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Effects of Petrosaspongiolide M, a Novel Phospholipase A_2 Inhibitor, on Acute and Chronic Inflammation[1]

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ABSTRACT

The marine product petrosaspongiolide M is a novel inhibitor of phospholipase A_2 (PLA_2), showing selectivity for secretory PLA_2 versus cytosolic PLA_2, with a potency on the human synovial enzyme (group II) similar to that of manoalide. This compound was more potent than manoalide on bee venom PLA_2 (group III) and had no effect on group I enzymes (*Naja naja* and porcine pancreatic PLA_2). Inhibition of PLA_2 was also observed in vivo in the zymosan-injected rat air pouch, on the secretory enzyme accumulated in the pouch exudate. Petrosaspongiolide M decreased carrageenan paw edema in mice after the oral administration of 5, 10, or 20 mg/kg. This marine metabolite (0.01–1.0 μmol/pouch) induced a dose-dependent reduction in the levels of prostaglandin (PG)E_2, leukotriene B_4, and tumor necrosis factor-α in the mouse air pouch injected with zymosan 4 h after the stimulus. It also had a weaker effect on cell migration. The inflammatory response of adjuvant arthritis was reduced by petrosaspongiolide M, which also inhibited leukotriene B_4 levels in serum and PGE_2 levels in paw homogenates. In contrast with indomethacin, this marine compound did not reduce PGE_2 levels in stomach homogenates. Petrosaspongiolide M is a new inhibitor of secretory PLA_2 in vitro and in vivo, with anti-inflammatory properties in acute and chronic inflammation.

The substrate for eicosanoid synthesis is arachidonic acid, which is frequently esterified in the *sn*-2 position of glycerophospholipids. Phospholipase A_2 (PLA_2) is a class of enzymes that hydrolyze the *sn*-2 acyl group, yielding free fatty acids and lysophospholipids. Secretory PLA_2 ($sPLA_2$) enzymes have been primarily divided into groups I (pancreatic enzyme, Elapidae and Hydrophidae snake venoms) and II (Crotalidae and Viperidae snake venoms, platelets, neutrophils, synovial and inflammatory fluids, and others). Other low-molecular-weight PLA_2s can be found in the venoms of bees and wasps and are included in group III (Glaser et al., 1993). Recently, a new $sPLA_2$ group (group V) has been reported to participate in immediate prostanoid generation in the cell line $P388D_1$ (Balboa et al., 1996). Cytosolic PLA_2s ($cPLA_2$s) (group IV) have been described in platelets, macrophage cell lines, and neutrophils (Clark et al., 1990; Serhan et al., 1996). On the other hand, a calcium-independent PLA_2 is present in the myocardium and other tissues. This enzyme may regulate the arachidonic acid turnover 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). The PLA_2 enzymes present in mammalian cells can participate in cell activation and signal transduction, although in pathological states, increased PLA_2 activity results in membrane alteration and excessive production of lipid mediators leading to tissue injury.

Arachidonic acid release is dependent on $cPLA_2$ activity in a number of cell systems, such as permeabilized human neutrophils (Bauldry and Wooten, 1996) or mouse peritoneal macrophages stimulated with zymosan or 12-*O*-tetradecanoylphorbol-13-acetate (Qiu and Leslie, 1994).

On the other hand, group II $sPLA_2$ can act as a signaling agent that mediates cell growth induced by interleukin-1β (Wada et al., 1997) or participate in signal transduction events such as CD11b/CD18 (MAC-1) expression, adhesion, or degranulation in human neutrophils (Takasaki et al., 1996). This group of enzymes 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 inflammatory fluids, $sPLA_2$ can associate with the cellular membrane to hydrolyze phospholipids and release arachidonic acid, resulting in the genera-

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ABBREVIATIONS: PLA_2, phospholipase A_2; $sPLA_2$, secretory phospholipase A_2; $cPLA_2$, cytosolic phospholipase A_2; COX-1, cyclooxygenase-1; COX-2, cyclooxygenase-2; 5-LO, 5-lipoxygenase; LPS, lipopolysaccharide; LT, leukotriene; iNOS, inducible nitric oxide synthase; NSAID, nonsteroidal anti-inflammatory drug; PG, prostaglandin; TNF-α, tumor necrosis factor-α; TXB_2, thromboxane B_2.

tion of inflammatory lipid mediators (Pfeilschifter et al., 1993). In addition, exocytosis of $sPLA_2$ could modulate the activity of $cPLA_2$ by initiating the formation of leukotriene $(LT)B_4$, which stimulates its own receptor, leading to the activation of $cPLA_2$ in neutrophils (Wijkander et al., 1995).

