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An anti-inflammatory ditriazine inhibiting leukocyte functions and expression of inducible nitric oxide synthase and cyclo-oxygenase-2

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Abstract

A ditriazine derivative (4,10-dichloropyrido[5,6:4,5]thieno[3,2-*d*′:3,2-*d*]-1,2,3-ditriazine (DTD)) inhibited neutrophil functions, including degranulation, superoxide generation, and leukotriene B_4 production, without any effect on 5-lipoxygenase activity. This compound reduced nitric oxide (NO) and prostaglandin E_2 production in mouse peritoneal macrophages stimulated with lipopolysaccharide, whereas no influence on the activity of inducible NO synthase, cyclo-oxygenase-2 or cyclo-oxygenase-1 was observed. DTD significantly reduced mouse paw oedema induced by carrageenan and also markedly reduced NO and prostaglandin E_2 levels in exudates from 24-h zymosan-stimulated mouse air pouch. Western blot analysis showed that DTD reduced the expression of inducible NO synthase and cyclo-oxygenase-2. Our results indicate that DTD exerts anti-inflammatory effects related to the inhibition of neutrophil functions and of NO and prostaglandin E_2 production, which could be due to a decreased expression of inducible NO synthase and cyclo-oxygenase-2.

Keywords: Ditriazines; Nitric oxide (NO); Eicosanoid; Macrophage; Neutrophil; Air pouch, mouse; Carrageenan paw oedema

1. Introduction

Prostaglandins and nitric oxide (NO) are ubiquitous mediator systems exerting numerous vascular and inflammatory effects. Production of prostaglandins or NO by the constitutive isoenzymes, cyclo-oxygenase-1, or endothelial NO synthase, is implicated in the physiological regulation of vascular tone and homeostatic functions. In contrast, cyclo-oxygenase-2 and inducible NO synthase are not generally expressed in resting cells, but are induced following appropriate stimulation with pro-inflammatory agents such as cytokines and lipopolysaccharide in many cell types including macrophages (Salvemini et al., 1993; MacMicking et al., 1997). The activity of these inducible enzymes results in overproduction of prostaglandins and NO, which play a key role in the pathophysiology of arthritis and other inflammatory conditions (Kaur and Halliwell, 1994; Vane et al., 1994; Kang et al., 1996).

It is known that simultaneous production of NO and superoxide by phagocytic cells leads to formation of peroxynitrite, a very reactive species mediating tissue injury (Demiryurek et al., 1998; Kröncke et al., 1997). In addition, NO is also able to enhance the production of a variety of mediators, such as tumour necrosis factor-α and interleukin-1α, which participate in the macrophage-dependent inflammatory response (Marcinkiewicz et al., 1995).

On the other hand, neutrophils are essential for host defense and their contribution to the propagation and maintenance of acute and chronic inflammation includes several mechanisms. Activated neutrophils also release granule constituents (Smith, 1994; Salvemini et al., 1996) and produce leukotrienes, which participate in the inflammatory response through stimulation of leukocyte functions and regulation of smooth muscle tone and vascular permeability (Lewis et al., 1990; Henderson, 1994). Thus,

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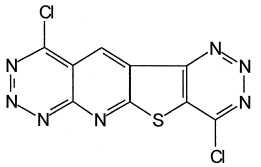

Fig. 1. Chemical structure of DTD.

the suppression of neutrophil functions could control the inflammatory response and has been implicated in the mechanism of action of some non-steroidal anti-inflammatory agents (Kankaanranta et al., 1994).

Little is known of the biological activity of 1,2,3-triazine derivatives and previous studies have focused mainly on their antifungal and antianaphylactic properties (Guerrera et al., 1993; Wagner et al., 1993). Nevertheless, there is increasing interest in the pharmacological potential of these types of heterocyclic compounds due to the recent finding by our group that a series of 8-cyanopyridothienotriazines were able to inhibit NO and prostaglandin E_2 synthesis in murine peritoneal macrophages stimulated with bacterial endotoxin (Quintela et al., 1999).

The present study was undertaken to examine the effects of a new ditriazine derivative, 4,10-dichloropyrido [5,6:4,5]thieno[3,2-*d*′:3,2-*d*]-1,2,3-ditriazine (DTD) (Fig. 1), on murine macrophage and human neutrophil functions as well as on several enzymes relevant to the inflammatory process. The results demonstrated the in vitro inhibitory effects on cell functions exerted by this compound, which also exhibited anti-inflammatory activity in vivo. This is the first report concerning the pharmacological properties of the compound.

2. Materials and methods

2.1. Preparation of human neutrophils

Venous blood was obtained, with informed consent, from healthy volunteers. Leukocytes were obtained and purified as previously was described (Bustos et al., 1995). Viability was greater than 95% according to the trypan blue exclusion test. 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 DTD on human neutrophils.

2.2. 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 $CaCl_2 \times 7H_2O$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 10 mM HEPES, 1 mM arginine, and 10 mM glucose) supplemented with 10% foetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and incubated at 37°C for 2 h. The adherent cells were used to perform the experiments described below. Cytotoxicity was assessed by the reduction of MTT (Gross and Levi, 1992).

