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Dysidotronic acid, a new sesquiterpenoid, inhibits cytokine production and the expression of nitric oxide synthase

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Abstract

In a previous study, we reported a new bioactive sesquiterpenoid, named dysidotronic acid, to be a potent, selective human synovial phospholipase A_2 inhibitor. Dysidotronic acid is a novel, non-complex manoalide analogue lacking the pyranofuranone ring. We now investigate the effect of this compound on cytokine, nitric oxide and prostanoid generation on the mouse macrophage cell line RAW 264.7, where it showed a dose-dependent inhibition with inhibitory concentration 50% values in the micromolar range. This effect was also confirmed in the mouse air pouch injected with zymosan. Dysidotronic acid inhibited the production of tumor necrosis factor alpha and interleukin-1 beta as well as the production of nitric oxide, prostaglandin E_2 and leukotriene B_4. Decreased nitric oxide generation was the consequence of inhibition of the expression of nitric oxide synthase, whereas PGE_2 and LTB_4 reduction was due to inhibition of arachidonic acid bioavailability through a direct inhibitory effect of dysodotronic acid on secretory phospholipase A_2. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Dysidotronic acid; Cytokine; Nitric oxide (NO); RAW 264.7; Zymosan; Air pouch, mouse

1. Introduction

Manoalide is a potent analgesic and anti-inflammatory sesterterpene isolated in 1980 from a marine sponge (De Silva and Scheuer, 1980). Since then, manoalide has been considered a very useful pharmacological tool and has become one of the best-known phospholipase A_2 inhibitors (Soriente et al., 1999). Several attempts have been made to develop analogues of manoalide for use in anti-inflammatory therapy (Glaser et al., 1995; García et al., 1999; De Rosa et al., 1998). In this regard, dysidotronic acid is a potent new selective human synovial phospholipase A_2 inhibitor (Giannini et al., 2000), and a non-complex manoalide analogue lacking the pyranofuranone ring that can offer new structural possibilities for developing pharmacological agents able to control inflammatory states.

Macrophages constitutively produce prostaglandins by means of the enzyme cyclo-oxygenase-1, whereas inflammatory mediators and cytokines stimulate the inducible enzymes cyclo-oxygenase-2 and inducible nitric oxide (NO) synthase, thus promoting high output of prostaglandin and NO (Barrios-Rodiles and Chadee, 1998; Sheffler et al., 1995). Reactive nitrogen intermediates such as NO play an important role in inflammatory and immune reactions. In this regard, NO can enhance the release of tumor necrosis factor-α (TNF-α) and interleukin-1α. Thus, in addition to acting as a powerful effector molecule mediating the cytotoxic activities of mouse macrophages, NO can play a role in enhancing the production of a variety of other inflammatory mediators, and thus can contribute both directly and indirectly to the immunopathology of macrophage-dependent inflammation (Marcinkiewicz et al., 1995). The present study was undertaken to determine whether this novel terpene lactone, called dysidotronic acid (Fig. 1), could modulate cytokine production as well as the generation of NO and prostaglandin E_2 on the mouse macrophage cell line RAW

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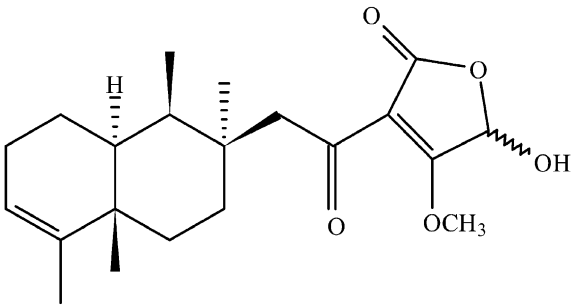

Fig. 1. Chemical structure of dysidotronic acid.

264.7, and on experimental inflammation using the mouse air pouch model injected with zymosan.