Arachidonic acid mobilization can be dependent on both types of PLA_2 in some systems, such as in delayed prostaglandin $(PG)D_2$ generation by cyclooxygenase-2 (COX-2) in rat peritoneal macrophages stimulated by lipopolysaccharide (LPS) (Naraba et al., 1998). Interestingly, in human monocytes stimulated by ionophore or zymosan, $cPLA_2$ would participate in the release of arachidonic acid for PG synthesis, whereas $sPLA_2$ would release the substrate for LT synthesis (Marshall et al., 1997).

A number of marine metabolites possess PLA_2 inhibitory properties in vitro (for a review, see Potts et al., 1992). Some of these compounds have demonstrated anti-inflammatory activity, mainly after topical application. Recently, we reported the inhibitory properties of the marine compound variabilin on $sPLA_2$ and $cPLA_2$ activities, as well as its acute anti-inflammatory activity in mice (Escrig et al., 1997). The current study was designed to assess the PLA_2 inhibitory properties of a new marine metabolite, petrosaspongiolide M (Fig. 1), isolated from the Caledonian marine sponge *Petrosaspongia nigra*. Our results indicate that petrosaspongiolide M is a potent inhibitor in vitro and in vivo of $sPLA_2$ possessing anti-inflammatory activity in models of acute and chronic inflammation.

Materials and Methods

Reagents. Petrosaspongiolide M was isolated from the Caledonian marine sponge *P. nigra* Bergquist according to known procedures (Randazzo et al., 1998). Antibody against LTB_4 was kindly provided by Zeneca Pharmaceuticals (Macclesfield, Cheshire, UK). Human synovial recombinant PLA_2 was a gift from Dr. R. M. Kramer (Lilly Research Laboratories, Indianapolis, IN. [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]thromboxane B_2 (TXB_2), L-[^{3}H]arginine, and the tumor necrosis factor-α (TNF-α) enzyme-linked immunosorbent assay kit were from Amersham Iberica (Madrid, Spain). Palmityl trifluoromethyl ketone was purchased from Cayman Chemical Co. (Ann Arbor, MI). *Mycobacterium butyricum* was obtained from Difco (Detroit, MI). The remainder of reagents were from Sigma Chemical Co. (St. Louis, MO). *Escherichia coli* strain CECT 101 was a gift from Prof. F. Uruburu (Department of Microbiology, University of Valencia, Valencia, Spain).

Assay of $sPLA_2$. $sPLA_2$ was assayed according to a modification of the method of Franson et al. (1974). *E. coli* strain CECT 101 were seeded onto medium containing 1% tryptone, 0.5% NaCl, and 0.6% sodium dihydrogen orthophosphate, pH 5.0, and grown for 6 to 8 h at 37°C in the presence of 5 μCi/ml [^{3}H]oleic acid (specific activity, 10 Ci/mmol). 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% BSA, pH 8.0), resuspended in saline, and autoclaved for 30 to 45 min. At least 95% of the radioactivity was incorporated into phospholipids. *Naja naja* venom enzyme, porcine pancreatic enzyme, bee venom enzyme, and human recombinant synovial enzyme 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-labeled membranes and was terminated by the addition of 100 μl of 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.

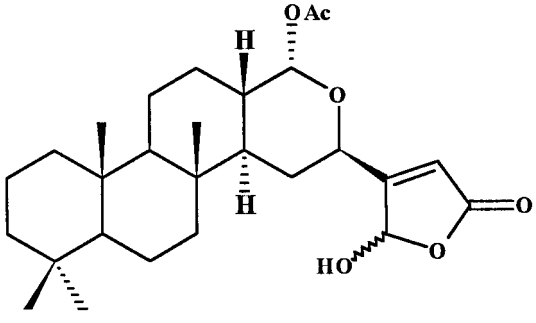

Fig. 1. Chemical structure of petrosaspongiolide M.

