoid levels in serum, paw or stomach homogenates and did not induce toxic effects.

6 Cacospongionolide B is a new inhibitor of $sPLA_2$ *in vitro* and *in vivo*, with anti-inflammatory properties in acute and chronic inflammation. This marine metabolite was active after oral administration and able to modify TNFα levels, and may offer an interesting approach in the search for new anti-inflammatory agents.

Keywords: Inflammation; phospholipase A_2; rat and mouse air pouch; adjuvant arthritis; manoalide; cacospongionolide B

Abbreviations: COX, cyclo-oxygenase; $cPLA_2$, cytosolic PLA_2; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; iNOS, inducible nitric oxide synthase; IP_3, inositol triphosphate; IL-1β, interleukin-1β; LDH, lactate dehydrogenase; LTB_4, leukotriene B_4; LPS, lipopolysaccharide; 5-LO, 5-lipoxygenase; MAC-1, CD11b/CD18; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NSAID, non-steroidal anti-inflammatory drug; PTK, palmityl trifluoromethyl ketone; PBS, phosphate buffered saline; PLA_2, phospholipase A_2; PLC, phospholipase C, PG, prostaglandin; PGD_2, prostaglandin D_2; PGE_2, prostaglandin E_2; $sPLA_2$, secretory phospholipase A_2; TPA, 12-*O*-tetradecanoylphorbol acetate; TXA_2, thromboxane A_2; TXB_2, thromboxane B_2; TNFα, tumour necrosis factor α

Introduction

The activation of different phospholipases is a critical step in the biosynthesis of lipid mediators. Phospholipase A_2 (PLA_2) is a class of enzymes that hydrolyze the acyl group from the *sn*-2 position of glycerophospholipids, yielding free fatty acids and lysophospholipids. These products or their metabolites are bioactive lipids modulating different cellular processes. Mammalian cells contain diverse PLA_2 which may play a distinct role in cell activation and signal transduction. Moreover, in pathologic states, increased PLA_2 activity causes alteration of membrane structure and function as well as an excessive production of lipid mediators and toxic species that contributes to tissue injury. Secretory PLA_2 ($sPLA_2$, groups I, II, III and V), cytosolic PLA_2 ($cPLA_2$, group IV) and calcium-independent PLA_2 have been studied (for review see Serhan *et al*. (1996); Dennis (1997)). Calcium-independent PLA_2 is present in the myocardium and other tissues. This enzyme may regulate the incorporation of arachidonic acid into membrane phospholipids in $P388D_1$ macrophages (Balsinde *et al*., 1995) and could participate in arachidonic acid release and cell spreading in murine peritoneal macrophages (Teslenko *et al*., 1997).

It has been reported that $cPLA_2$ play an important role in arachidonic acid release in a number of cell systems, e.g. human platelets stimulated with thrombin (Bartoli *et al*., 1994) or calcium ionophore (Riendeau *et al*., 1994), permeabilized human neutrophils (Bauldry & Wooten, 1996) or mouse peritoneal macrophages challenged with zymosan or 12-*O*-tetradecanoylphorbol acetate (TPA) (Qiu & Leslie, 1994). Inflammatory cytokines have been shown to induce $cPLA_2$, resulting in high levels of eicosanoids in airway epithelial cells (Wu *et al*., 1997), rheumatoid synovial fibroblasts (Hulkower *et al*., 1994) or mouse osteoblasts (Chen *et al*., 1997).

Group II $sPLA_2$ can act as a signalling agent that mediates cell growth induced by interleukin-1β (IL-1β) (Wada *et al*., 1997). In addition, it has a role in cell activation and contributes to the inflammatory response. $sPLA_2$ activation

*Author for correspondence.

may participate in signal transduction events such as CD11b/CD18 (MAC-1) expression on the surface of activated human neutrophils, and adhesion or degranulation (Takasaki *et al*., 1996; Jacobson & Schrier, 1993). This enzyme activity secreted at inflammatory sites becomes associated with cell surfaces and hydrolyzes phospholipids, thus releasing arachidonic acid, which enters the cell and participate in the increased generation of inflammatory lipid mediators (Pfeilschifter *et al*., 1993; Miyake *et al*., 1994). In fact, administration of different types of $sPLA_2$ can induce or amplify inflammatory responses in animals (Vishwanath *et al*., 1988; Tanaka *et al*., 1995; Cirino *et al*., 1994). Interestingly, inflammatory cytokines increase group II PLA_2 synthesis and secretion by rheumatoid synovial fibroblasts and other cell types (Pfeilschifter *et al*., 1993; Bomalaski & Clark, 1993). Thus, IL-1β induces an increase in group II $sPLA_2$ gene expression, but does not increase $cPLA_2$ gene expression or activity, and it provokes a parallel increase in prostaglandin E_2 (PGE_2) production by rabbit articular chondrocytes (Jacques *et al*., 1997). Group II $sPLA_2$ has been reported to release arachidonic acid in some systems and may provide the substrate for both cyclooxygenase (COX) and 5-lipoxygenase (5-LO) product formation in mouse bone marrow-derived mast cells (Fonteh *et al*., 1994). In contrast, PLA_2 secreted by guinea-pig peritoneal macrophages does not participate in the synthesis of PGE_2 accumulating in the media (Marshall *et al*., 1994).

On the other hand, exocytosis of $sPLA_2$ could modulate the activity of $cPLA_2$ by initiating the formation of leukotriene B_4 (LTB_4), which after release stimulates its own receptor, thus leading to activation of $cPLA_2$ in neutrophils (Wijkander *et al*., 1995). Exogenously added group I PLA_2 is also believed to be involved in arachidonic acid release (Hara *et al*., 1991), in some cases accompanied by induction of group II PLA_2, and recently a group V $sPLA_2$ has been reported to participate in immediate prostanoid generation in the mouse macrophage cell line $P388D_1$ (Balboa *et al*., 1996).

Arachidonic acid mobilization can be dependent on both types of PLA_2 in some systems, as in the case of delayed PGD_2 generation by COX-2 in rat peritoneal macrophages stimulated by lipopolysaccharide (LPS) (Naraba *et al*., 1998), as well as in receptor-stimulated $P388D_1$ macrophages (Balsinde & Dennis, 1996), and human umbilical vein endothelial cells (Murakami *et al*., 1993). In human monocytes stimulated by ionophore or zymosan, $cPLA_2$ would participate preferentially in the release of arachidonic acid for prostaglandin (PG) synthesis, whereas $sPLA_2$ probably releases the substrate for LT synthesis (Marshall *et al*., 1997).