2.3. Chemiluminescence

Neutrophils or peritoneal macrophages (2.5×10^6 cells/ml) were incubated with luminol (40 μM) or lucigenin (250 μM) and stimulated with 12-*O*-tetradecanoyl phorbol 13-acetate (TPA) 1 μM. Chemiluminescence was recorded with a Microbeta trilux counter (Wallac, Turku, Finland). Superoxide anions were also generated by the hypoxanthine/xanthine oxidase system to assess a possible direct scavenging activity of the test compound (Betts, 1985).

2.4. NADPH-oxidase activity

Fractions I and II from neutrophils were obtained as described by Thomas et al. (1993). 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).

2.5. 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 *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP, 10 nM) or platelet-activating factor (PAF) (0.5 μM) for 10 min. Elastase activity was estimated in supernatants, using *N*-*tert*-butoxy-carbonyl-L-alanine *p*-nitrophenyl ester (200 μM) as substrate and *p*-nitrophenol release was measured. Possible direct inhibitory effects on elastase activity were also assessed (Escrig et al., 1997).

2.6. Synthesis and release of leukotriene B_4 by human neutrophils

A suspension of human neutrophils (5×10^6 cells/ml) was preincubated with test compound or vehicle for 5 min and then stimulated with calcium ionophore A23187 (1 μM) for 10 min at 37°C. Leukotriene B_4 levels in supernatants were measured by radioimmunoassay (Moroney et al., 1988). High-speed ($100{,}000 \times g$) supernatants from sonicated human neutrophils were obtained and incubated under appropriate conditions with 10 μM arachidonic acid to assess 5-lipoxygenase activity (Tateson et al., 1988).

2.7. Nitrite and prostaglandin E_2 production in mouse peritoneal macrophages

Peritoneal macrophages (4×10^5/well) were incubated with *Escherichia coli* [serotype 0111:B4] lipopolysaccharide (10 μg/ml) at 37°C for 24 h in the presence of test compounds or vehicle. Nitrite and prostaglandin E_2 levels were determined in culture supernatants by a fluorimetric method (Misko et al., 1993) and by radioimmunoassay (Moroney et al., 1988), respectively. In another set of experiments, lipopolysaccharide-stimulated cells were collected to determine inducible NO synthase and cyclo-oxygenase-2 expression by Western blot analysis as described below.

2.8. Inducible NO synthase and cyclo-oxygenase-2 activity in intact cell

Twenty-four-hour lipopolysaccharide-stimulated macrophages (4×10^5/well) were washed and fresh medium supplemented with L-arginine (0.5 mM) and arachidonic acid (10 μM) was added for a further 2-h incubation with test compounds to assess the effects of compounds on induced enzyme activity. Supernatants were collected for the measurement of nitrite and prostaglandin E_2 accumulation for the last 2 h. Nitrite concentration, as reflection of NO released, was assayed fluorometrically and prostaglandin E_2 levels were assayed by radioimmunoassay.

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

Murine peritoneal macrophages stimulated with *E. coli* lipopolysaccharide (10 μg/ml) at 37°C for 24 h were collected and sonicated at 4°C in an ultrasonicator at maximum potency, microsomes were prepared by centrifugation at $2000 \times g$ for 5 min at 4°C followed by centrifugation of the supernatant at $100{,}000 \times g$ for 100 min at 4°C. Microsomes (40 μg protein/tube) were incubated for 30 min at 37°C in 50 mM Tris HCl, pH 7.4, with arachidonic acid (5 μM) 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 (Moroney et al., 1988).

2.10. Inducible NO synthase activity in broken cell preparations

High-speed ($100{,}000 \times g$) supernatants from peritoneal macrophages stimulated with *E. coli* lipopolysaccharide were obtained as described above. Aliquots of supernatants were used to determine NO synthase activity by monitoring the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline (Mitchell et al., 1991). Briefly, supernatants (100 μg protein/tube) were incubated at room temperature for 60 min with 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 adding 1.5 ml of a 1:1 suspension of DOWEX (50 W) in water. Radioactivity was measured in supernatants by liquid scintillation counting.

2.11. Cyclo-oxygenase-1 activity in human platelets microsomes

Human platelets were sonicated at 4°C in an ultrasonicator at maximum potency. Microsomes were prepared by centrifugation at $2000 \times g$ for 5 min at 4°C followed by centrifugation of the supernatant at $100{,}000 \times g$ for 100 min at 4°C. Microsomes (20 μg protein/tube) were incubated for 30 min at 37°C in 50 mM Tris HCl, pH 7.4, with arachidonic acid (5 μM) 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 tromboxane B_2 levels were determined by radioimmunoassay.

2.12. Phospholipase A_2 assay

Secretory phospholipase A_2 was assayed by using [^{3}H]oleate labeled membranes of *E. coli* (Franson et al., 1974). Bee venom and recombinant human synovial enzymes were used as sources of secretory phospholipase A_2. Cytosolic phospholipase A_2 was prepared from human monocytic U937 cells (Cell Collection, Department of Animal Cell Culture, C.S.I.C., Madrid, Spain) (Escrig et al., 1997). Cytosolic phospholipase A_2 activity was measured as the release of radiolabeled arachidonic acid according to the method of Clark et al. (1990).

