Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical Pharmacology 65 (2003) 1539–1549
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

LAAE-14, a new *in vitro* inhibitor of intracellular calcium mobilization, modulates acute and chronic inflammation

Rut Lucas[a], Mario Alves[b], Esther del Olmo[b], Arturo San Feliciano[b], Miguel Payá[a,*]

[a]*Departamento de Farmacología, Universidad de Valencia, Av. V. Andrés Estelles s/n, 46100 Burjassot, Valencia, Spain*
[b]*Departamento de Química Farmacéutica, Facultad de Farmacia, 37007 Salamanca, Spain*

Received 16 September 2002; accepted 5 February 2003

Abstract

A new lipidic acid-amido ether derivative (LAAE-14) able to reduce dose-dependently the calcium increases mediated either by calcium ionophore ionomycin, by the endoplasmic reticular Ca^{2+}-ATPase inhibitor thapsigargin, or by the chemotactic tripeptide *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP), in human neutrophils as well as in murine peritoneal macrophages, but not ATP, has been evaluated as a potential anti-inflammatory drug. This compound attenuated leukocyte activation by means of its inhibitory effect on the respiratory burst elicited in both types of cells by 12-*O*-tetradecanoyl phorbol 13-acetate, by inhibition of the degranulation process induced by cytochalasin B + fMLP or cytochalasin B + platelet activating factor, as well as by reduction of leukotriene B_4 synthesis induced by the calcium ionophore A23187. In addition, in zymosan-stimulated mouse peritoneal macrophages LAAE-14 caused a potent inhibition of nitrite and prostaglandin E_2 production. This compound exerted acute and chronic anti-inflammatory effects by oral route, that may be related with several mechanisms such as attenuation of leukocyte activation, inhibition of inducible nitric oxide synthase, cyclo-oxygenase-2 and cytosolic phospholipase A_2 expression as well as reduction in tumour necrosis factor-α production. Its anti-inflammatory profile is clearly correlated with its behavior as inhibitor of intracellular calcium mobilization. The profile and potency of this compound may have relevance for the inhibition of the inflammatory response at different levels and may represent a new approach to the development of new anti-inflammatory drugs.

Keywords: Intracellular calcium; Oxidative stress; Human neutrophils; Mouse peritoneal macrophages; TNFα; Adjuvant-arthritis

1. Introduction

The increase in the concentration of $[Ca^{2+}]_i$ is a powerful stimulus to cell activation [1]. In inflammatory leukocytes, such as neutrophils and macrophages, $[Ca^{2+}]_i$ plays a pivotal role because it is involved in the regulation of a great variety of functional responses including degranulation [2], superoxide anion, NO and TNFα production [2,3]. Therefore, a variety of inhibitors of Ca^{2+} influx have been investigated as potential therapeutic agents to attenuate leukocyte activation independently of the agonist hierarchy present in inflammation [4–6].

TNFα is a pro-inflammatory cytokine, that is recognized as a central mediator of endotoxaemia and other forms of inflammation [7]. In addition, the endogenous production of TNFα is a key intermediate in the induction of NO synthesis. The enhanced formation of NO by the macrophage type iNOS importantly contributes to tissue injury in human inflammatory arthritis [8], NO being an useful marker for monitoring rheumatoid arthritis in the early stages [9]. On the other hand, NO can be a modulator of COX-2 expression. Recently, it has been reported that NO donors induce COX-2 mRNA expression in rheumatoid synovial cells and suppression of this induction would take part in the anti-inflammatory action of glucocorticoids [10].

In the present study, we have evaluated the anti-inflammatory activity on acute and chronic inflammatory models of LAAE-14 (Fig. 1), a new LAAE-14 derivative inhibitor

* Corresponding author. Tel.: +34-96-3544946; fax: +34-96-3544943.
E-mail address: miguel.paya@uv.es (M. Payá).

Abbreviations: $[Ca^{2+}]_i$, intracellular free calcium; COX-2, cyclo-oxygenase-2; $cPLA_2$, cytosolic phospholipase A_2; fMLP, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; HBSS, Hank's balanced salt solution; iNOS, inducible nitric oxide synthase; LTB_4, leukotriene B_4; LAAE-14, lipidic acid-amido ether derivative; MTT, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; NO, nitric oxide; PAF, platelet activating factor; PGE_2, prostaglandin E_2; TPA, 12-*O*-tetradecanoyl phorbol 13-acetate; TNFα, tumour necrosis factor-α.

0006-2952/03/$ – see front matter © 2003 Elsevier Science Inc. All rights reserved.
doi:10.1016/S0006-2952(03)00120-5

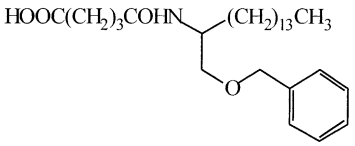


Fig. 1. Chemical structure of LAAE-14.

of calcium intracellular mobilization induced by ionomycin, thapsigargin and fMLP in human neutrophils as well as in murine macrophages. Our results demonstrate that LAAE-14 has substantial modulatory effects on the production of TNFα, NO and prostaglandins and shows that the development of inhibitors of intracellular Ca^{2+} influx could be a very interesting and effective approach to the development of anti-inflammatory drugs than a single mediator antagonist strategy.

2. Materials and methods

2.1. Reagents

LAAE-14 was prepared by acylation of the respective benzylic aminoether with glutaric anhydride, following procedures recently published [11]. [5,6,8,11,12,14,15(*n*)-^{3}H]PGE_2 and [5,6,8,9,11,12,14,15(*n*)-^{3}H]LTB_4 were from Amersham Biosciences. COX-2- and iNOS-specific polyclonal antisera were purchased from Cayman Chemicals. $cPLA_2$- and IgG-specific polyclonal antisera were purchased from Santa Cruz Biotechnology Inc. The peroxidase-conjugated IgG was purchased from Dako and the rest of reagents were from Sigma Chemical Co.

2.2. Preparation of human neutrophils

Leukocytes were obtained and purified as described previously [12]. Viability was greater than 95% by the trypan blue exclusion test. The mitochondrial-dependent reduction of MTT to formazan [13] was used to assess the possible cytotoxic effect of LAAE-14 on human neutrophils.

2.3. Isolation and culture of mouse peritoneal macrophages

Female Swiss mice weighing 25–30 g were used to obtain highly purified peritoneal macrophages. Cells were harvested by peritoneal lavage 4 days after i.p. injection of 1 mL of 10% thioglycolate broth. Cells were resuspended in culture medium (120 mM NaCl, 4.7 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 10 mM HEPES, 1 mM arginine, and 10 mM glucose) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin and incubated at 37° for 2 hr. The adherent cells were used to perform the following experiments. Cytotoxicity was assessed by the reduction of MTT [13].

2.4. Measurements of changes in intracellular calcium levels in human neutrophils and murine macrophages

Neutrophil suspensions (5×10^6 cells/mL) in HBSS pH 7.4 containing 1.26 mM Ca^{2+} and 0.42 mM Mg^{2+} were incubated at 37° for 45 min with 0.5 μM Fura-2/AM. Fura-2/AM-loaded cells were washed and reconstituted in HBSS (5×10^6 cells/mL) and kept in the dark at room temperature until used (within 1 hr). Then, 2.0 mL of cell suspension was added to appropriate cuvettes, placed in a thermostatically controlled holder with continuous stirring, and fluorescence was measured using a Perkin-Elmer spectrofluorimeter (LS-50B). The excitation ratio of 340:380 nm was recorded with an emission wavelength of 505 nm. $[Ca^{2+}]_i$ was calculated according to the equation, $[Ca^{2+}]_i = (F_0 - F_{min})/(F_{max} - F_0) \times 225$ (nM) as described [14]. On the other hand, freshly harvested murine peritoneal macrophages were obtained as previously described and adherent cells were loaded with Fura-2/AM (0.5 μM) for 1 hr. The cells were washed in HBSS (3×10^6 cells/mL) and kept in the dark at room temperature until used (within 1 hr).