2. Materials and methods

2.1. Materials

Dysidotronic acid was isolated from the sponge *Dysidea* sp. following known procedures recently published (Giannini et al., 2000). [5,6,8,11,12,14,15(*n*)-^{3}H]prostaglandin E_2, [5,6,8,9,11,12,14,15(*n*)-^{3}H]leukotriene B_4, [9,10-^{3}H]oleic acid and the enhanced chemiluminescence system were from Amersham Iberica (Madrid, Spain). Inducible NO synthase and cyclo-oxygenase-2 specific polyclonal antisera, *N*-(2-cyclohexyloxy-4-nitrophenyl)methanesulfonamide (NS398) and *N*-(3-(aminomethyl)benzyl)-acetamidine dihydrochloride (1400W) were purchased from Cayman Chem. (MI, USA). The peroxidase-conjugated goat anti-rabbit Immunoglobulin G (IgG) was purchased from Dako (Copenhagen, Denmark). The rest of reagents were from Sigma (MO, USA).

2.2. Preparation of human neutrophils

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 for 45–60 min. The upper phase was then collected and concentrated by centrifugation at $200 \times g$ for 10 min at room temperature. Contaminating erythrocytes were lysed by hypotonic treatment using ice-cold distilled water for 20 s. The cell pellets were gently resuspended in 10-ml ice-cold modified phosphate buffer saline free of Ca^{2+} and Mg^{2+}, and a solution of Ficoll-hypaque was carefully layered under the cell mixture to form a discontinuous gradient. The cell gradient mixture was centrifuged at $400 \times g$ for 40 min at room temperature. Neutrophils were separated and resuspended in phosphate buffer saline containing 1.26 mM Ca^{2+} and 0.9 mM Mg^{2+} (Bustos et al., 1995). Viability was greater than 95% as determined by the trypan blue exclusion test.

2.3. Cell viability assays

The mitochondrial dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan (Gross and Levi, 1992) was used to assess the possible cytotoxic effect of dysidotronic acid on human neutrophils as well as on the mouse macrophage cell line RAW 264.7.

2.4. Myeloperoxidase release by human neutrophils

Aliquots of 1.0 ml human neutrophils (2.5×10^6 cells/ml) were preincubated at 37°C for 5 min with 10 μl of dysidotronic acid dissolved in ethanol (or an equivalent volume of ethanol for the controls). After this, the tubes were stimulated for a further 10 min at 37°C using different stimuli: cytochalasin B (10 μM) and *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP, 10 nM), cytochalasin B (10 μM) and platelet activating factor (PAF, 5 μM). Myeloperoxidase activity was estimated in aliquots of supernatant (Suzuki et al., 1983). The direct effects on myeloperoxidase were also tested using aliquots of supernatants of cytochalasin B + fMLP-stimulated human neutrophils.

2.5. 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. Zymosan was prepared by incubating a 10-mg/ml sterile saline suspension of the material in a boiling water bath for 1 h. The boiled zymosan was washed twice and resuspended in sterile saline at 10 mg/ml, and stored at room temperature for no more than 3 days before use. Air pouch was produced in female Swiss mice (25–30 g) as previously described (Edwards et al., 1981; Posadas et al., 2000). Animals were anaesthetized with ethyl ether, and 10 ml of sterile air was injected into the subcutaneous tissue of the back. Three days later, 5 ml of sterile air was injected into the same cavity. On day 6, 1 ml of 1% w/v zymosan in saline + vehicle (10 μl ethanol: control group) or 1 ml of 1% w/v zymosan in saline + dysidotronic acid or dexamethasone (dissolved in 10 μl of ethanol) at the concentrations indicated in Results (treated groups) was injected into the air pouch. Another group received only 1 ml of saline + vehicle (saline group). Four or twenty-four hours after zymosan administration, the animals were sacrificed by cervical dislocation, and the exudate in the pouch was collected with 1 ml of saline. An additional dose of dysidotronic acid or dexamethasone was injected into the 24-h zymosan-injected air pouch, 8 h after zymosan injec-

tion. Leukocytes present in exudates were measured using a Coulter counter. After centrifugation of exudates at $400 \times g$ at 4°C for 10 min, the supernatants were used to measure prostaglandin E_2 and leukotriene B_4 levels by radioimmunoassay (Hoult et al., 1994), myeloperoxidase by a spectrophotometric technique (Suzuki et al., 1983) and TNF-α and interleukin-1β levels by time-resolved fluoroimmunoassay (Pennanen et al., 1995). The nitrite concentration in supernatants from 24 h, as a reflection of NO release, was assayed fluorometrically (Misko et al., 1993). The cell pellet from 24 h was used for inducible NO synthase and cyclo-oxygenase-2 Western blotting as described below. Protein was measured by the Lowry method using bovine serum albumin as standard.