Marine organisms are a rich source of molecules exhibiting PLA_2 inhibitory properties *in vitro*, mainly on secretory enzymes (for review, see Potts *et al*., 1992). Some of these compounds have been found to reduce experimental inflammatory responses, preferentially after topical application. We have examined the PLA_2 inhibitory activity of cacospongionolide B (Figure 1), a new marine metabolite isolated from the Mediterranean sponge *Fasciospongia cavernosa*. The results of our studies demonstrate that cacospongionolide B is a potent inhibitor of $sPLA_2$. We have also assessed its effects on models of acute and chronic inflammation.

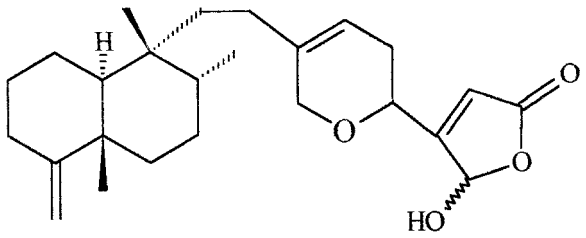

Figure 1 Chemical structure of cacospongionolide B.

Methods

$sPLA_2$ assay

$sPLA_2$ was assayed by using 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, BSA, pH 8.0), resuspended in saline and autoclaved for 30–45 min. At least 95% of the radioactivity was incorporated into phospholipids. *Naja naja* venom, porcine pancreatic, bee venom and human recombinant synovial enzymes were diluted in 10 μl of 100 mM Tris-HCl, 1 mM $CaCl_2$ buffer, pH 7.5. Supernatants (10 μl) of exudates from zymosan-injected rat air pouch (Payá *et al*., 1996) were also used as a source of $sPLA_2$. Enzymes were preincubated at 37°C for 5 min with 2.5 μl of test compound solution or its vehicle in a final volume of 250 μl. Incubation proceeded for 15 min in the presence of 10 μl of autoclaved oleate-labelled membranes and was terminated by addition of 100 μl ice-cold solution of 0.25% BSA in saline to a final concentration of 0.07% w/v. After centrifugation at 2500 × *g* for 10 min at 4°C, the radioactivity in the supernatants was determined by liquid scintillation counting.

$cPLA_2$ assay

$cPLA_2$ was prepared from human monocytic U937 cells (Cell Collection, Department of Animal Cell Culture, C.S.I.C., Madrid, Spain) grown in the above medium which were disrupted by sonication in 10 mM HEPES buffer pH 7.4, containing 0.32 M sucrose, 100 μM EDTA, 1 mM dithiothreitol, 2 mM phenylmethylsulphonylfluoride and 100 μM leupeptin. The homogenated cells were centrifuged at 2000 × *g* for 10 min at 4°C and the resulting supernatant was further centrifuged at 100,000 × *g* for 100 min at 4°C to obtain the cytosolic fraction. $cPLA_2$ activity was measured as the release of radiolabelled arachidonic acid according to the method of Clark *et al*. (1990). 1-Palmitoyl-2-[^{14}C]-arachidonyl-*sn*-glycero-3-phosphocholine (57.0 mCi mmol^{-1}, 2×10^6 c.p.m.) was dried under nitrogen, then suspended in 1 ml of 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% v/v 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 24 μg protein of cytosolic fraction from human monocytes) to a final volume of 100 μl of the assay mixture which contained 1 mM $CaCl_2$, 2 mM 2-mercaptoethanol, 150 mM NaCl, 40% glycerol, 1 mg ml^{-1} BSA and 50 mM HEPES pH 9.0. The substrate consisted of 5 μl of micelles (10^4 c.p.m.) containing dioleoyl glycerol at a molar ratio 2:1 (Kramer *et al*., 1987). Test compounds were dissolved in methanol and added to the reaction mixture just before the addition of the enzyme solution. The final concentration of methanol in the reaction mixture was less than 1%, which showed no effect on the

enzyme activity. The reaction was stopped after a 60 min incubation period at 37°C by mixing with 0.5 ml of isopropyl alcohol/heptane/0.5 M H_2SO_4 (10 : 5 : 1). Heptane (0.7 ml) and water (0.2 ml) were then added, and the solution was vigorously mixed for 15 s. The heptane phase was mixed with 100 mg silica gel 60 (Merck, 70-230 mesh) and centrifuged, and the radioactivity in each supernatant was measured (Zhang *et al*., 1991).

Preparation of human leukocytes

The citrated blood of healthy volunteers was centrifuged at $200 \times g$ for 15 min at room temperature. The platelet-rich plasma was removed, and the leukocytes contained in the residual blood were isolated by sedimentation with 2% (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 (PBS), and Ficoll-hypaque was layered under the cell mixture. The cell gradient mixture was centrifuged at $400 \times g$ for 40 min at 20°C. Neutrophils were separated and 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. The monocyte and lymphocyte layer was removed and pelleted by centrifugation. The cell pellet was resuspended in RPMI-1640 media, pH 7.4, with 10% foetal bovine serum, 2 mM L-glutamine, 50 u ml^{-1} penicillin and 50 $\mu g\ ml^{-1}$ streptomycin and was incubated at a cell density of $10^7\ ml^{-1}$ in 60/15 mm tissue culture dishes. The cells were allowed to adhere for 2 h at 37°C in a 5% CO_2 atmosphere incubator. The nonadherent cells were removed by vacuum suction of media followed by two washes with 1 ml of RPMI-1640. The adherent cells resulted in a greater than 90% pure monocyte population as assessed by differential staining.

Cytotoxicity assays

The cytoplasmic marker enzyme lactate dehydrogenase (LDH) (Bergmeyer & Bernt, 1974) and the mitochondrial dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan (Gross & Levi, 1992) were used to assess the possible cytotoxic effect of cacospongionolide B on human neutrophils.