2.5. Chemiluminescence

Neutrophils or peritoneal macrophages (2.5×10^6 cells/mL) were incubated with luminol (40 μM) and stimulated with TPA 1 μM. The chemiluminescence was recorded in Microbeta trilux counter (Wallac). Superoxide anions were also generated by the hypoxanthine/xanthine oxidase system [15].

2.6. NADPH-oxidase assay

Fractions I and II from human neutrophils were obtained as described previously [16]. The biochemical assay of NADPH oxidase activity of subcellular fractions from neutrophils was carried out by measuring the disappearance of NADPH (decrease in absorbance at 340 nm).

2.7. Elastase release by human neutrophils

Neutrophils (2.5×10^6 cells/mL) were preincubated with test compound or vehicle for 5 min and then stimulated with cytochalasin B (10 μM) and fMLP (10 nM) or PAF (0.5 μM) for 10 min at 37°. Elastase activity was estimated in supernatants as *p*-nitrophenol release. Possible direct inhibitory effects on elastase activity were also assessed [17].

2.8. Synthesis and release of LTB_4 by human neutrophils

A suspension of human neutrophils (5×10^6 cells/mL) was preincubated with test compound or vehicle and then stimulated with calcium ionophore A23187 (1 μM) for 10 min at 37°. LTB_4 levels in supernatants were measured

by radioimmunoassay [18]. High speed (100,000 *g*) supernatants from sonicated human neutrophils were obtained and incubated in appropriate conditions with 10 μM arachidonic acid to assess 5-LO activity [19].

2.9. Nitrite and PGE_2 production in mouse peritoneal macrophages

Peritoneal macrophages (4×10^5 cells/well) were pre-incubated with test compounds or vehicle for 30 min and then stimulated with zymosan (0.1 mg/mL) in 96-well culture plate at 37° for 18 hr in the presence of test compounds or vehicle. Nitrite and PGE_2 levels were determined in culture supernatants by a fluorimetric method [20] or by radioimmunoassay [18], respectively.

2.10. Mouse air pouch model

All studies were performed in accordance with European Union regulations for the handling and use of laboratory animals. The protocols were approved by the institutional Animal Care and Use Committee. Air pouch was performed in female Swiss mice (25–30 g) as previously described [17]. Test compound at 12.5, 25, and 50 mg/kg, dexamethasone (2 mg/kg) or indomethacin (5 mg/kg) was administered orally 30 min before zymosan injection. After 4 hr, the animals were killed by cervical dislocation, and the exudate in the pouch was collected with 1 mL of saline. Leukocytes present in exudate in the pouch were measured using a Coulter counter. After centrifugation of exudates (1200 *g* at 4° for 10 min), the supernatants were used to measure LTB_4 and PGE_2 levels by radioimmunoassay [18] and TNFα levels by time-resolved fluoroimmunoassay [21].

2.11. Carrageenan paw oedema model

The anti-inflammatory activity of LAAE-14 was assessed by the carrageenan paw oedema test in mice according to the method of Sugishita *et al.* [22]. LAAE-14 (12.5, 25, and 50 mg/kg), indomethacin (5 mg/kg) or vehicle (propylene glycol, glycerol, water; 4:1:5, v/v/v) was administered orally 30 min before injection of carrageenan (0.05 mL; 3%, w/v in saline) into the subplantar area of the right hind paw. The volumes of the injected and contralateral paws were measured at 1, 3, and 5 hr after induction of oedema by using a plethysmometer (Ugo Basile). The volume of oedema was expressed for each animal as the difference between the carrageenan-injected and contralateral paws.

2.12. Adjuvant-induced arthritis model

Adjuvant arthritis was elicited in female Lewis rats (175–200 g) by injection of 0.1 mL of *Mycobacterium butyricum* (10 mg/mL) in mineral oil into the base of the tail [23]. Paw volumes were measured at the beginning of the experiment by using a water displacement plethysmometer. The magnitude of the inflammatory response was evaluated by measuring the volume of both hind paws at day 17. Animals with oedema values of 1.1 mL larger than normal paws were then randomized into treatment groups. 12.5 mg/kg of LAAE-14 or vehicle (propylene glycol, glycerol, water; 4:1:5, v/v/v) was administered p.o. twice-daily and the oedema in hind paws was measured on days 17–24. Serum was collected on the last day of the experiment (day 25) for the determination of PGE_2 and nitrite content. Rats under anaesthesia were placed on a radiographic box at a distance of 90 cm from an X-ray source. Radiographic analysis of arthritic hind paws was performed by X-ray machine (Univet LX160) with a 40 kW exposition for 0.01 sec. Paws were oriented horizontally relative to detector. After death, hind paws were amputated above the ankle and homogenized in 2.5 mL of 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. After centrifugation at 1200 *g* for 15 min at 4°, supernatants were used for the determination of PGE_2, nitrite, and TNFα levels as above. Stomachs were homogenized in 2.0 mL of methanol and aliquots of supernatants were used to determine the content of PGE_2 by radioimmunoassay.

2.13. Western blot analysis

The supernatants from homogenized and centrifugated paws were sonicated and centrifugated at 10,000 *g* for 15 min at 4°. Supernatant protein was determined and 25 μg protein were loaded on 12% sodium dodecyl sulphate–polyacrilamide gel electrophoresis (SDS–PAGE) and transferred onto polyvinylidene difluoride membranes for 90 min at 125 mA. Membranes were blocked in phosphate buffer saline (0.02 M, pH 7.0)–Tween-20 (0.1%) containing 3%, w/v unfatted milk. Membranes were incubated with specific anti-iNOS or anti-COX-2 polyclonal antibody (1:1000 dilution); for IgG, membranes were incubated with specific anti-IgG polyclonal antibody (1:1000 dilution); for $cPLA_2$, membranes were incubated with specific anti-$cPLA_2$ monoclonal antibody (1:5000 dilution). After washing, membranes were incubated with peroxidase-conjugated goat–anti-rabbit IgG (1:20,000 dilution). The immunoreactive bands were visualized using an enhanced chemiluminescence system (ECL, Amersham Biosciences).

2.14. Statistical analysis

Statistical evaluation included ANOVA followed by Dunnett's *t*-test for multiple comparisons. *P*-values of $P < 0.05$ (*) or $P < 0.01$ (**) were taken as significant. Results are shown as mean ± SEM for N experiments. Inhibitory concentration 50% (IC_{50}) values were calculated from at least four significant concentrations.

3. Results

3.1. Effect on intracellular calcium levels in human neutrophils and murine peritoneal macrophages

Experiments were carried out to investigate the effect of LAAE-14 on $[Ca^{2+}]_i$ mobilization induced by different agonists using human neutrophils as well as murine macrophages. LAAE-14 did not produce at 10 μM any effect (Figs. 2A and 3A) on calcium increases induced by ATP, a known calcium mobilizing agonist [1,24], either in human neutrophils or in murine macrophages. The response to the calcium ionophore ionomycin and the endoplasmic reticular Ca^{2+}-ATPase inhibitor thapsigargin was rapid, but in contrast to ATP the increase in $[Ca^{2+}]_i$ was more sustained and had not diminished by 300 sec (Figs. 2B, C and 3B, C). LAAE-14 was able to reduce dose-dependently the calcium increases mediated by both agonists in both types of cells. However, in vehicle control experiments $[Ca^{2+}]_i$ did not change significantly during the 7-min pre-incubation period (data not shown) adopted in this study. As expected, dantrolene a well known inhibitor of calcium mobilization from calcium stored in the endoplasmic reticulum and mitochondria [4,25] reduced the increase in $[Ca^{2+}]_i$ mediated by thapsigargin in both types of cells (Figs. 2C and 3C). In addition, LAAE-14 reduced the increase in $[Ca^{2+}]_i$ mediated by fMLP in both cell types (Figs. 2D and 3D).