2.13. Mouse air pouch

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 produced in female Swiss mice (25–30 g) as previously described (Edwards et al., 1981; Posadas et al., 2000). Six days after the initial air injection, 1 ml of sterile saline or 1 ml of 1% w/v zymosan in saline was injected into the air pouch. In the 4-h zymosan-stimulated air pouch, DTD (0.01, 0.1 and 1 μmol/pouch) was administered at the same time as zymosan. In the 24-h zymosan-stimulated air pouch, DTD (0.01,0.05, 0.1 and 0.2 μmol/pouch) or dexamethasone (0.01 μmol/pouch) was injected 1 h before and 8 h after zymosan injection. After 4 or 24 h, the

animals were killed by cervical dislocation, and the exudate in the pouch was collected. Leukocytes present in exudates were measured using a Coulter counter. After centrifugation of exudates, the 4-h supernatants were used to measure myeloperoxidase activity (Suzuki et al., 1983), as well as leukotriene B_4 and prostaglandin E_2 levels by radioimmunoassay. In the 24-h exudates, the supernatants were used to measure nitrite (Misko et al., 1993) and prostaglandin E_2 levels and the cell pellets were used to determine cyclo-oxygenase-2 and inducible NO synthase expression by Western blot analysis as described below. Protein was measured by the Lowry method using bovine serum albumin as standard.

2.14. Western blot analysis

Inducible NO synthase or cyclo-oxygenase-2 protein expression was studied in the cytosolic or microsomal fractions, respectively, from lipopolisaccharide-stimulated peritoneal macrophages and cell pellets obtained by centrifugation of air pouch exudates. Equal amounts of protein 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 (1/1000); for cyclo-oxygenase-2, membranes were incubated with specific anti-cyclo-oxygenase-2 polyclonal antiserum (1/1000). Both membranes were incubated with peroxidase-conjugated goat anti-rabbit Immunoglobulin G (IgG) (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 (ECL, Amersham Iberica, Madrid, Spain).

2.15. Carrageenan paw oedema

The anti-inflammatory activity of DTD was assessed by the carrageenan paw oedema test in mice according to the method of Sugishita et al. (1981). DTD (6.3, 12.5 and 25 mg/kg), indomethacin (5mg/kg), or vehicle (tween 80/saline 1:99, v/v) was administered intraperitoneally 1 h before injection of carrageenan (0.05 ml; 3% w/v in saline) into the subplantar area of the right hind paw. The volumes of injected and contralateral paws were measured at 1, 3 and 5 h after induction of oedema by using a plethysmometer (Ugo Basile, Comerio, Italy). The volume of oedema was expressed for each animal as the difference between the carrageenan-injected and contralateral paws. After the last determination of paw oedema (5 h), the animals were killed by cervical dislocation and the right hind paws were homogenized in 2 ml of saline. Aliquots of supernatants were used to determine prostaglandin E_2 levels and elastase activity as above. Stomachs were homogenized in 2 ml of methanol and the content of prostaglandin E_2 was measured in supernatants after centrifugation.

2.16. Materials

DTD was prepared by diazotation of 3,6-diamino-2,5-dicyano-thieno[2,3-b]pyridines according to modified procedures from the literature (Quintela et al., 1998). L-[2,3,4,5-^{3}H]arginine monohydrochloride, [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, and [5,6,8,9,11,12,14,15(*n*)-^{3}H]thromboxane B_2 were from Amersham Iberica (Madrid, Spain). [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). 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 rest of reagents were from Sigma Chem. (MO, USA). Human recombinant synovial phospholipase A_2 and antibody against leukotriene B_4 were kindly provided by Zeneca Pharmaceuticals, Macclesfield, Cheshire, UK; *E. coli* strain CECT 101 was a gift from Prof. Uruburu, Department of Microbiology, University of Valencia, Spain.

2.17. Statistical analysis

The results are presented as means ± S.E.M.; *n* represents the number of experiments. Inhibitory concentration 50% (IC_{50}) or inhibitory dose 50% (ID_{50}) values were calculated from at least four concentrations ($n = 6$). The level of statistical significance was determined by analysis of variance (ANOVA) followed by Dunnett's *t*-test for multiple comparisons.

3. Results

3.1. Chemiluminescence by human neutrophils and murine peritoneal macrophages

DTD inhibited in a concentration-dependent manner the chemiluminescence response induced by stimulation of neutrophils with TPA, with an IC_{50} value and confidence limits of 3.9 (1.4–5.5) and 4.4 (1.5–6.7) μM when luminol or lucigenin was used as substrate, respectively (Fig. 2). In contrast, DTD showed a weak inhibition of the chemiluminescence generated by the hypoxanthine/xanthine oxidase system ($22.9 \pm 3.5^{**}$% inhibition at 10 μM), indicating a poor scavenging effect on reactive oxygen species generated in a cell-free system. In addition, this compound did not inhibit NADPH-oxidase in prepara-

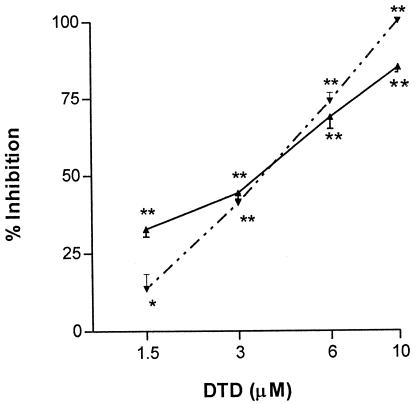

Fig. 2. Concentration–effect relationship for the inhibition by DTD of neutrophil activation: chemiluminiscence stimulated by TPA and measured with luminol (solid line) or lucigenin (dotted line). Data represent means ± S.E.M., $n = 6–10$. $^{*}P < 0.05$, $^{**}P < 0.01$.

tions of subcellular fractions of human neutrophils (data not shown). On the other hand, DTD also inhibited the chemiluminescence response in TPA-stimulated murine peritoneal macrophages, showing IC_{50} values of 1.4 (0.3–3.1) and 1.9 (1.6–2.2) μM for luminol and lucigenin, respectively. DTD did not cause cellular toxicity at the concentrations used as determined by reduction of MTT (data not shown).