Assay of $cPLA_2$. $cPLA_2$ was prepared from human monocytic U937 cells (Cell Collection, Department of Animal Cell Culture, Consejo Superior de Investigationes Científicas, Madrid, Spain) grown in the above medium that 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 phenylmethylsulfonyl fluoride, 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 radiolabeled 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, 2×10^6 cpm) was dried under nitrogen and 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 BSA, and 40% v/v glycerol. The suspension was sonicated to form mixed micelles of phospholipid and Triton X-100. The reaction was started by adding the enzyme solution (approximately 24 μg of 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 BSA, and 50 mM HEPES, pH 9.0. The substrate consisted of 5 ml of micelles (10^4 cpm) containing dioleoyl glycerol at a molar ratio of 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 of silica gel 60 (70–230 mesh; Merck) and centrifuged, and the radioactivity in each supernatant was measured.

Preparation of Human Leukocytes. The citrated blood of healthy volunteers was centrifuged at 200*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*g* for 10 min at 4°C. Contaminating erythrocytes were lysed by hypotonic treatment. The pellet was resuspended in PBS, and Ficoll-hypaque was layered under the cell mixture. The cell gradient mixture was centrifuged at 400*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% fetal bovine serum, 2 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin and was incubated at a cell

density of 10^7/ml 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.

Cell Viability Assays. The cytoplasmic marker enzyme lactate dehydrogenase (Bergmeyer and Bernt, 1974) and the mitochondria-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to formazan (Gross and Levi, 1992) were used to assess the possible cytotoxic effect of petrosaspongiolide M on human neutrophils.

Synthesis of LTB_4 by High-Speed Supernatants from Human Neutrophils. High-speed (100,000*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*g* at 4°C for 30 min. The LTB_4 levels in supernatants were measured by radioimmunoassay (Moroney et al., 1988).

COX-1. J774 cells (Cell Collection, Department of Animal Cell Culture, C.S.I.C, Madrid, Spain) were sonicated at 4°C in an ultrasonicator at maximum potency. Microsomes were prepared by centrifugation at 2,000 g for 5 min at 4°C followed by centrifugation of the supernatant at 100,000*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).

COX-2. Human monocytes or J774 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% fetal bovine serum, and incubated with LPS (10 μg/ml) 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 described above. Microsomes (40 μg of protein/tube) were used as a source of COX-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) to rats. After 24 h, the animals were sacrificed, and livers were excised and homogenated in 10 mM HEPES, pH 7.4, containing 0.32 M sucrose, 100 μM EDTA, 1 mM dithiothreitol, 1 mg/ml phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin (Knowles et al., 1990). The homogenate was centrifuged at 1200 *g* for 10 min at 4°C, followed by centrifugation of the supernatant at 100,000 *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 of 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 unlabeled and L-[^{3}H]arginine (10 μM, 1 μCi/ml). 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 the addition of 1.5 ml of a 1:1 suspension of Dowex (50W) in water. Radioactivity was measured in supernatants by liquid scintillation counting.

Rat and Mouse Air Pouch. Male Wistar rats (120–150 g) and female Swiss mice (25–30 g) were used. Air pouches were formed as described previously (Edwards et al., 1981). The animals were anesthetized with ethyl ether and given a 20-ml (rat) or 10-ml (mouse) injection of sterile air in the s.c. tissue of the back, and 3 days later, 10 ml (rat) or 5 ml (mouse) 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 plus 10 μl ethanol (control group), or 1 ml of 1% w/v zymosan in saline plus test drug (dissolved in 10 μl of ethanol-treated groups) was administered into the air pouch. Eight hours (rat) or 4 h (mouse) after administration, 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*g* at 4°C for 10 min, the rat supernatants were used to measure PLA_2 activity as above. Protein was quantified according to the Bradford technique (Bradford, 1976) using BSA as standard. The mouse supernatants were used to measure LTB_4 and PGE_2 levels as indicated above or TNF-α by enzyme-linked immunosorbent assay (Terencio et al., 1998).

Adjuvant Arthritis. Adjuvant arthritis was elicited in female Lewis rats (126–150 g) by injecting 0.1 ml of *M. butyricum* (10 mg/ml) 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 at 7:00 AM and off at 7:00 PM), and the room temperature was thermostatically regulated to 21 ±1°C. The magnitude of the initial inflammatory response was evaluated by measuring the volume of both paws at day 14. The edema was calculated as the mean increase in paw volume. Animals with edema paw volumes of at least 0.60 ml were then randomized into treatment groups. Petrosaspongiolide M (20 mg/kg), indomethacin (1 mg/kg), or vehicle (ethanol/Tween 80/distilled water, 5:5:90 v/v/v) was administered p.o. (1.0 ml) once daily on days 14 through 22. Serum was collected on the last day of the experiment (day 23) for the determination of PGE_2, TXB_2, and LTB_4. After death, paws from arthritic, treated groups and from nonarthritic normal animals were amputated above the ankle and homogenized in 2.5 ml of saline. After centrifugation at 10,000*g* for 15 min at 4°C, supernatants were used for the determination of PGE_2. Stomachs were homogenized in 2 ml of methanol, and aliquots of supernatants were used to determine the content of PGE_2 as above.