Phospholipase C (PLC)

Human neutrophils (1×10^7 cells ml^{-1}) were suspended in HBSS with Ca^{2+} and Mg^{2+} and preincubated with test drugs for 5 min at 37°C, before the reaction was initiated by adding 1 μM *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) for 5 min at 37°C. Inositol triphosphate (IP3) was quantified by the method of Palmer (1989).

Synthesis of LTB_4 by high speed supernatants from human neutrophils

High speed ($100{,}000 \times g$) supernatants from sonicated human neutrophils were obtained as previously described (Tateson *et al*., 1988). Aliquots (50 μg of protein/tube) in PBS containing 2 mM $CaCl_2$ were incubated with 5 μM arachidonic acid at 37°C for 5 min, in the presence of test compounds or vehicle. The samples were then heated at 90°C for 5 min and centrifuged at $10{,}000 \times g$ at 4°C for 30 min. The LTB_4 levels in supernatants were measured by radioimmunoassay (Moroney *et al*., 1988).

Cyclo-oxygenase-1

Human platelets were sonicated at 4°C in an ultrasonicator at maximum potency. Microsomes were prepared by centrifugation at $2000 \times g$ for 5 min at 4°C followed by centrifugation of the supernatant at $100{,}000 \times g$ for 100 min at 4°C. Microsomes (20 μg of 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 PGE_2 levels were determined by radioimmunoassay (Moroney *et al*., 1988).

Cyclo-oxygenase-2

Human monocytes cells were resuspended in RPMI-1640 culture medium containing aspirin (300 μM) and incubated at 37°C for 2 h. The cells were washed twice, resuspended in RPMI-1640 with 10% foetal bovine serum and incubated with *E. coli* lipopolysaccharide (10 $\mu g\ ml^{-1}$) at 37°C for 24 h (Grossman *et al*., 1995). After centrifugation the cells were sonicated at 4°C in an ultrasonicator at maximum potency, and microsomes were prepared as above. Microsomes (40 μg of 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).

Inducible nitric oxide synthase (iNOS) assay

NOS activity was induced by i.p. injection of LPS (2 mg kg^{-1}) to rats. After 24 h the animals were killed and livers were excised and homogenated in 10 mM HEPES, pH 7.4, containing sacarose (0.32 M), EDTA (100 μM), dithiothreitol (1 mM), phenylmethylsulfonyl fluoride (1 mg ml^{-1}) and leupeptin (10 $\mu g\ ml^{-1}$) (Knowles *et al*., 1990). The homogenate was centrifuged at $1200 \times g$ for 10 min at 4°C, followed by centrifugation of the supernatant at $100{,}000 \times g$ for 100 min at 4°C. NOS activity was determined in supernatants by monitoring the conversion of L-[^{3}H]-arginine to L-[^{3}H]-citrulline, (Mitchell *et al*., 1991). Samples (40 μg protein) were incubated at room temperature for 60 min with 100 μl of the above buffer in the presence of NADPH (1 mM) and a mixture of unlabelled and L-[^{3}H]-arginine (10 μM, 1 $\mu Ci\ ml^{-1}$). Incubations were terminated by the addition of 20 mM HEPES (1 ml, pH 5.5) containing 1 mM EGTA and 1 mM EDTA. L-[^{3}H]-citrulline was separated from arginine by adding 1.5 ml of a 1 : 1 suspension of Dowex (50 W) in water. Radioactivity was measured in supernatants by liquid scintillation counting.

Rat air pouch

Male Wistar rats (120–150 g) were used. Air pouches were formed as previously described (Edwards *et al*., 1981). The animals were anaesthetized with ethyl ether and given a 20 ml injection of sterile air in the subcutaneous tissue of the back, and 3 days later 10 ml of sterile air was injected into the same cavity. After 3 days 1 ml of sterile saline (saline group), 1 ml of 1% (w/v) zymosan in saline + 10 μl ethanol (zymosan control group) or 1 ml of 1% w/v zymosan in saline + test drug (dissolved in 10 μl of ethanol: treated groups), was administered into the air pouch. After 8 h, rats were sacrificed and the exudate was collected in 1 ml of saline. Leukocytes in exudate fluids were counted by Coulter counter. After centrifugation of the exudate at $1200 \times g$ at 4°C for 10 min, the supernatants were used to measure PLA_2 activity as above. Protein was

quantified by the Bradford technique (Bradford, 1976) using BSA as standard.

Mouse ear oedema

The protocols were approved by the institutional Animal Care and Use Commitee. All studies were performed in accordance with European Union regulations for the handling and use of laboratory animals. TPA (5 μg) dissolved in 20 μl of acetone was applied in 10 μl volumes to both inner and outer surfaces of the right ear of Swiss mice (20–25 g). Test compounds were applied topically in acetone before TPA administration. The left ear (control) received only acetone. The animals were killed by cervical dislocation after 4 h, 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 $10{,}000 \times g$ for 15 min at 4°C, the myeloperoxidase activity was measured in aliquots of supernatants. 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 (De Young *et al*., 1989).

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) were administered p.o. (0.5 ml) 1 h before the injection of carrageenin (0.05 ml; 3% w/v in saline) into the subplantar area of the right hind paws of groups of six 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 carrageenin-injected and contralateral paws.

Mouse air pouch

Female Swiss mice (25–30 g) were anaesthetized with ethyl ether, and 10 ml of sterile air was injected into the subcutaneous tissue of the back. Three days later, pouches were reinflated with 5 ml of sterile air. In 6 days-old air pouches, mice were administered saline (saline group), zymosan + vehicle (control group) or zymosan + test drug, as indicated in the rat air pouch method. Four hours after administration, the animals were killed by cervical dislocation, and the exudate in the pouch was collected with 1 ml of saline (Edwards *et al*., 1981). Leukocytes present in exudates were measured using a Coulter counter. After centrifugation of exudates at $1200 \times g$ at 4°C for 10 min, the supernatants were used to measure LTB_4 and PGE_2 levels as indicated above or TNFα by ELISA.