3.2. Effect on elastase release, chemiluminescence response, and LTB_4 production in human neutrophils

We assayed LAAE-14 in the degranulation process of human neutrophils activated by two different stimuli. Preincubation of isolated human neutrophils with the test compound elicited a concentration-dependent inhibition of cytochalasin B + fMLP and cytochalasin B + PAF-induced degranulation measured as elastase release. The IC_{50} values were 0.8 and 0.3 μM, respectively (Fig. 4A). Direct inhibitory effects on elastase activity were not observed (data not shown). Besides, LAAE-14 inhibited the chemiluminescence response induced by stimulation of human neutrophils or murine macrophages with TPA, with an IC_{50} values of 2.5 and 1.0 μM, respectively (Fig. 4B). In contrast, LAAE-14 did not inhibit the chemiluminescence generated by the hypoxanthine/xanthine oxidase system (data not shown) at 10 μM, indicating the absence of scavenging effect on reactive oxygen species in a cell-free system. In addition, this compound did not inhibit NADPH-oxidase in preparations of subcellular fractions of human neutrophils (data not shown). On the other hand, LAAE-14 completely abolished LTB_4 release by human neutrophils stimulated with calcium ionophore A23187. The concentration-dependent study showed an IC_{50} value of 2.4 μM. Nevertheless, LAAE-14 failed to modify LTB_4 synthesis by high-speed supernatants from human neutrophils at concentration up to 10 μM (data not shown). Thus, it appears that the reduction of LTB_4 release by LAAE-14 in intact neutrophils is not due to direct inhibition of 5-LO activity.

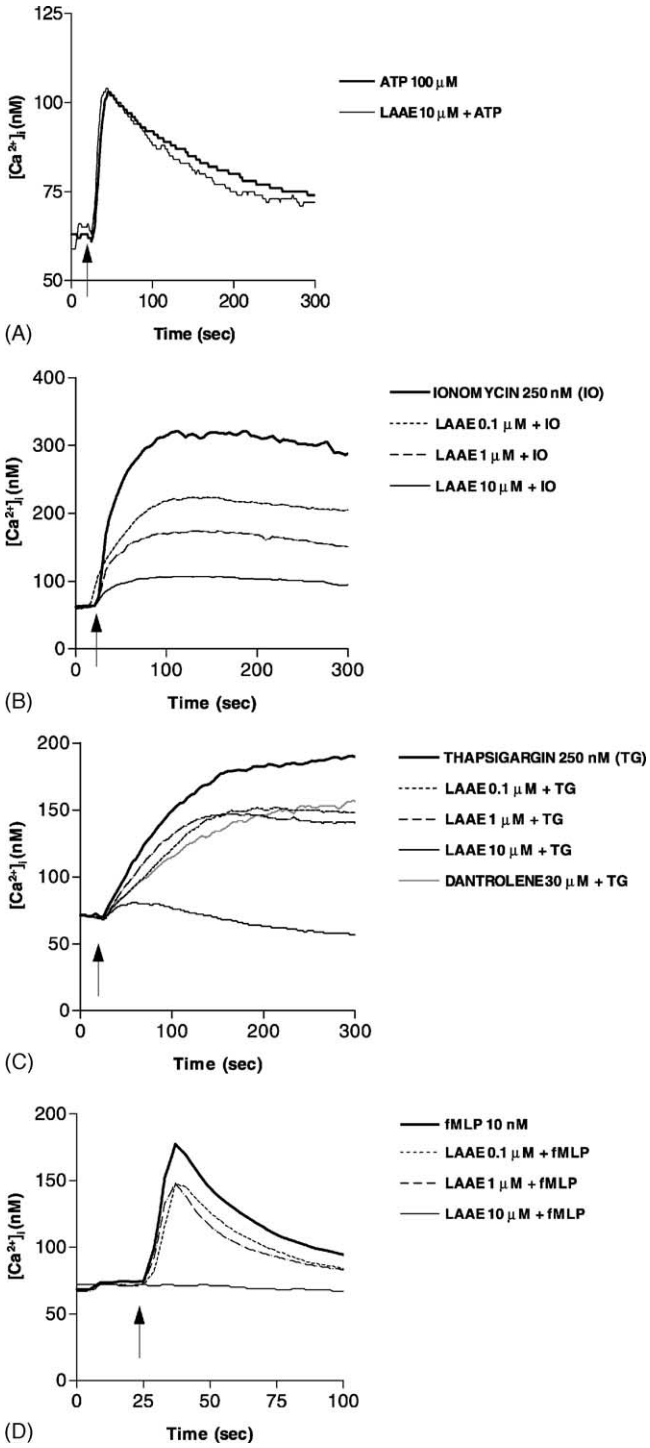
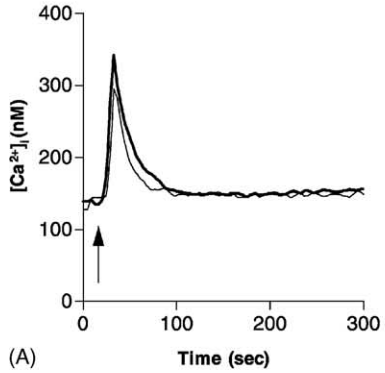
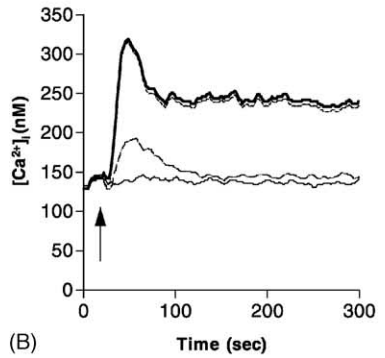
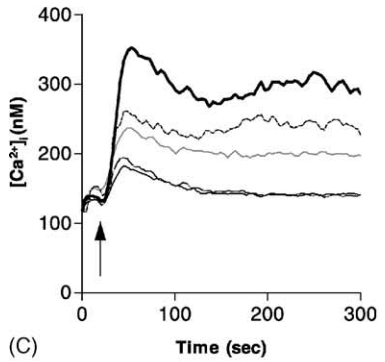


Fig. 2. Effect of LAAE-14 on $[Ca^{2+}]_i$ levels in Fura-2/AM-loaded human neutrophils exposed to different stimuli. (A) ATP, (B) ionomycin, (C) thapsigargin and (D) fMLP. The traces are from a single experiment and are typical of three separate cell preparations. LAAE-14 or dantrolene was preincubated previously for 7 min and stimuli were added at 20 sec (arrow) at the concentration shown at the right of each trace.