Statistical Analysis. The results are presented as mean ± S.E.M. IC_{50} values were calculated from at least four significant concentrations ($n = 6$). The level of statistical significance was determined by ANOVA followed by Dunnett's *t* test for multiple comparisons.

Results

Effect on PLA_2 and Other Enzyme Activities In Vitro. We first tested the in vitro effect of petrosaspongiolide M on $sPLA_2$ from different sources. As shown in Table 1, this marine compound preferentially inhibited the human synovial and the bee venom enzymes, showing a potency on the

TABLE 1
Effect of petrosaspongiolide M and manoalide on different secretory PLA_2 activities

Compound	*N. naja* Venom %I (10 μM)	Pancreas %I (10 μM) IC_{50}	Human Synovial %I (10 μM) IC_{50}	Bee Venom %I (10 μM) IC_{50}
Petrosaspongiolide M	11.5 ± 3.0	12.3 ± 6.0	68.6 ± 2.7** 4.3	1.0 ± 1.8** 0.6
Manoalide	17.0 ± 1.7*	32.3 ± 2.7** N.D.	93.2 ± 0.2** 3.9	62.5 ± 3.8** 7.5

Results are mean ± S.E.M. (n = 6). * $P < .05$, ** $P < .01$ compared with the enzyme control group.
% I, percentage of inhibition. IC_{50} values were determined for compounds that reach unless 50% inhibition at 10 μM.
N.D., not determined.

TABLE 2
Effect of petrosaspongiolide M and manoalide on $cPLA_2$, 5-LO, COX-2, COX-1, and iNOS activities in vitro

	$cPLA_2$	5-LO	COX-2	COX-1	iNOS
	pmol AA/mg min	*ng LTB_4/ml*	*ng PGE_2/ml*	*ng PGE_2/ml*	*pmol citrulline/mg min*
Control	5.6 ± 0.6	18.4 ± 0.8	1.7 ± 0.1	6.7 ± 0.8	31.2 ± 1.0
Petrosaspongiolide M	5.7 ± 0.7	9.8 ± 1.3**	2.1 ± 0.4	6.5 ± 0.7	25.9 ± 0.8
Manoalide	3.1 ± 0.7**	12.0 ± 1.0**	1.3 ± 0.3	6.6 ± 0.4	28.3 ± 1.6
Palmityl Trifluoromethyl Ketone	1.2 ± 0.6**	N.D.	N.D.	N.D.	N.D.
ZM 230,487	N.D.	4.1 ± 2.7**	N.D.	N.D.	N.D.
NS398	N.D.	N.D.	0.6 ± 0.1**	4.1 ± 0.6**	N.D.
Indomethacin	N.D.	N.D.	1.1 ± 0.1*	0.9 ± 0.2**	N.D.
N(nitro-L-arginine)methyl ester	N.D.	N.D.	N.D.	N.D.	11.8 ± 1.7**

Results are mean ± S.E.M. (n = 6–12). * $P < .05$, ** $P < .01$ compared with control group.
Petrosaspongiolide M, manoalide, and reference inhibitors were tested at 10 μM. N.D., not determined. Drugs were incubated with cytosolic fraction of U937 cells ($cPLA_2$), 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).

first enzyme comparable to that of the reference inhibitor manoalide. On the other hand, petrosaspongiolide M was more potent than manoalide on bee venom PLA_2, whereas it exerted no effect on the *N. naja* venom or porcine pancreatic enzymes. In contrast, petrosaspongiolide M had no inhibitory effects on $cPLA_2$, which was partially inhibited by manoalide and the specific inhibitor PTK at 10 μM (Table 2). We also tested the possible influence of petrosaspongiolide M on other enzymes involved in the synthesis of inflammatory mediators, such as COX-1, COX-2, 5-LO, and iNOS. Only 5-LO was inhibited by petrosaspongiolide M in a manner similar to manoalide. The first compound was devoid of significant cytotoxic effects on human neutrophils at concentrations up to 50 μM, as assessed by the release of lactate dehydrogenase and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method (data not shown).