Adyuvant arthritis

Adyuvant arthritis was elicited in female Lewis rats (126–150 g) by injecting 0.1 ml of *Mycobacterium butyricum* (10 mg ml^{-1}) in mineral oil into the base of the tail (Taurog *et al*., 1988). Paw volumes were measured at the beginning of the experiment by using a plethysmometer. Animals were housed in propylene cages with food and water *ad libitum*. The light cycle was automatically controlled (on 0700 h; off 1900 h) and the room temperature thermostatically regulated to 21 ± 1°C. The magnitude of the inflammatory response was evaluated by measuring the volume of both paws at day 13. The oedema was calculated as the mean increase in paw volume. Animals with oedema paw volumes 0.60 ml larger than normal paws were then randomized into treatment groups. Cacospongionolide B (20 mg kg^{-1}), indomethacin (5 mg kg^{-1}) or vehicle (ethanol, tween 80, distilled water: 5/5/90, v/v/v) were administered p.o. (1.0 ml) once-daily on days 13–17. Serum was collected on the last day of the experiment (day 18) for the determination of PGE_2, thromboxane B_2 (TXB_2) and LTB_4. After death, paws from arthritic, treated groups and non-arthritic normal animals were amputated above the ankle and homogenized in 2.5 ml saline. After centrifugation at $10{,}000 \times g$ for 15 min at 4°C, supernatants were used for the determination of PGE_2 and $sPLA_2$. Prior to evaluating the $sPLA_2$ content, a purification by acidic treatment was performed (Bolognese *et al*., 1995). Stomachs were homogenized in 2 ml of methanol and aliquots of supernatants were used to determine the content of PGE_2 as above.

Materials

Cacospongionolide B was isolated from the sponge *Faciospongia cavernosa* following known procedures (De Rosa *et al*., 1995). Antibody against LTB_4 was kindly provided by Zeneca Pharmaceuticals, Macclesfield, Cheshire, U.K. Human synovial recombinant PLA_2 was a gift from Dr R.M. Kramer (Lilly Research Laboratories, Indianapolis, U.S.A). [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). [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, [5,6,8,9,11,12,14,15(n)^{3}H]-TXB_2, L-[^{3}H]-argnine, IP_3 measurement kit and the TNFα ELISA kit were from Amersham Iberica, (Madrid, Spain). Palmityl trifluoromethyl ketone (PTK) was purchased from Cayman Chem. (MI, U.S.A.). *M. butyricum* was obtained from Difco (MI, U.S.A). The rest of reagents were from Sigma Chem.(MO, U.S.A). *E. coli* strain CECT 101 was a gift from Prof Uruburu, Department of Microbiology, University of Valencia, Spain.

Statistical analysis

The results are presented as means $\pm$ s.e.mean; n represents the number of experiments. Inhibitory concentration 50% (IC_{50}) values were calculated from at least four significant concentrations ($n = 6$). The level of statistical significance was determined by analysis of variance (ANOVA) followed by Dunnett's t-test for multiple comparisons.

Results

Effect on PLA_2 and other enzyme activities in vitro

The effect of cacospongionolide B on $sPLA_2$ was determined using different assay systems *in vitro*. As shown in Table 1, this marine compound preferentially inhibited the synovial and pancreatic secretory enzymes, and its potency on the human synovial enzyme was comparable to that of the reference inhibitor, manoalide. Other $sPLA_2$, including the enzyme

present in the inflammatory exudates of zymosan-injected rat air pouch and bee venom PLA_2, were inhibited by cacospongionolide B to a lesser extent, whereas it exerted no effect on the *Naja naja* venom enzyme. We also determined the concentration-dependent inhibition of human recombinant synovial PLA_2 of cacospongionolide B and manoalide at 10 μM, without and with 100 μg ml^{-1} BSA in reaction buffer, resulting a partial reduction in their potency (cacospongionolide B, 86.7$\pm$2.5% and 60.1$\pm$1.9% of inhibition without and with BSA, respectively, $n=6$, $P<0.01$ and manoalide, 93.2$\pm$0.2% and 53.1$\pm$5.2% of inhibition without and with BSA, respectively, $n=6$, $P<0.01$). This type of non-specific action has been reported previously in manoalide (Jacobson *et al*., 1990). In contrast, cacospongionolide B had no inhibitory effects on $cPLA_2$, which was partially inhibited by manoalide at 10 μM (Table 2). We also tested the possible influence of cacospongionolide B on other enzymes involved in the release or metabolism of arachidonic acid, such as PLC, COX-1, COX-2 and 5-LO, as well as on iNOS. Only 5-LO was slightly inhibited by cacospongionolide B in a manner similar to manoalide. In contrast, this reference compound also inhibited PLC. Cacospongionolide B did not exert significant cytotoxic effects on human neutrophils at concentrations up to 50 μM, as assessed by the release of LDH and the MTT method (data not shown). Otherwise manoalide showed a slight cytotoxicity effect at 50 μM, as assessed by the MTT method (18.9$\pm$3.0% of cytotoxicity, $n=6$, $P<0.01$).

To further characterize the inhibitory activity of cacospongionolide B on $sPLA_2$ we also studied the type of inhibition, which was apparently irreversible as assessed by the kinetic analysis of enzyme activity as a function of the human synovial PLA_2 concentration in the absence or presence of cacospongionolide B (Figure 2) because there was no significant difference in the slopes of the straight lines (Segel, 1975). This type of inhibition has been reported for other marine compounds such us manoalide (Jacobson *et al*., 1990).

Effect on $sPLA_2$ in vivo

Having established the inhibitory properties of cacospongionolide B on $sPLA_2$ *in vitro*, we tested the effects of this marine compound on an animal model in which high levels of secretory type II PLA_2 is generated (Payá *et al*., 1996). As shown in Table 3, cacospongionolide B administered into the air pouch dose-dependently inhibited the PLA_2 activity present

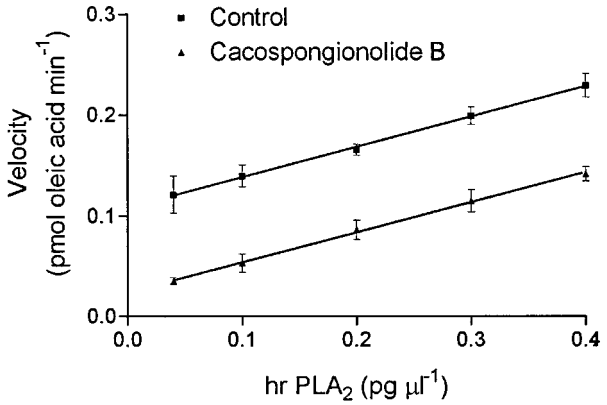

Figure 2 Activity of human synovial $sPLA_2$ as a function of enzyme concentration in the absence or presence of cacospongionolide B. Data are the means$\pm$s.e.mean of $n=6$. Different enzyme concentrations were preincubated with vehicle (control) or cacospongionolide B (1 μM) for 5 min at 37°C, and after addition of substrate, incubation proceeded for 15 min.