3.2. Elastase release by human neutrophils

We assayed DTD 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} and 95% confidence limits were 1.7 (0.6–3.4) and 1.9 (0.6–2.9) μM, respectively (Fig. 3). Direct inhibitory effects on elastase activity were not observed (data not shown).

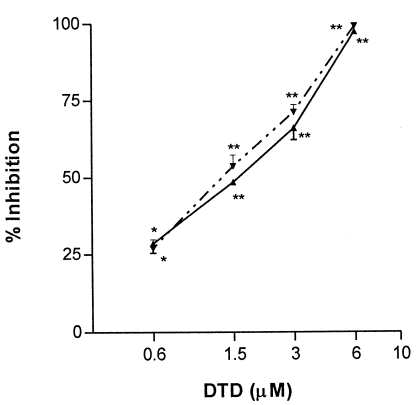

Fig. 3. Concentration–effect relationship for the inhibition by DTD of neutrophil activation: elastase release induced by cytochalasin B + fMPL (dotted line) or cytochalasin B + PAF (solid line). Data represent means ± S.E.M., $n = 6–10$. $^{*}P < 0.05$, $^{**}P < 0.01$.

3.3. Synthesis and release of leukotriene B_4 by human neutrophils

DTD at 10 μM completely abolished leukotriene B_4 release by human neutrophils stimulated with ionophore A23187. The concentration-dependent study showed an IC_{50} value of 2.9 (2.0–3.8) μM. Nevertheless DTD failed to modify leukotriene B_4 synthesis by high-speed supernatants from human neutrophils at concentrations up to 10 μM (data not shown). Thus, it appears that the reduction of leukotriene B_4 release by DTD in intact neutrophils is not due to direct inhibition of 5-lipoxygenase activity.

3.4. Production of nitrite and prostaglandin E_2 in stimulated mouse peritoneal macrophages

Incubation of 24-h lipopolysaccharide-stimulated mouse peritoneal macrophages with DTD caused a concentration-dependent inhibition of nitrite (as index of NO generation) and prostaglandin E_2 production. Table 1 shows the IC_{50} values of test compounds for nitrite and prostaglandin E_2, respectively. As expected, 1400W (selective inhibitor of inducible NO synthase activity) reduced nitrite levels and NS398 (cyclo-oxygenase-2 inhibitor) showed a high inhibitory potency on prostaglandin E_2 production, whereas dexamethasone inhibited both metabolites at nM concentrations. None of these compounds affected cellular viability, as assessed by mitochondrial reduction of MTT after 24 h (data not shown) indicating that they were not cytotoxic.

3.5. Inducible NO synthase and cyclo-oxygenase-2 activity in mouse peritoneal macrophages

The following experiments were designed to determine if the inhibition of nitrite and prostaglandin E_2 production in macrophages was due either to interference with enzyme induction or to direct inhibition of enzyme activities. Twenty-four-hour lipopolysaccharide-treated cells were washed and test products were added at 10 μM, followed by 2-h incubation in fresh culture medium supplemented

Table 1
IC_{50} values for inhibition of nitrite and prostaglandin E_2 accumulation in stimulated macrophages. Twenty-four-hour lipopolysaccharide-stimulated peritoneal macrophages produced 1018.0 ng nitrite/ml and 3.5 ng prostaglandin E_2/ml, compared to 90.5 ng nitrite/ml and 0.4 ng prostaglandin E_2/ml in untreated cells

Compound	IC_{50}^{a} nitrite	IC_{50}^{a} prostaglandin E_2
DTD	5.5 (4.6–6.6) μM	0.6 (0.5–1.3) μM
1400W	2.3 (1.3–3.2) μM	N.D.
NS 398	N.D.	3.1 (1.3–5.4) nM
Dexamethasone	35.8 (12.2–89.1) nM	1.0 (0.7–2.4) nM

[a] Values represent the concentration required to produce 50% inhibition of the response, along with the 95% confidence limits. N.D., not determined.

Table 2
Effect of DTD and enzyme inhibitors on inducible NO synthase and cyclo-oxygenase-2 activities in intact peritoneal macrophages after 24 h of lipopolysaccharide stimulation. All compounds were incubated at 10 μM for 2 h after the stimulation period
Data shown, means ± S.E.M. ($n = 6–9$). N.D., not determined. Basal: cells not stimulated with lipopolysaccharide.

	Inducible NO synthase (ng nitrite/ml)	Cyclo-oxygenase-2 (ng prostaglandin E_2/ml)
Basal	1.7 ± 0.3[b]	2.4 ± 0.2[b]
Control	46.6 ± 2.4	9.8 ± 0.6
DTD	45.6 ± 2.8	9.0 ± 1.0
1400W	17.2 ± 3.3[b]	N.D.
NS 398	N.D.	3.6 ± 0.4[b]

[b] $P < 0.01$.