2.6. Cell culture

The mouse macrophage cell line RAW 264.7 (European Collection of Cell Cultures) was cultured in Dulbecco's modified Eagle's medium (DMEM) containing L-glutamine 2 mM, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum. Macrophages were removed from the tissue culture flask using a cell scraper, centrifuged at $400 \times g$ for 10 min. Cells were resuspended at a concentration of 2×10^6/ml in a total volume of 200 μl and cultured in 96-well culture plate. Macrophages were co-incubated with test compounds at different concentrations and zymosan (0.1 mg/ml) at 37°C. TNF-α accumulation was measured in 5-h supernatants, meanwhile prostaglandin E_2 and nitrite production were determined in 18-h supernatants.

2.7. Inducible NO synthase and cyclo-oxygenase-2 activity in intact cells

After stimulation with zymosan (0.1 mg/ml) for 18 h, supernatants from mouse macrophage cell line RAW 264.7 were removed and cells were treated with Hank's buffer supplemented with L-arginine (0.5 mM) and arachidonic acid (10 μM) for a further 4-h incubation with test compounds at different concentrations. Supernatants were collected for the measurement of nitrite and prostaglandin E_2 as an index of inducible NO synthase and cyclo-oxygenase-2 activities.

2.8. Cyclo-oxygenase-2 activity in broken cell preparations

RAW 264.7 cultured cells were incubated with aspirin (300 μM) at 37°C for 2 h. The cells were washed twice, resuspended in DMEM with 10% fetal bovine serum and incubated with *Escherichia coli* lipopolysaccharide (serotype 0111:B4) (10 μg/ml) at 37°C for 24 h (Patel et al., 1999). The cells were pelleted at 4°C and disrupted by sonication in 10 mM Tris–HCl, pH 7.4, containing 0.32 M sucrose, 100 mM EDTA, 1 mM dithiothreitol, 2 mM phenylmethylsulphonylfluoride and 100 mM leupeptin. The homogenated cells were centrifuged at $2{,}000 \times g$ for 10 min at 4°C, and the resulting supernatant was further centrifuged at $100{,}000 \times g$ for 100 min at 4°C. Microsomes (1 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 (Brownlie et al., 1993). The reaction was stopped by boiling the samples for 5 min, and prostaglandin E_2 synthesis was determined by radioimmunoassay (Hoult et al., 1994).

2.9. Western blot analysis

Inducible NO synthase and cyclo-oxygenase-2 protein expression was studied in the cytosolic or microsomal fractions, respectively, from zymosan-stimulated RAW 264.7 cells and cell pellets obtained by centrifugation of air pouch exudates. Equal amounts of protein (20 μg) were loaded on 12.5% polyacrylamide gel electrophoresis-sodium dodecyl sulphate (PAGE-SDS) 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. For inducible NO synthase, membranes were incubated with specific anti-inducible NO synthase polyclonal antiserum (1/1,000); for cyclo-oxygenase-2, membranes were incubated with specific anti-cyclo-oxygenase-2 polyclonal antiserum (1/1,000). Both membranes were incubated with the peroxidase-conjugated goat anti-rabbit Immunoglobulin G (1/20,000) and peroxidase-conjugated rabbit anti-goat/sheep IgG (1/20,000), respectively. The immunoreactive bands were visualized using an enhanced chemiluminescence system.

2.10. Measurement of extracellular fatty acid release

RAW 264.7 cultured cells were plated at 10^6/well, and the cells were radiolabelled by including 5.0 μCi/ml [^{3}H]oleic acid during the overnight adherence period (20 h). Labelled fatty acid that had not been incorporated into cellular lipids was removed by washing the cells six times with serum-free medium containing 1mg/ml albumin (Balsinde et al., 2000). The cells were placed in serum-free medium and were stimulated with zymosan (0.3 mg/ml) for 24 h in the presence of 0.5 mg/ml bovine serum albumin. The supernatants were removed, cleared of detached cells by centrifugation, and assayed for radioactivity by liquid scintillation counting. Dysidotronic acid was added 30 min before the addition of zymosan.