Table 1 Effect of cacospongionolide B and manoalide on different secretory PLA_2 activities

Drug (10 μM)	N. Naja *venom* *%I*	*Pancreas* *%I*	*Pancreas* IC_{50} (μM)	*Human synovial* *%I*	*Human synovial* IC_{50} (μM)	*RAP+zymosan* *%I*	*RAP+zymosan* IC_{50} (μM)	*Bee venom* *%I*	*Bee venom* IC_{50} (μM)
Cacospongionolide B	0.0$\pm$0.0	64.2$\pm$2.1**	4.0	86.7$\pm$2.5**	4.3	36.9$\pm$1.4**	ND	35.4$\pm$1.2**	ND
Manoalide	17.0$\pm$1.7*	32.3$\pm$2.7**	ND	93.2$\pm$0.2**	3.9	8.4$\pm$0.5**	ND	62.5$\pm$3.8**	7.5

RAP: Rat air pouch. Results are the means$\pm$s.e.mean of $n=6$. *$P<0.05$; **$P<0.01$ with respect to the enzyme control group. IC_{50} values were determined for the compounds that reach 50% inhibition at 10 μM. ND: not determined.

Table 2 Effect of cacospongionolide B and manolide on hr-$sPLA_2$, $cPLA_2$, PLC, 5-LO, COX-2, COX-1 and iNOS activities *in vitro*

Group	*hr-$sPLA_2$* (pmol OA mg^{-1} min^{-1})	*$cPLA_2$* (pmol AA mg^{-1} min^{-1})	*PLC* (nmol IP_3 10^{-6} cells)	*5-LO* (ng LTB_4 ml^{-1})	*COX-2* (ng PGE_2 ml^{-1})	*COX-1* (ng PGE_2 ml^{-1})	*iNOS* (pmol citrulline mg^{-1} min^{-1})
Control	6093.8$\pm$245.8	5.4$\pm$0.5	2.4$\pm$0.3	22.6$\pm$2.1	1.5$\pm$0.1	6.4$\pm$0.6	30.9$\pm$0.9
Cacospongionolide B	782.2$\pm$174.3**	5.9$\pm$0.6	2.2$\pm$0.6	13.6$\pm$0.7**	1.4$\pm$0.4	5.8$\pm$0.6	28.9$\pm$0.6
Manoalide	358.9$\pm$200.0**	3.3$\pm$0.5**	1.2$\pm$0.1*	14.7$\pm$1.02**	1.2$\pm$0.2	6.5$\pm$0.3	27.3$\pm$1.5
PTK	ND	1.4$\pm$0.5**	ND	ND	ND	ND	ND
U73122	ND	ND	0.9$\pm$0.2*	ND	ND	ND	ND
ZM 230,487	ND	ND	ND	5.4$\pm$1.3**	ND	ND	ND
NS398	ND	ND	ND	ND	0.5$\pm$0.1**	4.0$\pm$0.5**	ND
Indomethacin	ND	ND	ND	ND	1.0$\pm$0.1*	0.8$\pm$0.1**	ND
L-NAME	ND	ND	ND	ND	ND	ND	10.4$\pm$1.6**

Results are the means$\pm$s.e.mean of $n=6-12$. *$P<0.05$; **$P<0.01$ with respect to control group. Cacospongionolide B, manoalide and reference inhibitors were tested at 10 μM. ND: not determined. Drugs were incubated with hr-$sPLA_2$, cystolic fraction of U937 cells ($cPLA_2$), human neutrophils stimulated with fMLP (PLC), high speed supernatants from human neutrophil homogenates (5-LO), microsomal fraction from human monocytes treated with LPS (COX-2), microsomal fraction from human platelets (COX-1) or cytosolic fraction of liver homogenates from LPS-injected rats (iNOS).

in the 8 h zymosan-injected rat air pouch, whereas the accumulation of leukocytes in the pouch was reduced by CB treatment only at the highest dose tested (1 μmol pouch^{-1}).

Effect on mouse ear oedema

Evaluation of the topical anti-inflammatory activity of cacospongionolide B was performed in the TPA-induced mouse ear oedema. Control animals showed an oedema of 17.8 ± 1.0 mg and myeloperoxidase levels of 0.852 ± 0.104 O.D.$_{620}$ units 4 h after TPA administration (Table 4). Topical treatment with cacospongionolide B resulted in a dose-dependent inhibition of TPA-induced ear oedema, in addition to a decrease in myeloperoxidase levels measured in ear homogenates. Indomethacin was more effective on myeloperoxidase than on oedema, which was also attenuated, but to a lesser extent than in animals treated with cacospongionolide B.

Effect on mouse paw oedema

Oral pretreatment (1 h before carrageenin) with 5, 10 or 20 mg kg^{-1} of cacospongionolide B significantly reduced oedema (Figure 3). This inhibitory effect was observed at the three time points considered, 1, 3 and 5 h after carrageenin, for the doses of 10 and 20 mg kg^{-1}, whereas the dose of 5 mg kg^{-1} caused significant inhibition at 1 and 3 h. Interestingly, cacospongionolide B was more effective than indomethacin in this model, mainly at 3 h carrageenin administration.