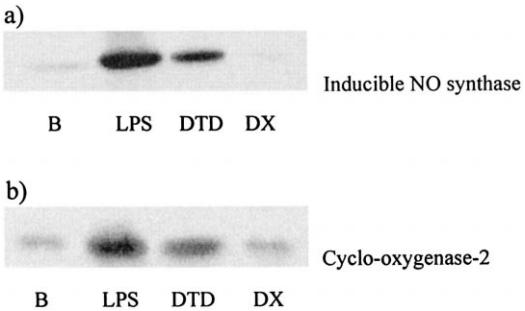

Fig. 4. Effect of ditriazine derivative (DTD) (10 μM) and dexamethasone (DX) (10 μM) on inducible NO synthase and cyclo-oxygenase-2 expression on 24-h lipopolysaccharide (LPS)-stimulated murine peritoneal macrophages. The figure is representative of three similar experiments. B (unstimulated cells).

with L-arginine and arachidonic acid. No significant reduction of either nitrite or prostaglandin E_2 levels was observed for DTD after this 2-h period (Table 2). Nevertheless, 1400W and NS398 caused a very significant reduction of nitrite (65%) and prostaglandin E_2 (63%) production, respectively.

3.6. Inducible NO synthase and cyclo-oxygenase-2 activity in broken cells preparations

To confirm the results obtained with intact cells, we examined the effects of this ditriazine derivative on inducible NO synthase and cyclo-oxygenase-2 activity in broken cell preparations (Table 3). DTD at 10 μM was inactive on all the enzymatic activities assayed. In contrast, 1400W and NS398 reduced significantly the production of citrulline (82% inhibition), and prostaglandin E_2 (58% inhibition), respectively, in these subcellular preparations.

3.7. Synthesis of thromboxane B_2 by human platelet microsomes

Synthesis of thromboxane B_2 by cyclo-oxygenase-1 present in microsomes from human platelets was significantly inhibited by the reference compound, indomethacin (79%), whereas DTD was inactive (Table 3), suggesting that this compound does not reduce prostaglandin E_2 generation by inhibition of cyclo-oxygenase-1 activity.

3.8. Phospholipase A_2 activity

To exclude the possibility that the effect on prostaglandin E_2 production could be related to interference with the release of arachidonic acid through phospholipase A_2 activities, DTD was assayed on different types of phospholipase A_2. This compound did not modify the amount of [^{3}H]oleic acid released from *E. coli* membranes by phospholipase A_2 activity belonging to groups II (human synovial) or III (bee venom) (data not shown). A lack of effect was also observed on cytosolic phospholipase A_2 activity obtained from human monocytic U937 cells (data not shown).

3.9. Inducible NO synthase and cyclo-oxygenase-2 protein expression in mouse peritoneal macrophages

Western blot assays of 24-h lipopolysaccharide-stimulated cells were performed to assess possible effects on inducible NO synthase or cyclo-oxygenase-2 protein expression. Fig. 4 shows a representative experiment where co-incubation of DTD at 10 μM with lipopolysaccharide

Table 3
Effect of DTD and enzyme inhibitors on inducible NO synthase and cyclo-oxygenase-2 activities in high speed supernatants or microsomes of 24-h lipopolysaccharide-stimulated macrophages, respectively, and on cyclo-oxygenase-1 activity in human platelet microsomes
Data shown, means ± S.E.M. ($n = 6–9$). N.D., not determined. All compounds were assayed at 10 μM.

	Inducible NO synthase (pmol citrulline/mg protein × min)	Cyclo-oxygenase-2 (ng prostaglandin E_2/mg protein)	Cyclo-oxygenase-1 (ng tromboxane B_2/mg protein)
Control	14.1 ± 1.0	16.6 ± 0.9	128.1 ± 5.1
DTD	13.8 ± 0.4	15.5 ± 1.8	122.4 ± 7.7
1400W	2.5 ± 0.4[b]	N.D.	N.D.
NS 398	N.D.	8.5 ± 0.8[b]	N.D.
Indomethacin	N.D.	N.D.	26.4 ± 1.2[b]

[b] $P < 0.01$.

Table 4
Effect of ditriazine derivative (DTD) (0.01–1.0 μmol/pouch) in the 4-h zymosan-stimulated air pouch
Data represent means ± S.E.M. ($n = 6$–12 animals).

Compound (μmol/pouch)	Leukocyte influx (10^6 cell/ml)	Myeloperoxidase (U/ml)	Prostaglandin E_2 (ng/ml)	Leukotriene B_4 (ng/ml)
Saline	3.6 ± 0.6[b]	42.2 ± 19.4[b]	0.5 ± 0.1[b]	0.9 ± 0.4[b]
Zymosan	23.9 ± 1.1	1522.0 ± 193.4	24.8 ± 1.4	56.5 ± 4.9
DTD (0.01)	20.5 ± 0.5	420.2 ± 79.0[b]	21.9 ± 2.3	33.6 ± 2.8[b]
DTD (0.1)	13.1 ± 1.1[b]	349.4 ± 28.7[b]	10.9 ± 0.4[b]	27.2 ± 2.8[b]
DTD (1.0)	7.2 ± 0.5[b]	230.3 ± 43.6[b]	7.8 ± 0.7[b]	22.1 ± 1.7[b]
Indomethacin (0.1)	18.9 ± 1.2	1127.8 ± 102.1	0.3 ± 0.0[b]	47.2 ± 0.6

[b] $P < 0.01$.