The kinetic analysis of enzyme activity as a function of the $sPLA_2$ concentration (human synovial enzyme) in the absence or presence of petrosaspongiolide M (Fig. 2) did not show any significant difference in the slopes of the straight lines, suggesting an irreversible inhibition (Segel, 1975), as reported for manoalide (Jacobson et al., 1990).

Effect on $sPLA_2$ In Vivo. To analyze further the inhibitory properties of petrosaspongiolide M on$sPLA_2$, we tested the effects of this marine compound on an animal model suitable to study inhibitors of secretory type II PLA_2 (Payá et al., 1996). As shown in Table 3, petrosaspongiolide M administered into the air pouch as well as by i.p. route dose-dependently inhibited the PLA_2 activity present in the 8-h zymosan-injected rat air pouch, whereas the accumulation of leukocytes in the pouch was reduced by petrosaspongiolide M treatment only at the highest dose tested (1 μmol/pouch or 10 mg/kg i.p.).

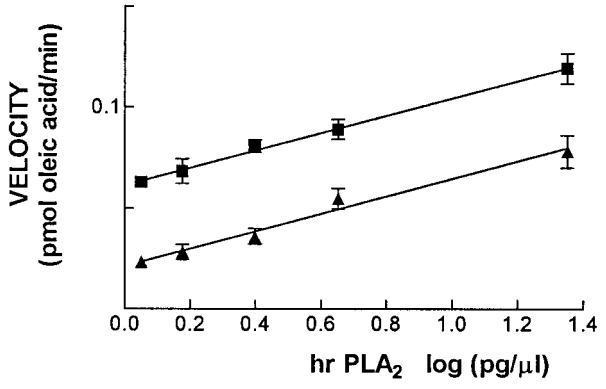

Fig. 2. Activity of human synovial $sPLA_2$ as a function of enzyme concentration in the absence or presence of petrosaspongiolide M. ■, Control; ▲, 1 μM petrosaspongiolide M. Data represent mean ± S.E.M. ($n = 6$). Different enzyme concentrations were preincubated with vehicle (control) or petrosaspongiolide M (1 μM) for 5 min at 37°C, and after the addition of substrate, incubation proceeded for 15 min.

Effect on Mouse Paw Edema. Intraplantar injection of carrageenan to mice caused an inflammatory reaction with control edema values of 147.1 ± 8.1 μl (1 h), 178.3 ± 8.2 μl (3 h), and 161.0 ± 7.2 μl (5 h) ($n = 6$) after the stimulus. Oral pretreatment (1 h before carrageenan) with 5, 10, or 20 mg/kg petrosaspongiolide M reduced hind paw swelling (Fig. 3). This inhibitory effect was observed at the three time points considered for the dose of 20 mg/kg, whereas a significant inhibitory effect was achieved at 10 mg/kg at the 3- and 5-h determinations. Petrosaspongiolide M was as effective as indomethacin in this model, and the percentages of edema inhibition by this nonsteroidal anti-inflammatory drug (NSAID) administered p.o. at 10 mg/kg were 38.4 ± 3.6 ($n = 6$, $P < .01$), 48.1 ± 2.3 ($n = 6$, $P < .01$), and 43.3 ± 1.9 ($n = 6$, $P < .01$) at 1, 3, and 5 h after carrageenan, respectively.

Effect on 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. Petrosaspongiolide M inhibited cell accumulation in exudates at the doses of 1 and 0.1 μmol/pouch (Fig. 4a). This inflammatory response also showed high levels of PGE_2, LTB_4, and TNF-α in the air pouch exudates of control animals injected with zymosan (Fig. 4, b–d). Treatment with petrosaspongiolide M resulted in a significant decrease in PGE_2, LTB_4, and TNF-α levels at the doses of 1 and 0.1 μmol/pouch, being ineffective at the

TABLE 3
Effect of petrosaspongiolide M on cellular accumulation and secretory PLA_2 in 8-h zymosan-injected rat air pouch

	Total Cells	$sPLA_2$
	$\times 10^6$/ml	*pmol OA/ml*
Zymosan	189.0 ± 11.6	444.3 ± 19.7
Saline	19.3 ± 1.9**	206.4 ± 14.3**
Zymosan + Petrosaspongiolide M		
0.1 μmol/pouch	183.0 ± 15.2	414.5 ± 38.1
0.5 μmol/pouch	210.8 ± 15.2	375.5 ± 16.1**
1 μmol/pouch	113.4 ± 19.8**	214.5 ± 14.3**
Zymosan + Petrosaspongiolide M		
1 mg/kg i.p.	188.7 ± 9.1	454.7 ± 26.6
5 mg/kg i.p.	175.8 ± 19.4	379.1 ± 14.0
10 mg/kg i.p.	2.3 ± 0.5**	161.3 ± 15.2**

Results are mean ± S.E.M. (n = 6). * $P < .05$, ** $P < .01$ compared with the zymosan control group.