Effect on the mouse air pouch

An important increase in leukocyte migration was observed in zymosan-injected animals in comparison with the saline-injected group 4 h after the induction of inflammation. Cacospongionolide B blocked cell accumulation in exudates at the dose of 1 μmol pouch^{-1}, but it was ineffective at lower doses (Figure 4a). This inflammatory response also showed high levels of PGE_2, LTB_4 and TNFα in the air pouch exudates of control animals injected with zymosan (Figure 4b, c and d). Treatment with cacospongionolide B resulted in a significant decrease in PGE_2 levels at a dose as low as 1 nmol pouch^{-1}, whereas LTB_4 levels were significantly reduced at higher doses (0.5 and 1 μmol pouch^{-1}). In this model, TNFα levels were very sensitive to cacospongionolide B; 1 nmol pouch^{-1} of this compound achieved a significant reduction in TNFα and at 1 μmol pouch^{-1} the levels of this cytokine were abolished. As expected, the 5-LO inhibitor ZM 230,487 (0.1 μmol pouch^{-1}) strongly reduced LTB_4 levels (87.3 ± 5.8% of inhibition, $n=6$, $P<0.01$) and cell migration (36.7 ± 3.1% of inhibition, $n=6$, $P<0.01$), whereas the COX inhibitor indomethacin (0.1 μmol pouch^{-1}) decreased PGE_2 levels (89.2 ± 4.8% of inhibition, $n=6$, $P<0.01$). Dexamethasone (2 mg kg^{-1} i.p.) inhibited cell migration (37.8 ± 3.3% of inhibition, $n=6$, $P<0.01$) as well as PGE_2 and LTB_4 (74.8 ± 3.6% and 82.8 ± 4.0 of inhibition, respectively, $n=6$, $P<0.01$) and TNFα (75.7 ± 7.1% of inhibition, $n=6$, $P<0.01$) levels in exudates. The effects of cacospongionolide B this experimental model were also confirmed after oral administration at a single dose of 20 mg kg^{-1}, which reduced PGE_2, LTB_4 and TNFα levels (58.6 ± 4.8, 64.7 ± 8.2 and 59.5 ± 7.1% of inhibition, respectively; $n=6$, $P<0.01$) to a similar extent, and had a lower effect on cell accumulation in exudates (38.5 ± 4.1% of inhibition, $n=6$, $P<0.01$).

Table 3 Effect of cacospongionolide B on cellular accumulation and secretory PLA_2 in the 8 h zymosan-injected rat air pouch

Group	*Total cells* ($\times 10^6$ ml^{-1})	*sPLA$_2$* (pmol OA ml^{-1})
Zymosan	144.8 ± 8.8	312.2 ± 15.8
Saline	25.3 ± 6.3**	54.4 ± 11.0**
Zymosan + cacospongionolide B		
0.01 μmol pouch^{-1}	140.2 ± 5.0	244.3 ± 10.2*
0.1 μmol pouch^{-1}	135.9 ± 10.2	223.7 ± 9.8**
1 μmol pouch^{-1}	62.3 ± 8.6**	180.3 ± 24.1**

Results are the means $\pm$ s.e.mean of $n=6$. *$P<0.05$; **$P<0.01$ with respect to the zymosan control group. OA: Oleic acid.

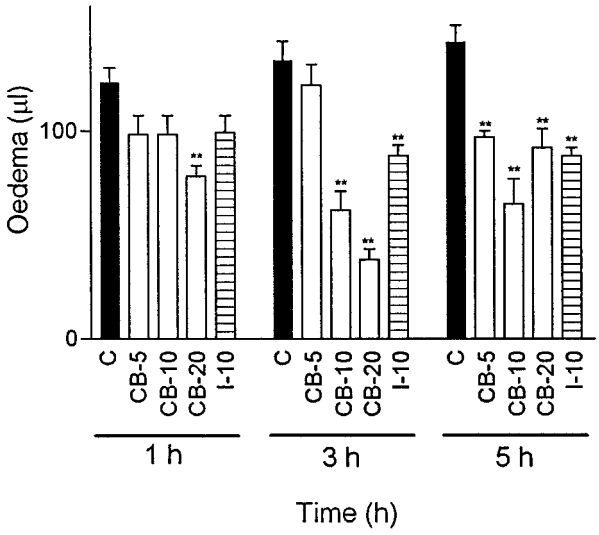

Figure 3 Effect of cacospongionolide B (CB) and indomethacin (I) on mouse paw oedema induced by carrageenin. Data represent means $\pm$ s.e.mean of $n=6$. *$P<0.05$; **$P<0.01$, significantly different from control (C). Cacospongionolide B (5–20 mg kg^{-1}) and indomethacin (10 mg kg^{-1}) were administered p.o. 1 h before the injection of carrageenin.

Table 4 Topical inhibitory effect of cacospongionolide B and indomethacin on mouse ear oedema induced by application of TPA

Drug		*% Oedema inhibition*	*% Myeloperoxidase inhibition*
Cacospongionolide B	50 μg ear^{-1}	43.4 ± 4.8*	64.3 ± 7.2**
Cacospongionolide B	100 μg ear^{-1}	54.6 ± 4.5**	70.9 ± 6.3**
Indomethacin	62.5 μg ear^{-1}	14.0 ± 1.3	46.6 ± 1.7**
Indomethacin	125 μg ear^{-1}	37.0 ± 5.0*	77.4 ± 10.4**

Percentages of inhibition are the means $\pm$ s.e.mean of $n=6$. *$P<0.05$; **$P<0.01$ with respect to the control group.