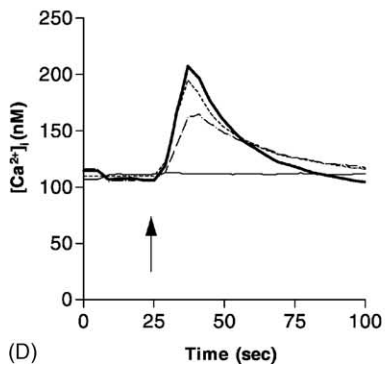
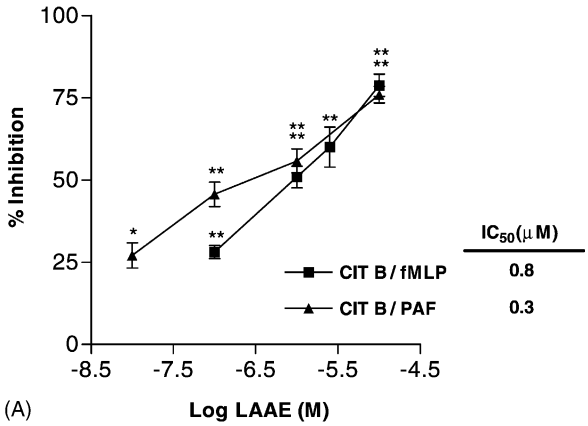
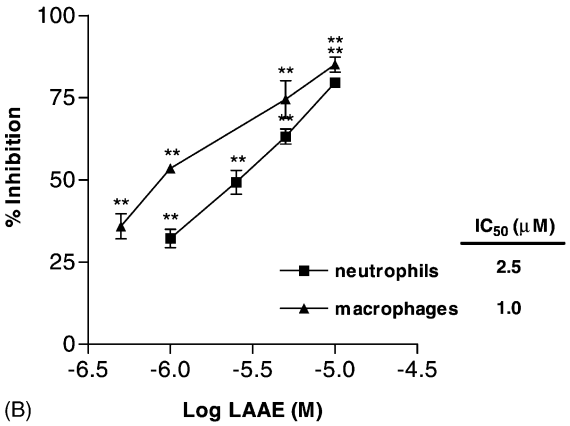


Fig. 3. Effect of LAAE-14 on $[Ca^{2+}]_i$ levels in Fura-2/AM-loaded murine peritoneal macrophages exposed to different stimuli. (A) ATP, (B) ionomycin, (C) Thapsigargin and (D) fMLP. The traces are from a single experiment and are typical of three separate cell preparations. LAAE-14 or dantrolene was preincubated previously for 7 min and stimuli were added at 20 sec (arrow) at the concentration shown at the right of each trace.





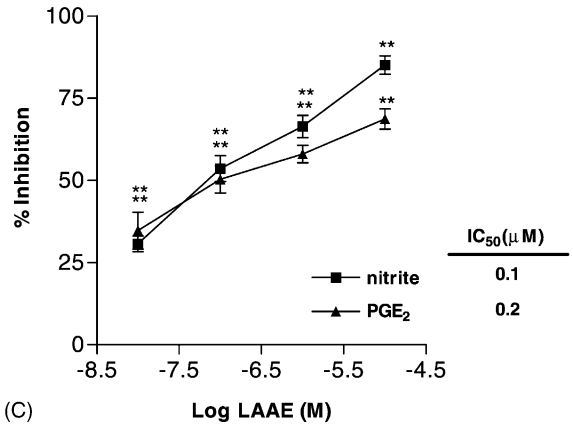


Fig. 4. Concentration–effect relationship for the inhibition by LAAE-14 of different leukocyte functions. (A) Inhibition of elastase release induced by cytochalasin B + fMLP or cytochalasin B + PAF on human neutrophils (unstimulated cells = $1.0 \pm 0.1^{**}$ nmol *p*-nitrophenol/mL/min; fMLP-stimulated cells = 5.3 ± 0.1 nmol *p*-nitrophenol/mL/min; PAF-stimulated cells = 6.9 ± 0.1 nmol *p*-nitrophenol/mL/min), (B) Inhibition of superoxide production induced by TPA on human neutrophils or on murine peritoneal macrophages (unstimulated cells = $293.2 \pm 28.6^{**}$ and $14.3 \pm 0.7^{**}$ L.c./min/10^6 cells, respectively; stimulated cells = 4384.5 ± 165.6 and 72.1 ± 6.7 L.c./min/10^6 cells, respectively; L.c., Luminescence counts), (C) Nitrite and PGE_2 inhibition in zymosan-stimulated murine peritoneal macrophages (unstimulated cells = $65.5 \pm 6.8^{**}$ and $1.2 \pm 0.2^{**}$ ng/mL, respectively; zymosan-stimulated cells = 416.8 ± 35.2 and 7.1 ± 0.6 ng/mL, respectively). Data represent mean ± SEM, N = 6–10. (*) $P < 0.05$, (**) $P < 0.01$ with respect to the stimulated control group.

3.3. Effect on nitrite and PGE_2 production in zymosan-stimulated murine peritoneal macrophages

Incubation of 18 hr zymosan-stimulated mouse peritoneal macrophages with LAAE-14 caused a concentration-dependent inhibition of nitrite (as an index of NO generation) and PGE_2 production (Fig. 4C). The IC_{50} values for both parameters were in the submicromolar range.

3.4. Effect on cell migration, PGE_2, LTB_4, and TNFα in the mouse air pouch injected with zymosan. Effect on oedema in carrageenan mouse paw model

As shown in Fig. 5, the number of leukocytes present in the pouch exudate collected 4 hr after zymosan challenge was significantly reduced by oral administration of LAAE-14 at the doses of 12.5, 25, and 50 mg/kg. PGE_2 and LTB_4 were reduced strongly at 25 and 50 mg/kg. Indomethacin (5 mg/kg), assayed as reference compound, potently reduced PGE_2 levels without affecting LTB_4 content. TNFα levels were strongly reduced at the three doses assayed. Dexamethasone (2 mg/kg) markedly reduced all the parameters studied.

After oral administration, LAAE-14 (12.5, 25, and 50 mg/kg) caused a dose-dependent inhibition in carrageenan-induced paw oedema at 1, 3, and 5 hr after induction of inflammation (Fig. 6). The greatest effect was observed at the highest dose assayed, with inhibition percentages of oedema greater than 75% during all the period evaluation. Indomethacin (5 mg/kg p.o.) was assayed as reference compound, showing the highest reduction at 3 hr (67.2%) after the administration of carrageenan.

3.5. Effect on adjuvant-induced arthritis

We have evaluated the anti-inflammatory effect of LAAE-14 in a chronic inflammatory model, the rat adjuvant-induced arthritis. As shown in Fig. 7A, oral administration of this compound (12.5 mg/kg) on days 17–24 after adjuvant injection, significantly reduced paw oedema. At the end of the experiment (day 25) paw swelling was reduced in LAAE-14-treated animals by 61.9% relative to the paw volume of vehicle-treated animals (control). The dexamethasone-treated group showed a 73.9% reduction in paw volume at that day. The LAAE-14 daily treatment did not affect the body weight progression as it did the dexamethasone group, specially the last 2 days of treatment (Fig. 7B). LAAE-14 reduced significantly the levels of nitrite in serum and in homogenized rat paws (Fig. 8A), and PGE_2 content in serum and in homogenized rat paws but not in stomach ones, as it did the dexamethasone group (Fig. 8B). TNFα content in homogenized rat paws was reduced significantly in both groups (Fig. 8C). We also determined by Western blot analysis the modulatory effect of LAAE-14 and dexamethasone on the expression of different proteins known to be implicated in chronic