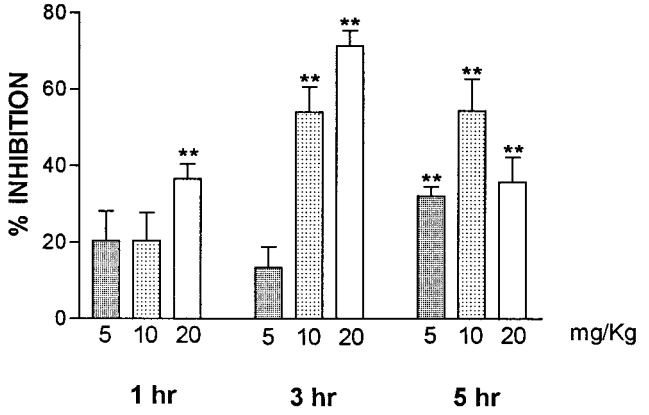

Fig. 3. Effect of petrosaspongiolide M on mouse paw edema induced by carrageenan. Data represent mean ± S.E.M. ($n = 6$). *$P < .05$, **$P < .01$. Petrosaspongiolide M was administered p.o. 1 h before the injection of carrageenan.

lowest dose assayed. In this model, TNF-α levels were very sensitive to petrosaspongiolide M, and at 1 μmol/pouch, the levels of this cytokine were abolished. As expected, the 5-LO inhibitor ZM 230,487 (0.1 μmol/pouch) strongly reduced LTB_4 levels and cell migration, whereas the COX inhibitor indomethacin (0.1 μmol/pouch) decreased PGE_2 levels. Dexamethasone (2 mg/kg i.p.) inhibited cell migration as well as eicosanoid and TNF-α levels in exudates (Table 4). The effects of petrosaspongiolide M on this experimental model were also confirmed after oral administration at a single dose of 20 mg/kg. At this dose, petrosaspongiolide M reduced PGE_2, LTB_4, and TNF-α levels (74.4 ± 4.9%, 79.8 ± 9.1%, and 88.3 ± 8.3% of inhibition, respectively; $n = 6$, $P < .01$), whereas it exerted a lower inhibitory effect on cell accumulation in exudates (51.8 ± 8.2% of inhibition, $n = 6$, $P < .01$).

Effect on Adjuvant Arthritis. The anti-inflammatory properties of this marine compound were also tested on a model of chronic inflammation, the established adjuvant-induced arthritis. The administration of 20 mg/kg/day on days 14 through 22 after adjuvant injection to animals with developed arthritis significantly reduced mean paw edema on days 18 through 23 after adjuvant (Fig. 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 23), with respect to the nonarthritic control. Petrosaspongiolide M did not modify either PGE_2 in serum and stomach homogenates or TXB_2 in serum. Interestingly, petrosaspongiolide M reduced LTB_4 levels in serum and PGE_2 levels in paw homogenates. On the other hand, indomethacin was very effective in edema 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, which was absent in the rats treated with petrosaspongiolide M.