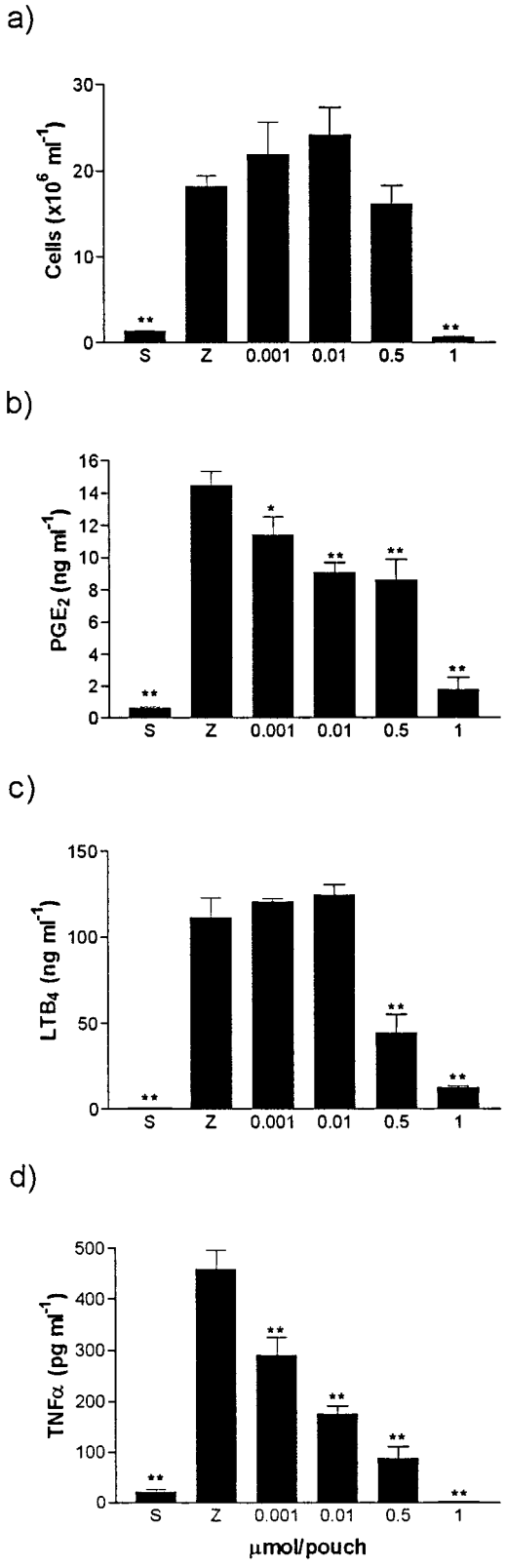

Figure 4 Effect of cacospongionolide B on the mouse air pouch injected with zymosan. Data represent means ± s.e.mean of $n=6$. $^{*}P<0.05$; $^{**}P<0.01$ with respect to the zymosan control group. Cacospongionolide B was injected into the air pouch at the same time as zymosan. S = saline, Z = zymosan. (a) Number of cells present in exudates 4 h after zymosan. (b) PGE_2 levels in exudates. (c) LTB_4 levels in exudates. (d) TNFα levels in exudates.

Effect on adjuvant arthritis

We tested the effects of cacospongionolide B on a model of chronic inflammation, the established adjuvant-induced arthritis, to further characterize its anti-inflammatory properties. Administration of 20 mg kg^{-1} daily on days 13–17 after adjuvant injection, to animals with developed arthritis, significantly reduced mean paw oedema on the two measures performed, on days 15 and 18 after adjuvant (Figure 5). As shown in Table 5, arthritic animals showed a significant increase in eicosanoid levels in different tissues measured at the end of the experiment (day 18), with respect to the non-arthritic control. Cacospongionolide B did not modify eicosanoid levels in serum, stomach and paw homogenates. Interestingly, an increase in $sPLA_2$ activity was detected in paw homogenates of arthritic rats which was significantly inhibited in animals treated with this marine metabolite. Indomethacin was very effective in oedema reduction, and with it a striking inhibition of prostanoid levels was obtained in serum, stomach and paw homogenates. Nevertheless, the stomachs of the animals treated with this NSAID showed redness and perforations, and suppurative peritonitis was observed in two animals. All these toxic effects were absent in the rats treated with cacospongionolide B.

Discussion

Our results indicate that cacospongionolide B is a new inhibitor of $sPLA_2$ with a strong effect on human synovial PLA_2 (group II), and a somewhat milder effect on the pancreatic enzyme (group I) and bee venom PLA_2 (group III). Acting in an irreversible way, this marine metabolite showed *in vitro* a selectivity of inhibition for $sPLA_2$ versus $cPLA_2$, and a potency on the human synovial enzyme similar to that of manoalide. Several reports on the structure-activity relationship of manoalide indicate that the most likely initial reaction between manoalide and PLA_2 is the formation of a Schiff base between a lysine residue and the aldehyde generated upon opening of the manoalide γ-lactone ring (Jacobson *et al*., 1990;

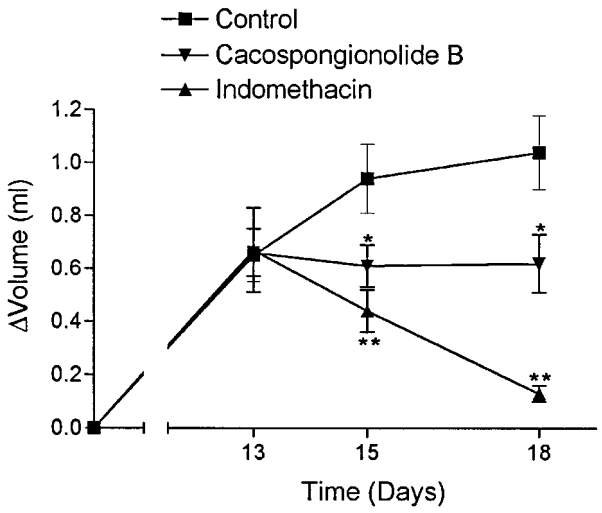

Figure 5 Effect of cacospongionolide B (20 mg kg^{-1}) p.o. and indomethacin (5 mg kg^{-1}) p.o. on the development of adjuvant-induced arthritis in female Lewis rats. Values are the means ± s.e.mean of $n=6$. $^{*}P<0.05$; $^{**}P<0.01$ with respect to the vehicle-treated arthritic rats.

Table 5 Effect of cacospongionolide B and indomethacin on some parameters associated with the development of inflammatory process in adjuvant-induced arthritis

		Serum eicosanoids			*Stomach*	*Paw*	
Group	*Treatment*	PGE_2 (ng ml^{-1})	TXB_2 (ng ml^{-1})	LTB_4 (ng ml^{-1})	PGE_2 (ng ml^{-1})	PGE_2 (ng ml^{-1})	$sPLA_2$ (pmol OA mg^{-1} min^{-1})
Arthritic rats	Vehicle	5.5±0.9	284.6±38.9	51.7±8.4	45.7±5.2	96.8±9.7	10.3±1.1
Normal rats	Vehicle	3.7±0.8	28.4±4.2**	13.2±2.0**	25.0±2.2**	31.3±3.5**	2.2±0.9**
Arthritic rats	Cacospongionolide B	4.8±0.4	166.8±45.4	45.0±3.0	34.7±2.5	76.0±2.2	6.6±1.0*
Arthritic rats	Indomethacin	2.4±0.5*	13.2±8.7**	63.2±10.2	15.9±3.3**	16.0±3.3**	12.1±1.0

Results are the means±s.e.mean of $n=6$. *$P<0.05$; **$P<0.01$ with respect to arthritic control group. OA: Oleic acid. All the samples were collected on the last day of the experiment (day 18). Vehicle, cacospongionolide B (20 mg kg^{-1}) or indomethacin (5 mg kg^{-1}) were administered p.o. once-daily on days 13–17.