(10 μg/ml) for 24 h, caused a reduction in the expression of both isoenzymes. As expected, dexamethasone at 10 μM was very effective.

3.10. Mouse air pouch

As shown in Table 4, the number of leukocytes present in the pouch exudate collected 4 h after zymosan challenge was significantly reduced by intrapouch administration of DTD at 0.1 and 1 μmol/pouch. Prostaglandin E_2 levels were also reduced at the same doses. Myeloperoxidase activity and leukotriene B_4 levels were strongly reduced even at the lowest dose assayed (0.01 μmol/pouch), which was unable to affect prostaglandin E_2 levels and cell migration. Indomethacin, assayed as reference compound, potently reduced prostaglandin E_2 levels without affecting the other parameters measured.

After 24 h following zymosan injection, nitrite and prostaglandin E_2 levels were greatly increased in the mouse pouch exudates. Intrapouch administration of DTD at doses of 0.01, 0.05, 0.1 and 0.2 μmol/pouch, resulted in a dose-dependent reduction of both metabolites in the exudates (Fig. 5a) without affecting cell accumulation (data not shown). ID_{50} values were 0.080 μmol/pouch and

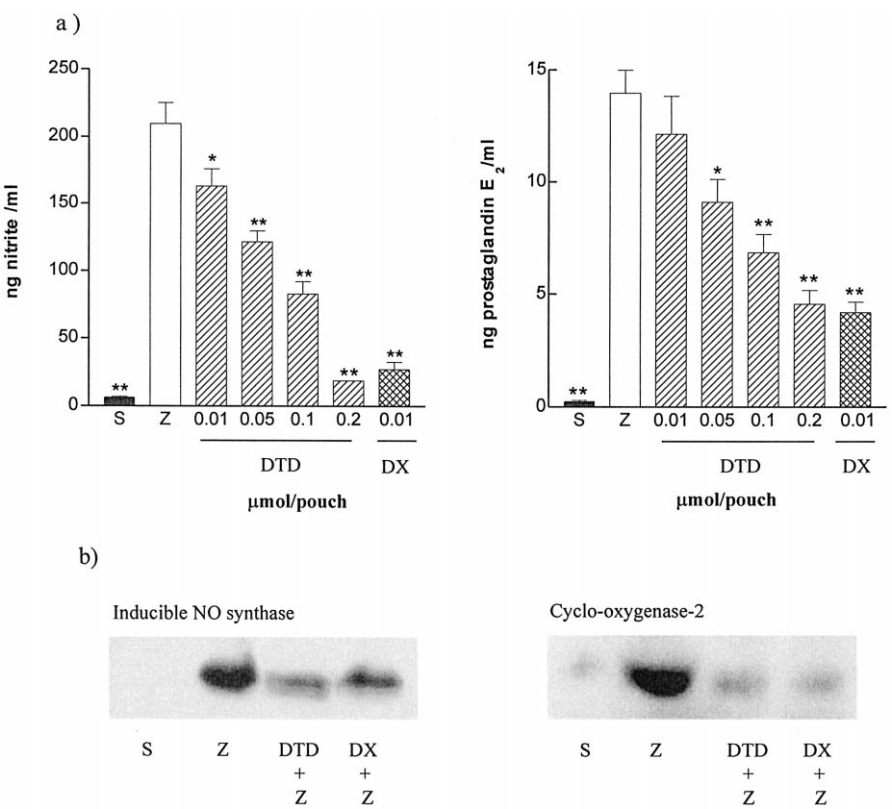

Fig. 5. Effect of ditriazine derivative (DTD) (0.01–0.2 μmol/pouch) and dexamethasone (DX) (0.01 μmol/pouch) in the 24-h zymosan-injected air pouch. Drugs were injected in the pouch at times indicated in Section 2. (a) Nitrite and prostaglandin E_2 levels in exudates. Data represent means ± S.E.M. ($n = 6$–12 animals); $^{*}P < 0.05$, $^{**}P < 0.01$. (b) Western blot analysis of inducible NO synthase and cyclo-oxygenase-2 protein expression in cells from exudates. Saline (S), zymosan (Z), zymosan + ditriazine (DTD + Z) (0.2 μmol/pouch), and zymosan + dexamethasone (DX + Z) (0.01 μmol/pouch). The figure is representative of three experiments.

0.129 μmol/pouch for nitrite and prostaglandin E_2, respectively. Dexamethasone was assayed as reference compound and at the dose of 0.01 μmol/pouch markedly reduced nitrite (87%) and prostaglandin E_2 (70%) levels.

Western blot analysis (Fig. 5b) for inducible NO synthase and cyclo-oxygenase-2 was carried out, respectively on cytosolic and microsomal fraction of leukocytes obtained by centrifugation of air pouch exudates from the 24 h assay. Zymosan injection caused a strong protein expression of inducible NO synthase and cyclo-oxygenase-2 with respect to saline-injected animals, whereas in animals treated with DTD (0.2 μmol/pouch) a clear decrease in the expression of both proteins was observed. As expected, dexamethasone potently inhibited the expression of both enzymes.