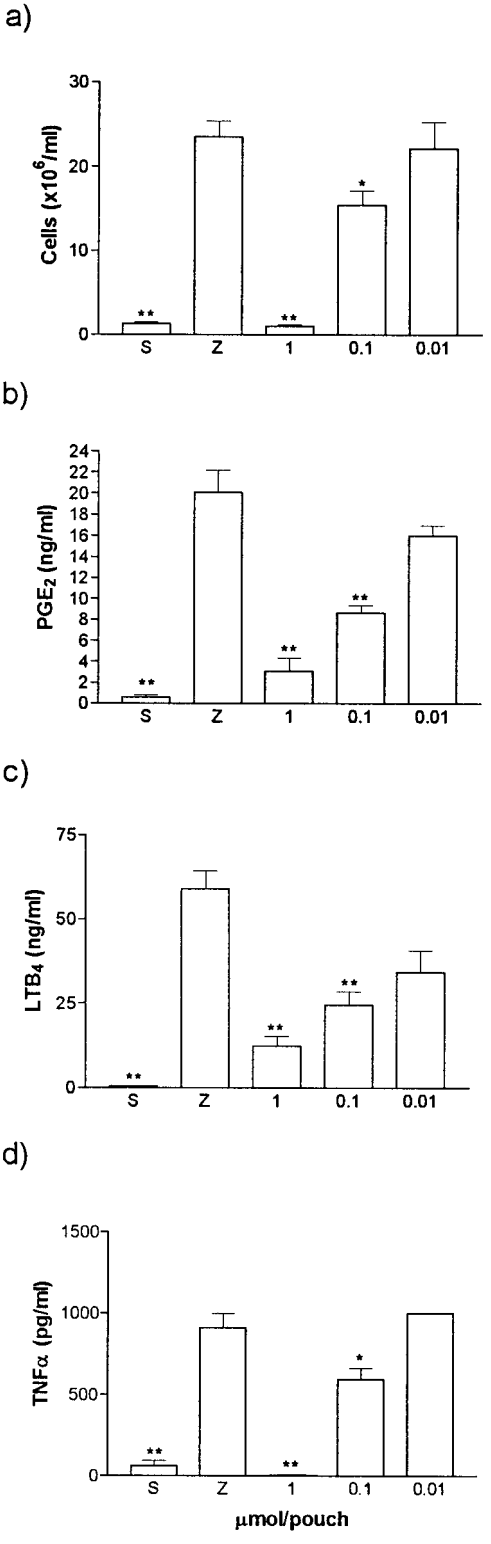

Fig. 4. Effect of petrosaspongiolide M on the mouse air pouch injected with zymosan. Data represent mean ± S.E.M. ($n = 6$). *$P < .05$, **$P < .01$ with respect to zymosan control group. Petrosaspongiolide M was injected into the air pouch at the same time as zymosan. S, saline; Z, zymosan. a, number of cells present in exudates 4 h after zymosan. b, PGE_2 levels in exudates. c, LTB_4 levels in exudates. d, TNF-α levels in exudates.

Discussion

In this study, we used in vitro assays to show that petrosaspongiolide M is a new inhibitor of $sPLA_2$, acting on human synovial PLA_2 (group II) with a potency similar to that of manoalide and bee venom PLA_2 (group III), whereas it exerted no effect on the *N. naja* venom or porcine pancreatic enzymes (group I). Interestingly, petrosaspongiolide M also inhibits group II $sPLA_2$ in vivo in the 8-h zymosan-injected rat air pouch (Payá et al., 1996), even at a dose (0.5 μmol/

TABLE 4
Effect of reference drugs on zymosan-injected mouse air pouch

Group	Total Cells	PGE_2	LTB_4	TNF-α
	$\times 10^6/ml$	ng/ml		pg/ml
Zymosan	23.5 ± 1.9	20.1 ± 2.1	59.0 ± 5.5	909.4 ± 91.2
Saline	1.3 ± 0.2**	0.6 ± 0.2**	0.5 ± 0.3**	61.4 ± 32.0**
Indomethacin (0.1 μmol/pouch)	15.7 ± 1.9	2.9 ± 1.8**	60.4 ± 2.3	941.3 ± 14.9
ZM 230,487 (0.1 μmol/pouch)	13.1 ± 1.9*	22.6 ± 2.4	7.1 ± 2.5**	892.2 ± 12.5
Dexamethasone (2 mg/kg i.p.)	12.9 ± 1.5**	5.9 ± 1.7**	15.7 ± 2.6**	210.6 ± 42.0**

Results are mean ± S.E.M. (n = 6). * $P < .05$, ** $P < .01$ compared with the zymosan control group.

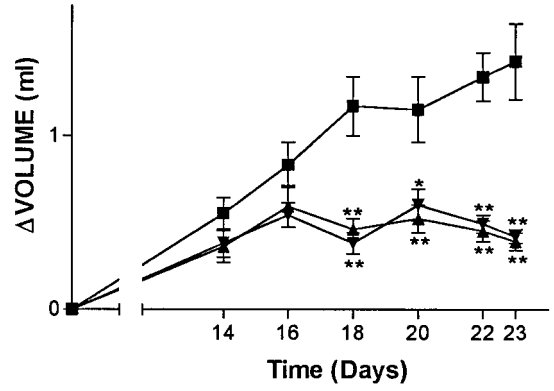

Fig. 5. Effect of petrosaspongiolide M on the development of adjuvant-induced arthritis in female Lewis rats. Values are the mean ± S.E.M. ($n = 6$). *$P < .05$, **$P < .01$ with respect to the vehicle-treated arthritic rats (■). ▼, 20 mg/kg petrosaspongiolide M; ▲, 1 mg/kg indomethacin.

pouch) that did not affect neutrophil accumulation into the inflammatory exudate.