2.11. Statistical analysis

The results are presented as means ± S.E.M.; 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

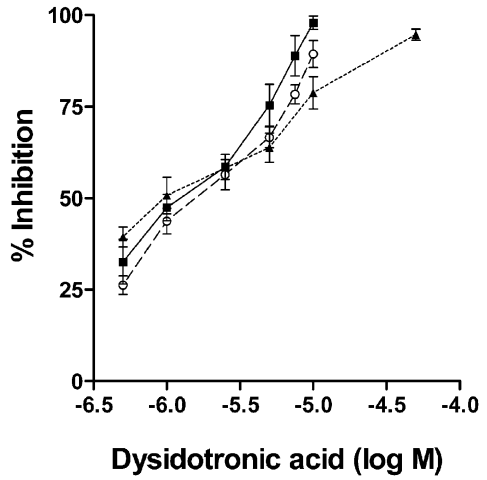

Fig. 2. Concentration–effect relationship for the inhibition by dysidotronic acid of TNF-α (■), prostaglandin E_2 (▲) and nitrite (○) production by dysidotronic acid in zymosan-stimulated RAW 264.7 cells. TNF-α accumulation was measured in 5-h supernatants (non-stimulated cells: 69.2 ± 19.5 pg/ml and zymosan-stimulated cells: 728.0 ± 77.3 pg/ml, respectively), while prostaglandin E_2 and nitrite production were determined in 18-h supernatants (non-stimulated and zymosan-stimulated cell values are reflected in Table 1). Results are the means ± S.E.M., $n = 6–10$. $^{*}P < 0.05$, $^{**}P < 0.01$.

significance was determined by analysis of variance (ANOVA) followed by Dunnett's *t*-test for multiple comparisons.

3. Results

3.1. Production of TNF-α, nitrite and prostaglandin E_2 in zymosan-stimulated RAW 264.7 cells

The effect of dysidotronic acid (Fig. 1) on TNF-α, NO and prostanoid generation on mouse macrophage cell line RAW 264.7 stimulated with zymosan was determined (Fig. 2). After 5 h of macrophage stimulation with zymosan, this compound inhibited TNF-α production dose-dependently with an IC_{50} value of 1.3 μM and after 18-h stimulation it inhibited nitrite and prostaglandin E_2 production, showing IC_{50} values of 1.7 and 1.1 μM, respectively. Dysidotronic acid did not affect the cellular viability, as assessed by mitochondrial reduction of MTT after 18-h treatment on mouse macrophage cell line RAW 264.7 ($E_{492\ nm}$ values of 0.914 ± 0.050 and 0.965 ± 0.040 for non-treated and dysotronic-treated cells, respectively) as well as on human neutrophils ($E_{492\ nm}$ values of 0.684 ± 0.024 and 0.657 ± 0.019 for non-treated and dysotronic-treated cells, respectively). This indicates that it was not cytotoxic.

3.2. Inducible NO synthase and cyclo-oxygenase-2 activity in intact cells

To establish whether the inhibition of nitrite and prostaglandin E_2 production was due to an interaction with the enzyme induction by zymosan or to a direct action of dysidotronic acid on inducible NO synthase and cyclo-oxygenase-2 activities, this compound as well as the reference compounds were added to cells which previously had expressed inducible NO synthase and cyclo-oxygenase-2 and were then incubated for 4 h in fresh culture medium supplemented with L-arginine and arachidonic acid. Dysidotronic acid, which produced a significant reduction in nitrite production (89%) in the induction phase, did not affect nitrite content in the post-induction phase, whereas the selective inducible NO synthase inhibitor 1400W caused a marked inhibition of nitrite production (Table 1). In contrast, dysidotronic acid produced a significant inhibition of prostaglandin E_2 production in both experimental conditions, with a profile similar to that of the selective cyclo-oxygenase-2 inhibitor NS398. In addition, dexamethasone, an inhibitor of inducible NO synthase and cyclo-oxygenase-2 gene expression, significantly modified the levels of both metabolites in the induction phase. This suggests that dysidotronic acid could display a dual inhibitory profile, acting as an inducible NO synthase expression inhibitor in addition to inhibiting prostaglandin E_2 production.