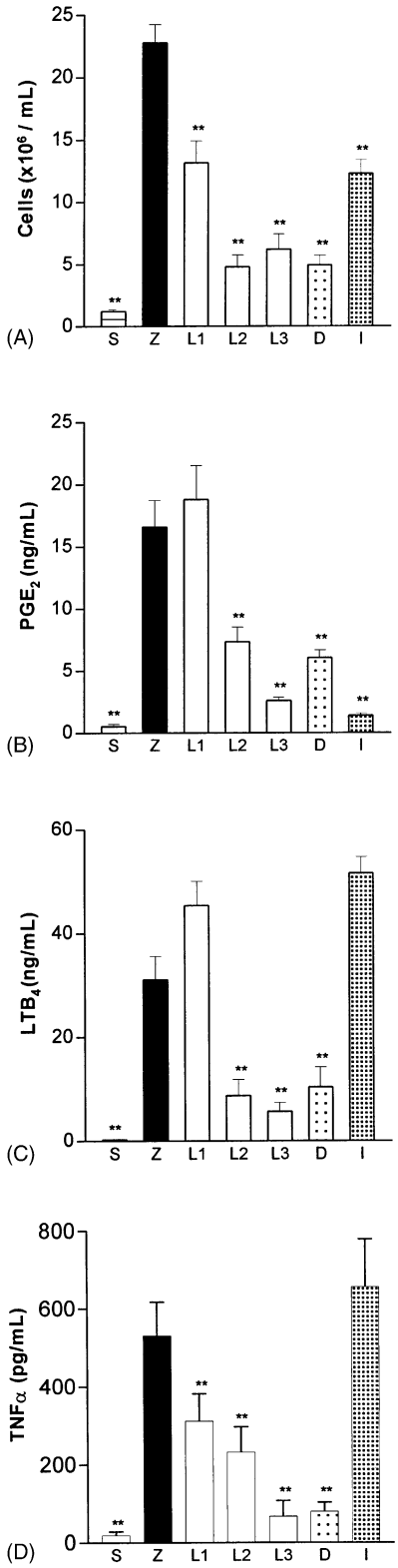


Fig. 5. Effect of LAAE-14 in the 4 hr mouse air pouch injected with zymosan. Data represent mean ± SEM of 6–10 animals; (*) $P < 0.05$, (**) $P < 0.01$ with respect to the zymosan control group. LAAE-14 was administered orally 30 min before zymosan stimulation. S, saline; Z, zymosan; L, LAAE-14 at 12.5, 25, and 50 mg/kg, respectively; D, dexamethasone at 2 mg/kg; I, indomethacin at 5 mg/kg. (A) Number of cells present in exudates 4 hr after zymosan, (B) PGE_2 levels in exudates, (C) LTB_4 levels in exudates, (D) TNFα levels in exudates.

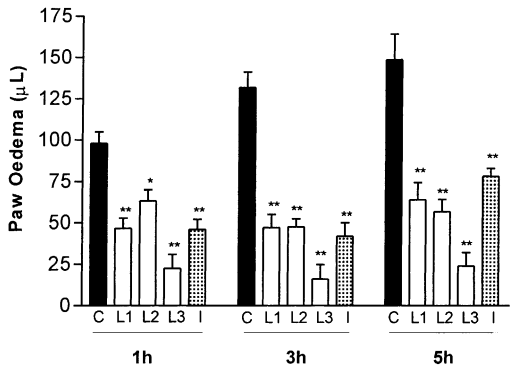


Fig. 6. Inhibitory effect of LAAE-14 on carrageenan mouse paw oedema, 1, 3, and 5 hr after the induction of inflammation. C, oedema control group; L, LAAE-14 at 12.5, 25, and 50 mg/kg, respectively; I, indomethacin at 5 mg/kg. Products were administered orally 30 min before the injection of carrageenan. Data represent mean ± SEM of six animals; (*) $P < 0.05$, (**) $P < 0.01$ with respect to the carrageenan control group.

inflammatory processes like iNOS, COX-2, $cPLA_2$ and IgG (Fig. 9). As shown in Fig. 9, LAAE-14 and dexamethasone practically abolished the expression of iNOS and reduced significantly the expression of COX-2, $cPLA_2$ and IgG content in homogenized rat paws. A radiographic examination of hind paws from rats 25 days post-adjuvant injection revealed bone matrix resorption and osteophyte formation at the joint margin (Fig. 10A). LAAE-14 and dexamethasone markedly reduced the degree of bone resorption, soft tissue swelling and osteophyte formation (Fig. 10B and C). Bone erosion was also scored by densitometric analysis on an arbitrary scale (Fig. 10D) to facilitate its evaluation.

4. Discussion

The cellular signaling events leading to the systemic inflammation are complex. It is generally accepted that

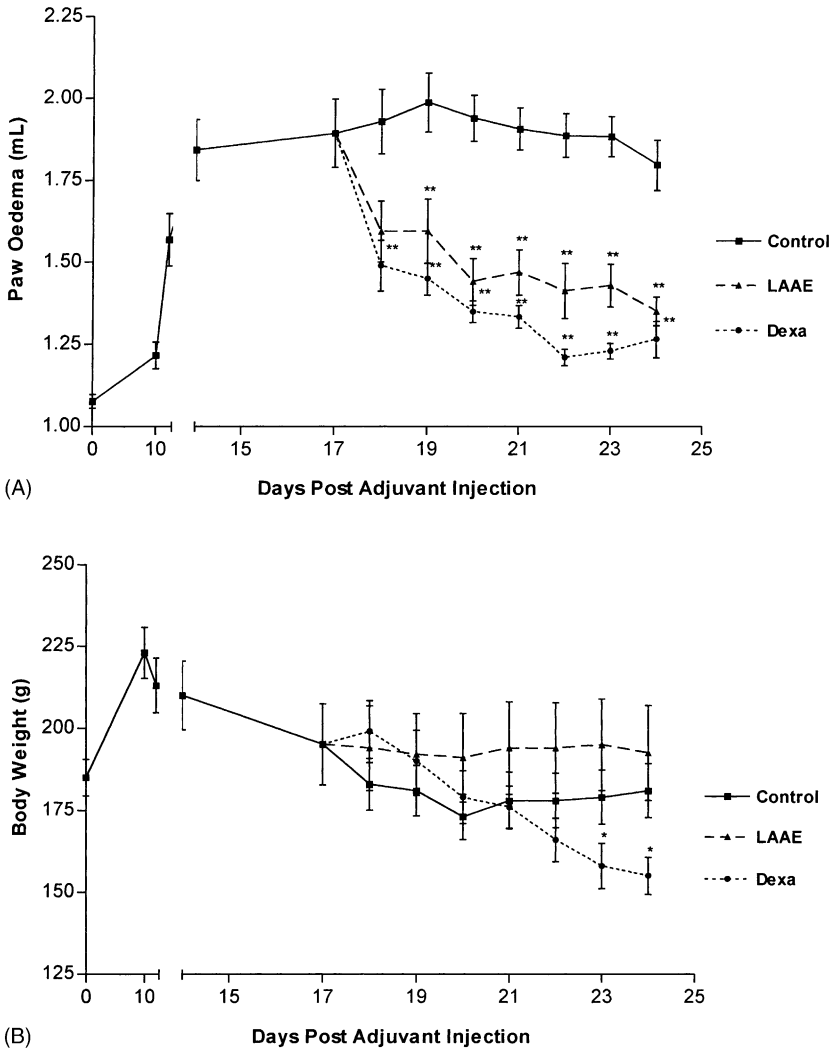


Fig. 7. Effect of LAAE-14 (12.5 mg/kg) and dexamethasone (2 mg/kg) on the development of adjuvant-induced arthritis in Lewis rats. Compounds were administered twice-daily on days 17–24 and paw tissues were recovered on day 25 post-adjuvant injection for analysis. Data represent mean ± SEM of six animals; (*) $P < 0.05$, (**) $P < 0.01$ with respect to the arthritic control group. (A) Paw oedema progression, (B) body weight progression.