3.11. Carrageenan paw oedema

After i.p. administration, DTD caused a dose-dependent reduction in carrageenan-induced oedema at 3 and 5 h after induction of inflammation (Fig. 6). The greatest effect was observed at 3 h, with percent inhibitions of 47.5%, 70% and 80.1% at the doses of 6.3, 12.5 and 25 mg/kg, respectively. Indomethacin (5 mg/kg i.p.) was assayed as reference compound, showing a significant reduction in swelling at 3 (55.2%) and 5 h (42.3%) after the administration of carrageenan.

The last evaluation of oedema (5 h) was followed by killing of the animals and the paws injected with carrageenan were homogenized to determine the levels of elastase and prostaglandin E_2 (Table 5). The results indicate that elastase activity was significantly and dose dependently decreased by the three doses of DTD assayed. Indomethacin also significantly reduced elastase activity in homogenates of inflamed paws. This reference compound strongly reduced the levels of prostaglandin E_2 at the dose

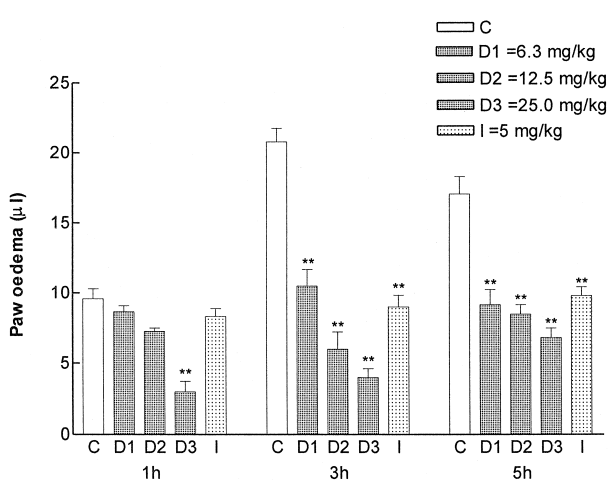

Fig. 6. Effect of ditriazine derivative (five-dotted square) and indomethacin (dotted square) on carrageenan mouse paw oedema, 1, 3 and 5 h after the induction of inflammation. Control (□). $^{*}P < 0.05$, $^{**}P < 0.01$ ($n = 6$–12 animals).

Table 5
Inhibition by ditriazine derivative (DTD) and indomethacin of elastase activity (control value 167.6 ± 8.6 nmol *p*-nitrophenol released/ml) and prostaglandin E_2 levels in homogenates of inflamed paws or stomachs (control values 95.8 ± 8.2 and 19.7 ± 1.7 ng/ml, respectively)
Data represent means ± S.E.M. ($n = 6$–12 animals).

Dose (mg/kg) i.p	% Inhibition		
	Inflamed paws		Stomachs
	Elastase	Prostaglandin E_2	Prostaglandin E_2
DTD (6.3)	34.9 ± 3.5[b]	14.9 ± 8.2	0.0 ± 0.0
DTD (12.5)	52.6 ± 5.3[b]	31.3 ± 5.9[a]	12.7 ± 2.0
DTD (25.0)	64.3 ± 2.5[b]	41.5 ± 2.8[b]	23.9 ± 1.4
Indomethacin (5.0)	45.0 ± 4.5[b]	97.2 ± 1.4[b]	83.4 ± 0.5[b]

[b] $P < 0.01$.
[a] $P < 0.05$.

assayed, whereas DTD reduced the levels of this prostanoid at the doses of 25 and 12.5 mg/kg. On the other hand, the content of prostaglandin E_2 in stomach homogenates (Table 5) was not significantly affected by the administration of DTD, in contrast to indomethacin, which clearly reduced the levels of this metabolite.

4. Discussion

It is generally accepted that 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 mediate tissue injury (Smith, 1994). Accordingly, inhibition of neutrophil functions can participate in the mechanism of action of a number of drugs, including some non-steroidal anti-inflammatory agents (Kankaanranta et al., 1994). In the present work, the respiratory burst elicited in human neutrophils by TPA was potently inhibited by DTD, showing a minor scavenging action in the cell-free system. DTD also reduced the degranulation induced by cytochalasinB + fMLP or cytochalasinB + PAF, as well as the leukotriene B_4 synthesis induced by ionophore A23187, thus exerting inhibitory effects on neutrophil functions triggered by structurally divergent agonists. These effects showed a good correlation with in vivo results obtained in the 4-h zymosan-stimulated mouse air pouch model. DTD reduced myeloperoxidase activity and leukotriene B_4 levels even at the lowest dose (0.01 μmol/pouch), that did not affect cell migration, indicating an inhibitory effect on degranulation and leukotriene B_4 synthesis. In contrast, prostaglandin E_2 levels were decreased only at doses inhibiting migration, with similar percentages of inhibition for both responses, suggesting that the reduction in this eicosanoid was due to a lower accumulation of cells in exudates. As a consequence, DTD may either prevent or

slow the progression of neutrophil-mediated tissue injury. In this respect, there are reports of the modification of neutrophil functions by some non-steroidal anti-inflammatory drugs in cells stimulated by chemotactic factors. These drugs may interact with the GTP/GDP exchange at a regulatory G protein, resulting in inhibition of signal transduction (Abramson et al., 1994). In contrast, our compound DTD, seems to affect cell activation at a site common to different signaling pathways as it inhibited responses induced by fMLP, PAF, TPA or ionophore A23187.