Table 1
Effect of dysidotronic acid, dexamethasone, NS-398 and 1400W on inducible NO synthase and cyclo-oxygenase-2 activities on macrophage RAW 264.7 cells. In the induction phase, cells were co-incubated with zymosan and test compounds for 18 h. In the post-induction phase, the cells were stimulated with zymosan for 18 h and after washing the cells, test compounds were added and incubated for 4 h in the presence of L-arginine (0.5 mM) and arachidonic acid (10 μM). N.D., not determined. Results are the mean ± S.E.M. of six independent experiments assayed in duplicate

	18-h treatment (induction phase)		4-h treatment (post-induction phase)	
	NO_2^- (ng/ml)	Prostaglandin E_2 (ng/ml)	NO_2^- (ng/ml)	Prostaglandin E_2 (ng/ml)
Non-stimulated cells	13.4 ± 2.6[a]	2.7 ± 0.3[a]	67.0 ± 3.6[a]	0.5 ± 0.1[a]
Zymosan-stimulated cells	308.8 ± 16.5	15.0 ± 0.7	253.0 ± 25.3	6.3 ± 0.5
Dysidotronic acid (10 μM)	46.0 ± 6.0[a]	6.7 ± 0.9[a]	245.6 ± 14.3	3.7 ± 0.6[a]
Dexamethasone (1 μM)	155.0 ± 7.3[a]	4.7 ± 0.3[a]	N.D.	N.D.
1400W (10 μM)	N.D.	N.D.	126.5 ± 18.4[a]	6.0 ± 0.6
NS-398 (1 μM)	N.D.	N.D.	225.9 ± 25.8	3.9 ± 0.8[b]

[a] $P < 0.01$ compared with zymosan-stimulated cells.
[b] $P < 0.05$ compared with zymosan-stimulated cells.

Table 2
Effect of dysidotronic acid on the 4-h zymosan-injected air pouch model of acute inflammation. Results are the mean ± S.E.M. ($n = 6$). Dysidotronic acid was administered at 100 nmol/pouch into the air pouch at the same time as zymosan. Myeloperoxidase activity is expressed as miliUnits of $E_{620\ nm}$

	Saline	Zymosan	Zymosan + dysidotronic acid
Migration (10^6 cells/ml)	2.3 ± 0.5[a]	21.9 ± 2.2	16.0 ± 4.3[b]
Myeloperoxidase ($E_{620\ nm}$)	65.0 ± 4.3[a]	367.5 ± 83.5	169.4 ± 13.3[b]
Leukotriene B_4 (ng/ml)	0.1 ± 0.1[a]	15.1 ± 1.4	9.8 ± 1.6[b]
Prostaglandin E_2 (ng/ml)	0.2 ± 0.1[a]	17.8 ± 1.5	15.0 ± 1.4
IL-1β (pg/ml)	30.0 ± 3.1[a]	5205.0 ± 423.0	3815.0 ± 387.0[b]
TNF-α (pg/ml)	2.1 ± 1.3[a]	146.4 ± 15.0	48.7 ± 9.6[a]

[a] $P < 0.01$ compared with zymosan control group.
[b] $P < 0.05$ compared with zymosan control group.

3.3. Mouse air pouch

To corroborate in vivo the inhibitory behaviour of this compound, we administered it into the pouch in the zymosan-injected mouse air pouch (Posadas et al., 2000). This model of experimental inflammation in mice is characterised by an early acute phase (4 h after zymosan administration) mediated mainly by eicosanoids, with high levels of prostaglandin E_2 produced by cyclo-oxygenase-1, and a second phase (12–24 h) with participation of NO and prostaglandin E_2 accompanied by co-induction of both inducible NO synthase and cyclo-oxygenase-2. Table 2 shows that dysodotronic acid, at 100 nmol/pouch in the early acute phase (4 h after zymosan administration), inhibited cell accumulation (30%) and consequently leukotriene B_4 production (30%) and myeloperoxidase content (65%). In addition, it also reduced interleukin-1β (27%) and TNF-α production (67%) but did not affect prostaglandin E_2 content during this time period. As shown in Table 3, in the 24-h zymosan-injected air pouch model of inflammation, this compound produced a surprising reduction in nitrite (53%) and prostaglandin E_2 content (44%) and showed a profile similar to that of dexamethasone. Neither dysidotronic acid nor dexamethasone affected cell accumulation at this time.