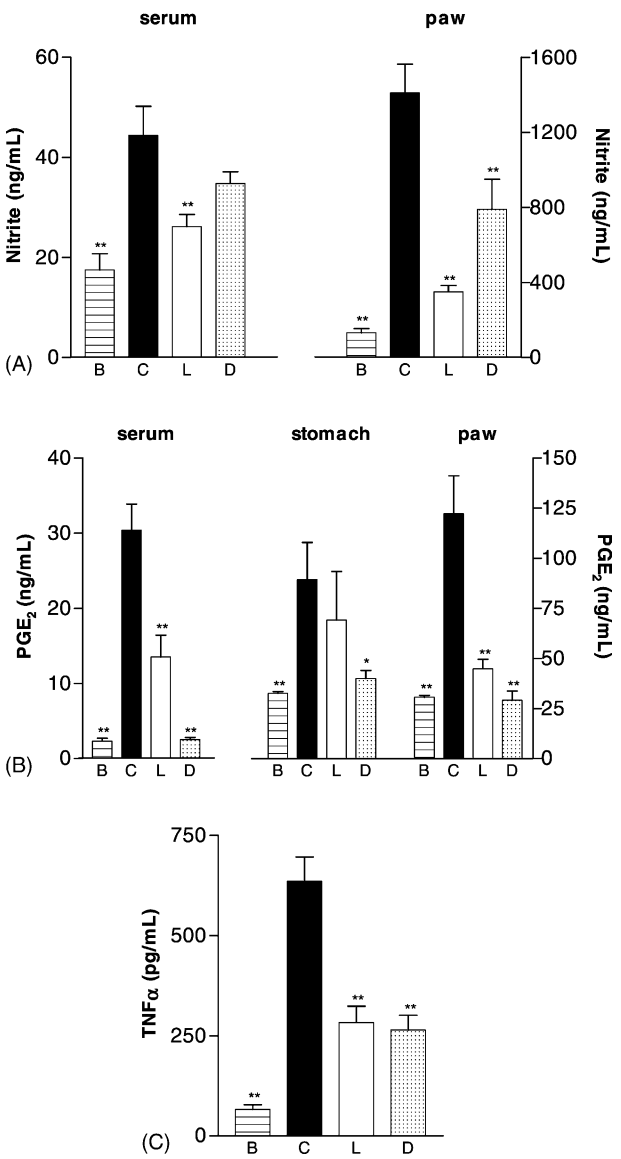


Fig. 8. Effect of LAAE-14 (12.5 mg/kg) and dexamethasone (2 mg/kg) on nitrite, PGE_2, and TNFα levels in serum and homogenized stomachs and paws from adjuvant-induced arthritic rats. Compounds were administered twice-daily on days 17–24 and paw tissues were recovered on day 25 post-adjuvant injection for analysis. (A) Nitrite levels in serum and paws, (B) PGE_2 levels in serum, stomach and paws, (C) TNFα levels in paw. B, non-arthritic group; C, arthritic control group; L, LAAE-14; D, dexamethasone. Data represent mean ± SEM of six animals; (*) $P < 0.05$, (**) $P < 0.01$ with respect to the arthritic control group.

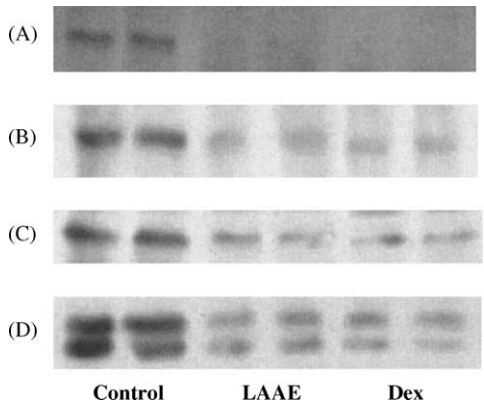


Fig. 9. Effect of LAAE-14 (12.5 mg/kg) and dexamethasone (2 mg/kg) on (A) iNOS expression, (B) COX-2 expression, (C) $cPLA_2$ expression and (D) IgG expression in homogenized rat paws from adjuvant-induced arthritic rats. Compounds were administered twice-daily on days 17–24 and paw tissues were recovered on day 25 post-adjuvant injection for analysis. Dex, dexamethasone. The figure is representative of three similar experiments.

recruitment and activation of leukocytes contribute to tissue damage in inflammation. Neutrophils migrate to the site of inflammation and upon activation by different stimuli, generate large amounts of reactive oxygen species, and release granular enzymes such as elastase and myeloperoxidase, which participate in tissue injury [26]. There are a multitude of intracellular processes which are linked to (or precede) the production of inflammatory mediators. The fluctuation of $[Ca^{2+}]_i$ is a powerful stimulus to cell activation [1]. In inflammatory cells such as neutrophils or macrophages, which are not voltage activated, receptor-mediated $[Ca^{2+}]_i$ influx pathways appear to be an important and abundant mechanism for calcium entry [27]. In this regard, it is known that neutrophil elastase promotes rapid exocitosis by producing $[Ca^{2+}]_i$ oscillations [2] and that the oxidative burst and nitrite formation in activated macrophages [28] are mediated by mobilization of $[Ca^{2+}]_i$. A synergistic co-operation between thapsigargin, a Ca^{2+}-ATPase inhibitor of endoplasmic reticulum, and TPA a protein kinase activator, has been reported for the induction of NO in murine peritoneal macrophages [29].

In the present work, we have shown that LAAE-14 a new LAAE-14, inhibited potently the $[Ca^{2+}]_i$ mobilization which was induced either by calcium ionophore ionomycin, by thapsigargin or by fMLP in human neutrophils as well as in murine peritoneal macrophages. Interestingly, LAAE-14 did not affect the transient increases in $[Ca^{2+}]_i$ induced by the physiological agonist ATP, suggesting that LAAE-14 could not act as a potential chelator of intracellular Ca^{2+}. The respiratory burst elicited in both types of cells by TPA was potently inhibited by LAAE-14, showing no sign of scavenging action in a cell-free system. In this regard, using membranes prepared from neutrophils stimulated with TPA, LAAE-14 did not inhibit this preassembled NADPH oxidase, suggesting that this compound could act at on a previous step, by interfering with the assembly of the active NADPH oxidase. LAAE-14 also reduced the degranulation induced by cytochalasin B + fMLP or cytochalasin B + PAF, as well as the LTB_4 synthesis induced by the calcium ionophore A23187. In addition, in zymosan-stimulated mouse peritoneal macrophages LAAE-14 caused a potent inhibition of nitrite and PGE_2 production. The inhibitory *in vitro* effects observed by this compound on different leukocyte functions are close related with the inhibitory profile on $[Ca^{2+}]_i$ mobilization exerted by LAAE-14. In the 4 hr zymosan-stimulated mouse air pouch model of inflammation, this compound reduced cell migration as well as

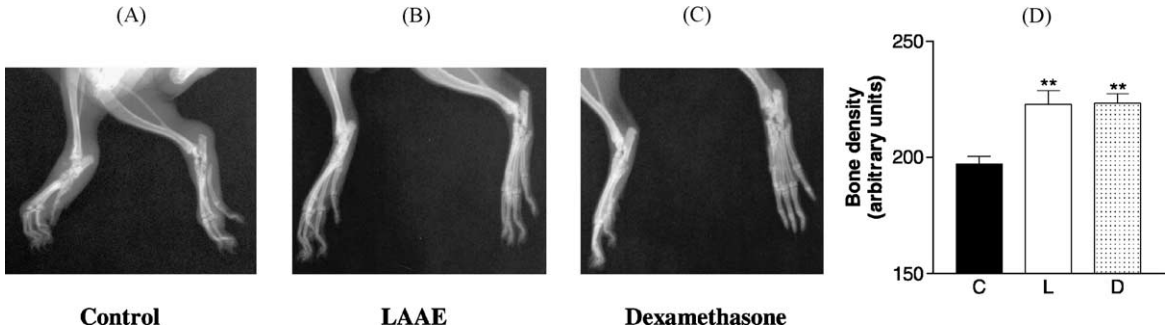


Fig. 10. Radiographic progression of adjuvant-induced arthritis in the tibiotarsal joint of rats. Compounds were administered twice-daily on days 17–24 and paw tissues were recovered on day 25 post-adjuvant injection for radiographic examination. (A) Control, (B) LAAE-14 (12.5 mg/kg), (C) Dexamethasone (2 mg/kg), (D) Densitometric analysis of bone erosion. C, arthritic control group; L, LAAE-14; D, dexamethasone. The figures are representative of three similar experiments.