Glaser *et al*., 1989). Manoalide and cacospongionolide B are very close chemically, differing from each other by the hydroxylic substitution in the dihydropyran ring and in the hydrophobic region of the molecule that is cyclic in cacospongionolide B structure. Sharing both compounds a non-specific action on lysine-containing proteins, the *in vitro* loss of potency observed is lower for cacospongionolide B. Interestingly, we have demonstrated that cacospongionolide B also inhibits group II $sPLA_2$ *in vivo*, in the 8 h zymosan-injected rat air pouch, a model sensitive to this type of inhibitor (Payá *et al*., 1996). This effect was not due to inhibition of neutrophil accumulation in the air pouch exudate, which is the main source of this secretory enzyme (Payá *et al*., 1996), for inhibition of this activity was also observed at doses that did not affect cell migration. The bioavailability by oral route of this compound was also confirmed in the adjuvant-induced arthritis model of chronic inflammation.

A potential protective role of group II $sPLA_2$ inhibitors can be inferred from studies showing that this type of enzymes participates in the inflammatory process, which leads to the generation of mediators and production of tissue injury in different pathological states. High levels of this enzyme are known to be present in synovial fluids, articular cartilage and blood from patients with rheumatic diseases (Pruzanski *et al*., 1987; Bomalaski & Clark, 1993). In addition, recent studies suggest that group II $sPLA_2$ may play a broad role in tissue injury. Elevated levels of this enzyme, for example, have been associated with a poor clinical outcome in the acute respiratory distress syndrome (Arbibe *et al*., 1997). This enzyme activity has also been detected in human atherosclerotic plaques and it could increase the atherogenicity of LDL by hydrolyzing the phospholipids present in this lipoprotein (Eckey *et al*., 1997). A high expression of group II $sPLA_2$ has been demonstrated in colon biopsies from patients of ulcerative colitis (Haapamaki *et al*., 1997), and its levels are also increased in effusions from cancer patients, associated with a high mRNA expression in carcinoma cells (Abe *et al*., 1997).

On the other hand, $sPLA_2$ could also play a role in cellular defence against infection, as this enzyme activity is bactericidal against *E. coli* (Weiss *et al*., 1994), *L. monocytogenes* (Weiss *et al*., 1994; Harwig *et al*., 1995) and *S. aureus* (Weinrauch *et al*., 1996). In addition, it has been suggested that $cPLA_2$, $sPLA_2$ and diacylglycerol/monoacylglycerol lipase participate in arachidonic acid release in rat NK cells, which plays a crucial role in the lytic activity of these cells (Cifone *et al*., 1997).

Cacospongionolide B can act as a topical anti-inflammatory agent and has also shown oral efficacy. Our data thus demonstrate that a selective inhibitor of $sPLA_2$ is able to decrease the inflammatory reaction in models of acute and chronic inflammation. The production of eicosanoids derived from the COX and 5-LO pathways was reduced by cacospongionolide B in an acute inflammatory response, the mouse air pouch. This effect is probably the consequence of a reduction in substrate availability, since this compound is not an inhibitor of COX and is only a weak inhibitor of 5-LO. This weak effect would explain the slightly higher inhibition of LTB_4. In contrast, the fact that in the model of chronic inflammation, cacospongionolide B did not significantly affect the levels of eicosanoids suggests the participation of other pathways in the regulation of arachidonic acid availability. It has been suggested that inhibition of $cPLA_2$ interferes with cellular activation and therefore results in the control of adjuvant arthritis (Amandi-Burgermeister *et al*., 1997). This model of chronic inflammation is a complex response involving different mediators, and therefore there is a possibility of multiple interactions, e.g. inhibition of iNOS results in the control of the inflammatory features (Connor *et al*., 1995). Our results indicate that inhibition of $sPLA_2$ can also interfere with this model of chronic inflammation without producing toxic effects.

Interestingly, cacospongionolide B inhibited TNFα levels in the mouse air pouch dose-dependently. This effect is absent in COX and 5-LO inhibitors and is similar to that of dexamethasone. The molecular mechanism of this inhibitory effect remains to be established. Inflammatory cytokines induce the enzymes responsible for arachidonic acid release and metabolism, thus leading to increased levels of eicosanoids, and they are involved in the chronification of the inflammatory response. Eicosanoids, in turn, may regulate in part the synthesis of inflammatory cytokines, although there are differences depending on the cell type and the experimental conditions. In certain cases, LTB_4 can increase the generation of IL-1β (Rola-Pleszczynski & Lemaire, 1985), whereas PGE_2 inhibits the production of TNFα and IL-1β in human monocytes (Caughey *et al*., 1997). In contrast, thromboxane A_2 (TXA_2) facilitates cytokine production in these cells, and the balance between different eicosanoids may determine the resulting effect on cytokines (Caughey *et al*., 1997).

Of the anti-inflammatory drugs in use, NSAID can not prevent the progression of chronic inflammation. This group is able to affect cytokine synthesis by human monocytes stimulated by TPA at high concentrations only (Jiang *et al*., 1998), and in addition, the inhibition of PG synthesis can potentiate the expression of TNFα induced by LPS (Bondeson & Sundler, 1996). Glucocorticoids are potent agents for the treatment of chronic inflammatory diseases, although severe side effects limit the long term administration required in chronic disorders. These drugs exert complex effects on inflammation, with inhibition of G protein-dependent activation of $cPLA_2$ activity (Croxtall *et al*., 1995) and the expression of $cPLA_2$, $sPLA_2$ and COX-2, thus leading to inhibition of

eicosanoid biosynthesis. These anti-inflammatory agents also inhibit the biosynthesis of other enzymes, cytokines and adhesion molecules, and act on gene regulation by different mechanisms (Goppelt-Struebe, 1997). The inhibition of cytokine generation by compounds active after oral administration, such us cacospongionolide B, can offer an interesting approach to the search for new anti-inflammatory agents, since this type of drugs can have disease modifying properties (Geiger *et al*., 1994).

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