Table 3
Effect of dysidotronic acid on the 24-h zymosan-injected air pouch model of acute inflammation. Dysidotronic acid (DA) and dexamethasone (DEX) were administered at 100 and 10 nmol/pouch, respectively, into the air pouch at the same time as zymosan. An additional dose of dysidotronic acid or dexamethasone was injected into the air pouch 8 h after zymosan injection. Results are the mean ± S.E.M. ($n = 6$). Z, zymosan.

	Saline	Zymosan	Z + DA	Z + DEX
Migration (10^6 cells/ml)	1.3 ± 0.4[a]	21.2 ± 1.6	26.0 ± 2.2	21.5 ± 3.2
Nitrite (ng/ml)	0.0 ± 0.0[a]	58.4 ± 4.1	27.8 ± 4.9[b]	26.5 ± 3.7[a]
Prostaglandin E_2 (ng/ml)	0.3 ± 0.0[a]	33.2 ± 3.8	18.9 ± 4.0[b]	13.8 ± 2.8[a]

[a] $P < 0.01$ compared with zymosan control group.
[b] $P < 0.05$ compared with zymosan control group.

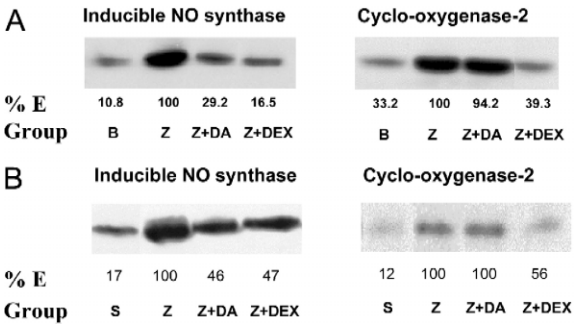

Fig. 3. Effect of dysidotronic acid on inducible NO synthase and cyclo-oxygenase-2 expression and densitometric analysis. (A) RAW 264.7 cells. The figure is representative of three similar experiments. B: normal cells. Z: zymosan-stimulated cells. Z + DA: zymosan-stimulated cells treated with dysidotronic acid at 10 μM. Z + DEX: zymosan-stimulated cells treated with dexamethasone at 1 μM. (B) Cells from exudates of zymosan-injected (24 h) mouse air pouch. The figure is representative of three similar experiments. S: saline-injected air pouch cells. Z: zymosan-injected air pouch cells. Z + DA: zymosan-injected air pouch cells treated with dysidotronic acid at 100 nmol/pouch. Z + DEX: zymosan-injected air pouch cells treated with dexamethasone at 10 nmol/pouch.

3.4. Inducible NO synthase and cyclo-oxygenase-2 protein expression

Western blot analysis for inducible NO synthase and cyclo-oxygenase-2 proteins using RAW 264.7 cells shows clearly that dysidotronic acid only inhibited inducible NO synthase expression (Fig. 3A), whereas dexamethasone, as expected, reduced the expression of both inducible proteins. These results were also confirmed when the cells from exudates of the 24-h zymosan-injected air pouch model of inflammation were used (Fig. 3B).