PGE_2, LTB_4 and TNFα content in the pouch exudate. In this respect, there are reports about activation of mouse peritoneal macrophages showing that zymosan induced both a transient increase in $[Ca^{2+}]_i$ and stoichiometric phosphorylation of $cPLA_2$ on Ser-505 and thus is a potent inducer of arachidonic acid release [30–32]. Besides, other authors [33,34], indicate that increases in $[Ca^{2+}]_i$ play an important role in the control of monokine secretion and that they are required for TNFα and IL-6 protein release by macrophages.

The induction of iNOS and COX-2 greatly increases the synthesis of NO and prostaglandins, which contribute to the pathophysiology of various inflammatory processes. Both pro-inflammatory inducible enzymes are involved in paw swelling induced by carrageenan in mice and rats [35,36]. We have shown that LAAE-14 exerted potent anti-inflammatory effects in the mouse paw oedema model induced by carrageenan. Furthermore, NO has been shown in *in vitro* and *in vivo* studies to increase the production of pro-inflammatory prostaglandins [37,38]. On the other hand, overproduction of prostaglandins by COX-2 expression has been reported for chronic inflammatory conditions such as rheumatoid arthritis [39]. In the present study, using the rat adjuvant arthritis model, we have shown that LAAE-14, by oral route, inhibited the production of NO and prostaglandins. Western blot analysis showed that iNOS and COX-2 expression was reduced, being coupled with a reduction in NO and prostaglandin levels in arthritic paw homogenates. This compound also diminished serum PGE_2 levels without affecting PGE_2 generation in stomach of arthritic rats, which is related with COX-1 activity. The model of adjuvant-induced arthritis is characterized by a chronic swelling in multiple joints, with influx of inflammatory cells, erosion of joint cartilage and bone destruction and remodeling [40]. This inflammatory disease is also characterized by increased NO production and cytokine activation [41]. LAAE-14, in addition with its potent anti-oedematogenic profile, exhibited an important protection on weight loss when compared with the respective control group or with the reference dexamethasone group.

In inflammatory arthritis, there is evidence indicating that the affected tissues produce large amount of radical oxygen species, NO and IgG which react to produce covalent cross-linked aggregates that behave as pro-inflammatory immunocomplexes [42,43]. In this regard, we have also shown that oral administration of LAAE-14 reduced significantly the IgG content in this experimental model. On the other hand, phospholipases A_2, which cleave fatty acid from the *sn*-2 position of membrane phospholipid, play a key role in controlling the release of arachidonic acid which is the precursor of pro-inflammatory lipid mediators (i.e. prostaglandins and leukotrienes). The importance of the arachidonic-acid selective 85-kDa $cPLA_2$ in mediating agonist-induced release of arachidonic acid is well recognized [44]. This $cPLA_2$ is regulated post-translationally by both phosphorilation and calcium [31]. We have also shown by Western blot analysis, that LAAE-14 in arthritic rats reduced the content of a 85-kDa $cPLA_2$ in membranes, which could be related with its *in vitro* inhibitory profile on $[Ca^{2+}]_i$ mobilization. Finally, our results indicate that this compound showed radiographic signs of protection against bone resorption and osteophyte formation in inflamed joints.

In summary, the present study demonstrated that LAAE-14 exerts acute and chronic anti-inflammatory effects that may be related with several mechanisms such as attenuation of leukocyte activation, inhibition of inducible NO synthase, COX-2 and $cPLA_2$ expression, as well as reduction in TNFα production, probably as a consequence of its behavior as inhibitor of $[Ca^{2+}]_i$ mobilization. The profile and potency of this compound may have relevance for the inhibition of the inflammatory response at different levels and may represent a new approach to the development of new anti-inflammatory drugs.

Acknowledgments

This work was supported by Grant from CICYT (SAF2001-2639), Spanish Ministerio de Ciencia y Tecnología. R. Lucas was the recipient of a scholarship from FPI program.

References

[1] Li SW, Westwick J, Poll CT. Receptor-operated Ca^{2+} influx channels in leukocytes: a therapeutic target? Trends Pharmacol Sci 2002;23:63–70.

[2] Maizieres M, Kaplan H, Millot JM, Bonnet N, Manfait M, Puchelle E, Jacquot J. Neutrophil elastase promotes rapid exocytosis in human airway gland cells by producing cytosolic Ca^{2+} oscillations. Am J Respir Cell Mol Biol 1998;18:32–42.

[3] Chen YJ, Lin-Shiau SY. The role of endoplasmic reticular $Ca^{(2+)}$ stores in cell viability and tumor necrosis factor-alpha production of the murine macrophage RAW 264.7 cell line. J Biomed Sci 2000;7:122–7.

[4] Hasko G, Szabo C, Nemeth ZH, Lendvai B, Vizi ES. Modulation by dantrolene of endotoxin-induced interleukin-10, tumour necrosis factor-alpha and nitric oxide production *in vivo* and *in vitro*. Br J Pharmacol 1998;124:1099–106.

[5] Choi SY, Ha H, Kim KT. Capsaicin inhibits platelet-activating factor-induced cytosolic Ca^{2+} rise and superoxide production. J Immunol 2000;165:3992–8.

[6] Kokoska ER, Smith GS, Miller TA. Nonsteroidal anti-inflammatory drugs attenuate proliferation of colonic carcinoma cells by blocking epidermal growth factor-induced Ca^{2+} mobilization. J Gastrointest Surg 2000;4:150–61.

[7] Beutler BA. The role of tumor necrosis factor in health and disease. J Rheumatol 1999;26(suppl 57):16–21.

[8] Grabowski PS, Wright PK, Van't H, Helfrich MH, Ohshima H, Ralston SH. Immunolocalization of inducible nitric oxide synthase in synovium and cartilage in rheumatoid arthritis and osteoarthritis. Br J Rheumatol 1997;36:651–5.

[9] Nakamura H, Ueki Y, Sakito S, Matsumoto K, Yano M, Miyake S, Tominaga Y, Eguchi K. Clinical effects of actarit in rheumatoid arthritis: improvement of early disease activity mediated by reduction of serum concentrations of nitric oxide. Clin Exp Rheumatol 2000;18: 445–50.

[10] Honda S, Migita K, Hirai Y, Ueki Y, Yamasaki S, Urayama S, Kawabe Y, Fukuda T, Kawakami A, Kamachi M, Kita M, Ida H, Aoyagi T, Eguchi K. Induction of COX-2 expression by nitric oxide in rheumatoid synovial cells. Biochem Biophys Res Commun 2000;268:928–31.