A high level of group II $sPLA_2$ accumulates in inflammatory fluids and serum of patients from inflammatory diseases (Abe et al., 1997), and this group of enzymes may play a role in tissue injury in different pathological states. Group II $sPLA_2$ has also been implicated in cancer (Abe et al., 1997). The inhibition of $sPLA_2$ could control the excessive production of lipid mediators and exert protective effects in inflammatory disorders.

Petrosaspongiolide M reduced the production of eicosanoids derived from the COX and 5-LO pathways in an acute inflammatory response, the mouse air pouch. This effect probably reflects an inhibition of $sPLA_2$ resulting in diminished substrate availability, because this compound is not an inhibitor of COX and is only a weak inhibitor of 5-LO. In addition, petrosaspongiolide M significantly affected the levels of PGE_2 in the inflamed tissue during chronic inflammation, but it did not modify the content of this eicosanoid in serum or stomach. Thus, this marine compound, unlike NSAID, selectively reduced PGE_2 abnormally elevated during inflammation without a reduction of protective levels in other tissues.

It has been reported that the inhibition of $cPLA_2$ results in the control of adjuvant arthritis (Amandi-Burgermeister et al., 1997). We have shown that an inhibitor of $sPLA_2$ can also be effective in chronic inflammation without producing toxic effects. Furthermore, the results of this study support a role for $sPLA_2$ in inflammatory responses.

Inflammatory cytokines are involved in the chronification of the inflammatory response through enzymatic induction resulting in increased levels of eicosanoids. Those cytokines also increase group II $sPLA_2$ synthesis and secretion by rheumatoid synovial fibroblasts and other cell types (Pfeilschifter et al., 1993). Interestingly, petrosaspongiolide M inhibited TNF-α levels in the mouse air pouch in a dose-dependent manner. This profile is different from that of COX and 5-LO inhibitors and closer to that of dexamethasone.

Of the anti-inflammatory drugs in use, NSAIDs cannot prevent the progression of chronic inflammation. In addition, the strong inhibition of PG synthesis produced by NSAIDs can potentiate the expression of TNF-α in some systems (Bondeson and Sundler, 1996). Glucocorticoids control the inflammatory response through complex mechanisms. These drugs inhibit G protein-dependent activation of $cPLA_2$ activity (Croxtall et al., 1995), as well as the expression of $cPLA_2$, $sPLA_2$, and COX-2 and the biosynthesis of other enzymes, cytokines, and adhesion molecules (Goppelt-Struebe, 1997). Glucocorticoids are potent agents for chronic disorders, although severe side effects limit the long-term treatment. We have shown that petrosaspongiolide M is a new inhibitor of $sPLA_2$, active after oral administration, which inhibits eicosanoid and TNF-α generation. These effects could be responsible for the anti-inflammatory activity of this marine metabolite in acute and chronic models of inflammation.

TABLE 5
Effect of petrosaspongiolide M and indomethacin on some parameters associated with development of inflammatory process in adjuvant-induced arthritis

Group	Treatment	Serum Eicosanoids			Stomach PGE_2	Paw PGE_2
		PGE_2	TXB_2	LTB_4		
		ng/ml			ng/ml	
Arthritic rats	Vehicle	10.5 ± 1.5	259.3 ± 38.0	58.4 ± 4.7	59.3 ± 3.3	95.4 ± 10.3
Normal rats	Vehicle	3.7 ± 0.8**	28.4 ± 4.2**	13.2 ± 2.0**	25.0 ± 2.2**	31.3 ± 3.5**
Arthritic rats	Petrosaspongiolide M	11.3 ± 3.0	251.9 ± 54.0	39.1 ± 4.2**	74.5 ± 7.4	44.8 ± 1.9**
Arthritic rats	Indomethacin	4.7 ± 1.2*	46.9 ± 42.7**	53.6 ± 3.3	38.5 ± 3.3**	42.6 ± 4.8**

Results are mean ± S.E.M. (n = 6). * $P < .05$, ** $P < .01$ compared with the arthritic control group.
All samples were collected on the last day of the experiment (day 23). Vehicle, petrosaspongiolide M (20 mg/kg), or indomethacin (1 mg/kg) was administered p.o. once daily on days 14 through 22.

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