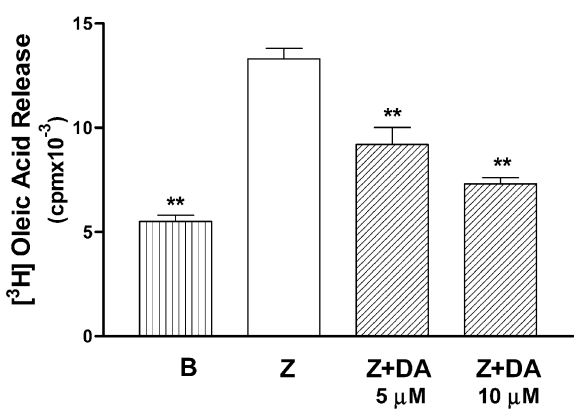

Fig. 4. Effect of dysidotronic acid on [^{3}H]oleic acid release in zymosan-activated RAW 264.7 macrophages. The figure is representative of three similar experiments. B: normal cells. Z: zymosan-stimulated cells. Z + DA: zymosan-stimulated cells treated with dysidotronic acid at 5 and 10 μM, respectively. $^{*}P < 0.05$, $^{**}P < 0.01$.

3.5. Cyclo-oxygenase-2 activity in broken cell preparations

To explain the reduction in prostaglandin E_2 production mediated by dysidotronic acid, we evaluated the potential enzymatic inhibitory effect on cyclo-oxygenase-2 using microsomes from lipopolysaccharide-activated RAW 264.7 cells as the enzyme source. This compound did not have any effect at this level (25.8 ± 1.8 and 28.6 ± 1.4 ng/ml of prostaglandin E_2 for control and dysidotronic group, respectively).

3.6. Measurement of extracellular fatty acid release

Fig. 4 shows that by labelling RAW 264.7 cells with [^{3}H]oleic acid, it was possible to show that this compound inhibited the release of this fatty acid in response to zymosan in a dose-dependent manner, thus producing a 76% fatty acid release reduction at 10 μM. It should be noted that this percentage coincides with that observed for prostaglandin E_2 reduction in this cell system (Fig. 2).

3.7. Myeloperoxidase release by human neutrophils

The influence of dysidotronic acid on the myeloperoxidase degranulation process was studied using human neutrophils. This molecule potently inhibited this cellular function in the nanomolar range. As seen in Fig. 5, the myeloperoxidase release mediated by stimulation of human neutrophils with either fMLP or PAF was reduced dose-dependently, showing IC_{50} values of 562 and 371 nM, respectively. In this regard, it is interesting to note that dysidotronic produced a reduction on myeloperoxidase

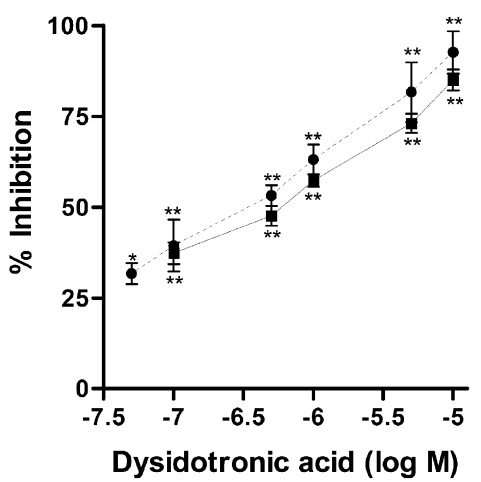

Fig. 5. Concentration–effect relationship for the inhibition by dysidotronic acid of myeloperoxidase release process on stimulated human neutrophil cells with 10 μM of cytochalasin B (CB) and 10 nM of fMLP (■), or 10 μM of CB and 5 μM of PAF (●). Results are the means ± S.E.M., $n = 6$–10. $^{*}P < 0.05$, $^{**}P < 0.01$ (non-stimulated neutrophils: $E_{620\ nm}$ values of 0.075 ± 0.007; CB + fMLP-stimulated neutrophils: $E_{620\ nm}$ values of 0.670 ± 0.025; CB + PAF-stimulated neutrophils: $E_{620\ nm}$ values of 0.907 ± 0.057).

levels in the 4-h zymosan-injected air pouch model of inflammation. Direct inhibitory effects on myeloperoxidase activity were not observed (data not shown).