[11] Lucas R, Ubeda A, Payá M, Alves M, del Olmo E, Lopez JL, San Feliciano A. Synthesis and enzyme inhibitory activities of a series of lipidic diamine and aminoalcohol derivatives on cytosolic and secretory phospholipases A_2. Bioorg Med Chem Lett 2000;10:285–8.

[12] Bustos G, Ferrándiz ML, Sanz MJ, Payá M, Alcaraz MJ. A study of the novel anti-inflammatory agent florifenine. Topical anti-inflammatory activity and influence on arachidonic acid metabolism and neutrophil functions. Naunyn Schmiedebergs Arch Pharmacol 1995;351:298–304.

[13] Gross SS, Levi R. Tetrahydrobiopterin synthesis. An absolute requirement for cytokine-induced nitric oxide generation by vascular smooth muscle. J Biol Chem 1992;267:25722–9.

[14] Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 1985;260:3440–50.

[15] Betts WH. Detecting oxy radicals by chemiluminescence. In: Greenwald RA, editor. Handbook of methods for oxygen radical research. Boca Raton, FL: CRC Press; 1985. p. 197–201.

[16] Thomas PD, Mao GD, Rabinovitch A, Poznansky MJ. Inhibition of superoxide-generating NADPH oxidase of human neutrophils by lazaroids (21-aminosteroids and 2-methylaminochromans). Biochem Pharmacol 1993;45:241–51.

[17] Escrig V, Ubeda A, Ferrándiz ML, Darias J, Sánchez JM, Alcaraz MJ, Payá M. Variabilin: a dual inhibitor of human secretory and cytosolic phospholipase A_2 with anti-inflammatory activity. J Pharmacol Exp Ther 1997;282:123–31.

[18] Hoult JR, Moroney MA, Payá M. Actions of flavonoids and coumarins on lipoxygenase and cyclooxygenase. Methods Enzymol 1994;234: 443–54.

[19] Tateson JE, Randall RW, Reynolds CH, Jackson WP, Bhattacherjee P, Salmon JA, Garland LG. Selective inhibition of arachidonate 5-lipoxygenase by novel acetohydroxamic acids: biochemical assessment *in vitro* and *ex vivo*. Br J Pharmacol 1988;94:528–39.

[20] Misko TP, Schilling RJ, Salvemini D, Moore WM, Currie MG. A fluorometric assay for the measurement of nitrite in biological samples. Anal Biochem 1993;214:11–6.

[21] Pennanen N, Lapinjoki S, Palander A, Urtti A, Monkkonen J. Macrophage-like RAW 264 cell line and time-resolved fluoroimmunoassay (TRFIA) as tools in screening drug effects on cytokine secretion. Int J Immunopharmacol 1995;17:475–80.

[22] Sugishita E, Amagaya S, Ogihara Y. Anti-inflammatory testing methods: comparative evaluation of mice and rats. J Pharmacobio-Dynam 1981;4:565–75.

[23] Taurog JD, Argentieri DC, McReynolds RA. Adjuvant arthritis. Methods Enzymol 1988;162:339–55.

[24] Zhang Y, Palmblad J, Fredholm BB. Biphasic effect of ATP on neutrophil functions mediated by P_{2U} and adenosine A_{2A} receptors. Biochem Pharmacol 1996;51:957–65.

[25] Song SK, Karl IE, Ackerman JJ, Hotchkiss RS. Increased intracellular Ca^{2+}: a critical link in the pathophysiology of sepsis? Proc Natl Acad Sci USA 1993;90:3933–7.

[26] Smith JA. Neutrophils, host defense, and inflammation: a double-edged sword. J Leukoc Biol 1994;56:672–86.

[27] Meldolesi J, Clementi E, Fasolato C, Zacchetti D, Pozzan T. Ca^{2+} influx following receptor activation. Trends Pharmacol Sci 1991; 12:289–92.

[28] Hotchkiss RS, Bowling WM, Karl IE, Osborne DF, Flye MW. Calcium antagonists inhibit oxidative burst and nitrite formation in lipopolysaccharide-stimulated rat peritoneal macrophages. Shock 1997;8:170–8.

[29] Jun CD, Yoon HJ, Park YC, Lee SY, Kang SS, Kim HM, Chung HT. Synergistic cooperation between thapsigargin and phorbol ester for induction of nitric oxide synthesis in murine peritoneal macrophages. Free Radic Biol Med 1996;20:769–76.

[30] Berger F, Borchard U, Hafner D, Weis T. Activation of a potassium outward current by zymosan and opsonized zymosan in mouse peritoneal macrophages. Naunyn Schmiedebergs Arch Pharmacol 1994;349:594–601.

[31] Qiu ZH, Gijon MA, de Carvalho MS, Spencer DM, Leslie CC. The role of calcium and phosphorylation of cytosolic phospholipase A_2 in regulating arachidonic acid release in macrophages. J Biol Chem 1998;273:8203–11.

[32] Gijón MA, Leslie CC. Regulation of arachidonic acid release and cytosolic phospholipase A_2 activation. J Leukoc Biol 1999;65:330–6.

[33] West MA, Clair L, Bellingham J. Role of calcium in lipopolysaccharide-stimulated tumor necrosis factor and interleukin-1 signal transduction in naive and endotoxin-tolerant murine macrophages. J Trauma 1996;41:647–52.

[34] Marriott I, Bost KL, Mason MJ. Differential kinetics for induction of interleukin-6 mRNA expression in murine peritoneal macrophages: evidence for calcium-dependent and independent-signalling pathways. J Cell Physiol 1998;177:232–40.

[35] Ianaro A, O'Donnell CA, Di Rosa M, Liew FY. A nitric oxide synthase inhibitor reduces inflammation, down-regulates inflammatory cytokines and enhances interleukin-10 production in carrageenin-induced oedema in mice. Immunology 1994;82:370–5.

[36] Sautebin L, Ialenti A, Ianaro A, Di Rosa M. Endogenous nitric oxide increases prostaglandin biosynthesis in carrageenin rat paw oedema. Eur J Pharmacol 1995;286:219–22.

[37] Salvemini D, Misko TP, Masferrer JL, Seibert K, Currie MG, Needleman P. Nitric oxide activates cyclooxygenase enzymes. Proc Natl Acad Sci USA 1993;90:7240–4.

[38] Salvemini D, Manning PT, Zweifel BS, Seibert K, Connor J, Currie MG, Needleman P, Masferrer JL. Dual inhibition of nitric oxide and prostaglandin production contributes to the antiinflammatory properties of nitric oxide synthase inhibitors. J Clin Invest 1995;96:301–8.

[39] Kang RY, Freire Moar J, Sigal E, Chu CQ. Expression of cyclooxygenase-2 in human and an animal model of rheumatoid arthritis. Br J Rheumatol 1996;35:711–8.
[40] Winder CV, Lembke LA, Stephens MD. Comparative bioassay of drugs in adjuvant-induced arthritis in rats: flufenamic acid, mefenamic acid and phenylbutazone. Arthritis Rheum 1969;12:472–82.
[41] van't Hof RJ, Ralston SH. Nitric oxide and bone. Immunology 2001;103:255–61.
[42] Uesugi M, Yoshida K, Jasin HE. Inflammatory properties of IgG modified by oxygen radicals and peroxynitrite. J Immunol 2000;165:6532–7.
[43] Clancy RM, Amin AR, Abramson SB. The role of nitric oxide in inflammation and immunity. Arthritis Rheum 1998;41:1141–51.
[44] Balsinde J, Balboa MA, Insel PA, Dennis EA. Regulation and inhibition of phospholipase A_2. Annu Rev Pharmacol Toxicol 1999;39:175–89.