4. Discussion

Manoalide can be considered a very useful pharmacological tool, and is by far the best known phospholipase A_2 inhibitor (Soriente et al., 1999). Mammalian cells contain diverse phospholipase A_2, which may play a distinct role in cell activation and signal transduction (Balsinde et al., 1999). Group II secretory phospholipase A_2 can act as a signalling agent that mediates cell growth induced by interleukin-1β (Wada et al., 1997) and contributes to the inflammatory response. This enzyme secreted at inflammatory sites becomes associated with cell surfaces and hydrolyzes phospholipids, thus releasing arachidonic acid, which enters the cell and participates in the increased generation of inflammatory lipid mediators (Pfeilschifter et al., 1993; Miyake et al., 1994). In fact, administration of different types of secretory phospholipase A_2 can induce inflammatory responses in animals (Vishwanath et al., 1988; Cirino et al., 1994). Interestingly, inflammatory cytokines increase group II phospholipase A_2 synthesis and secretion from rheumatoid synovial fibroblasts and other cell types (Pfeilschifter et al., 1993; Bomalaski and Clark, 1993) as well as other pro-inflammatory mediators like NO (Nègre-Salvayre and Salvayre, 1992). On the other hand, inflammatory cytokines are involved in the chronification of the inflammatory response through enzymatic induction of diverse inducible proteins such as inducible NO synthase and cyclo-oxygenase-2 (Klein et al., 1998; Cirino, 1998). Previous results indicate that dysidotronic acid is a novel inhibitor of human synovial phospholipase A_2, with a slight higher selectivity and potency for this enzyme than the reference inhibitor manoalide (Giannini et al., 2000). In chemical structure, dysidotronic acid is very closely related but lacks the dihydropyran ring in addition to some other chemical features. We have demonstrated that this compound inhibits dose-dependently cytokine production measured as TNF-α, NO determined indirectly by nitrite content and prostanoid generation measured as prostaglandin E_2 on mouse macrophage cell line RAW 264.7. On the other hand, it modified some leukocyte functions such as the myeloperoxidase degranulation process in human neutrophils. These results were confirmed in the mouse air pouch model of acute inflammation using zymosan as stimulus. It can be suggested that NO reduction was the consequence of inhibition of the expression of inducible NO oxide synthase, whereas prostaglandin E_2 reduction was not due to a direct inhibitory action on cyclo-oxygenase-2 or to an effect on expression. We attribute this behaviour to an inhibitory effect of dysidotronic acid on secretory phospholipase A_2 that would limit the

bioavailability of arachidonic acid. Whether dysidotronic acid inhibits inducible NO synthase gene expression by interaction of nuclear factors or by other mechanisms remains to be determined. It is interesting to note that manoalide has been reported as an inhibitor of cyclo-oxygenase-2 expression on human monocytes (Glaser and Lock, 1995). Recently, it has been described that cyclolinteinone, a manoalide-related compound, is able to down-regulate cyclo-oxygenase-2 and inducible NO synthase protein expression by blocking nuclear factor-kappaB activation in J774 macrophages (D'Acquisto et al., 2000).

Inducible NO synthase inhibition attenuates inflammatory response in different experimental models such as chronic granulomatous colitis in rats (Yamada et al., 1993), granuloma formation by subcutaneous implantation in rats of carrageenin-soaked sponges (Iuvone et al., 1994) and the zymosan air pouch model in mice (Posadas et al., 2000). We have also shown in this last model that inhibiting the enhanced expression of inducible NO synthase can control prostaglandin E_2 overproduction. In this regard, dysidotronic acid, by limiting iNOS expression, could attenuate prostaglandin E_2 production.

Of the anti-inflammatory drugs in use, the glucocorticoids constitute a group of potent agents for the treatment of chronic inflammation. This group is able to affect cytokine synthesis and consequently the expression of cyclo-oxygenase-2 and inducible NO synthase through the anti-inflammatory protein, lipocortin 1 (Goppelt-Struebe, 1997; Minghetti et al., 1999). At therapeutic concentrations, dexamethasone markedly inhibited NO-mediated cyclo-oxygenase-2 expression in synovial cells (Honda et al., 2000), suggesting that NO is an important modulator of cyclo-oxygenase-2 expression. In summary, the present study demonstrated that dysidotronic acid exerts inhibition of cytokine generation with a characteristic inhibitory profile on inducible NO synthase expression and prostaglandin E_2 production. Thus, this molecule could be of potential interest in the search for a new type of anti-inflammatory drug.

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