The induction of NO synthase and cyclo-oxygenase-2 greatly increases the synthesis of NO and prostaglandins, which contribute to the pathophysiology of various inflammatory processes. In addition, NO has been implicated in septic shock (Cobb and Danner, 1996). Inducible NO synthase inhibition results in modulation of the inflammatory response in different models such as subcutaneous granuloma formation in rats (Iuvone et al., 1994) and delayed paw swelling induced by carrageenan in mice (Ianaro et al., 1994). In humans, the production of NO by activated macrophages or neutrophils can be an index of bronchial inflammation and a mechanism for increasing asthmatic inflammation (Alving et al., 1993). Furthermore, NO has been shown, in in vitro and in vivo studies, to increase the production of pro-inflammatory prostaglandins (Salvemini et al., 1993, 1995). On the other hand, overproduction of prostaglandins by cyclo-oxygenase-2 expression in vivo has been reported for chronic inflammatory conditions such as rheumatoid arthritis (Kang et al., 1996) and experimental models of inflammation (Seibert et al., 1994; Vane et al., 1994). In the present study, we have shown that DTD inhibited the production of NO and prostaglandin E_2 in murine peritoneal macrophages stimulated by lipopolysaccharide. The inhibition was dose-dependent without any evidence of a cytotoxic effect. Nevertheless, this compound was ineffective when inducible NO synthase and cyclo-oxygenase-2 were already expressed. Western blot analysis of mouse peritoneal macrophages lysates showed that inducible NO synthase and cyclo-oxygenase-2 protein expression was reduced by the presence of DTD during lipopolysaccharide treatment, indicating that this compound inhibits the induction rather than the activity of both enzymes. This hypothesis was confirmed by the fact that DTD was inactive on inducible NO synthase and cyclo-oxygenase-2 activity in a cell-free system (broken cell preparations). In addition, DTD, at μM concentrations, did not modify the arachidonic acid pathway by a direct action on the activity of enzymes such as phospholipase A_2, 5-lipoxygenase, or cyclo-oxygenase-1.

We have recently investigated the participation of inducible NO synthase and cyclo-oxygenase-2 metabolites in the zymosan-stimulated mouse air pouch model of inflammation (Posadas et al., 2000). In the present work, we used two different sets of experiments, the 4-h zymosan-stimulated mouse air pouch to determine the acute response, and the 24-h zymosan-stimulated mouse air pouch, that allowed us to assess the effects of DTD on inducible NO synthase and cyclo-oxygenase-2. This compound exhibited an inhibitory behaviour well correlated with its in vitro effects on peritoneal macrophages. Nitrite and prostaglandin E_2 levels measured in exudates from 24-h zymosan-stimulated air pouch were inhibited dose dependently. Western blot analysis showed an inhibitory effect of DTD on protein levels for inducible NO synthase and cyclo-oxygenase-2 in the leukocytes migrating into the air pouch, indicating that the reduction of nitrite and prostaglandin E_2 in the late phase of this response was due to inhibition of enzyme expression.

In another model of inflammation, the mouse paw oedema induced by carrageenan, DTD also exerted potent inhibitory effects. Interestingly, we have shown that DTD reduced the elastase content in the inflamed paw, an index of migration, which correlated with the results obtained with the 4-h zymosan-stimulated mouse air pouch. In addition, the inhibition of cyclo-oxygenase-2 expression by DTD, demonstrated in vivo (24-h zymosan-stimulated mouse air pouch) and in vitro (mouse peritoneal macrophages stimulated with lipopolysaccharide), may account for the anti-inflammatory effects of this compound on mouse paw oedema, as evidenced by the observed reduction of prostaglandin E_2 levels in the inflamed paw.

NO or prostaglandin E_2 overproduction can be controlled by NO synthase or cyclo-oxygenase-2 inhibitors, respectively. Nevertheless, at the doses normally used they can also inhibit constitutive isoforms, which leads to detrimental effects. Drugs such as glucocorticoids, able to inhibit inducible NO synthase and cyclo-oxygenase-2 expression are potent anti-inflammatory agents (Goppelt-Struebe, 1997; Di Rosa et al., 1990). Our results indicate that DTD can control NO and prostaglandin E_2 overproduction by selective inhibition of the enhanced expression of both enzymes, thus providing a possible strategy in the treatment of inflammatory diseases.

On the other hand, DTD inhibited the oxidative burst in human neutrophils and murine peritoneal macrophages. Reactive oxygen species and reactive nitrogen intermediates have been implicated in the synthesis of different pro-inflammatory mediators and thus it is known that these species do not operate solely as end-stage effector molecules, but also as mediators regulating cytokine gene expression (Remick and Villarete, 1996). Besides, reactive oxygen species can participate in the activation of nuclear factors such as nuclear factor-κB (NF-κB) (Pinkus et al., 1996; Piette et al., 1997). This transcription factor is essential in the enhanced expression of inducible NO synthase (Xie et al., 1994) and cyclo-oxygenase-2 (Lo et al., 1998) genes in lipopolysaccharide-treated macrophages. Further studies are required to find if the inhibition of enzyme expression by DTD is related to an effect on the generation of reactive oxygen species and/or on the regulation of transcription factors such as NF-κB.

In summary, the present study demonstrated that DTD exerts acute anti-inflammatory effects that may be related to several mechanisms such as reduction of leukocyte activation and inhibition of inducible NO synthase and cyclo-oxygenase-2 expression. The profile and potency of this compound may have relevance for the inhibition of the inflammatory response, representing a new approach for the modulation of different inflammatory pathologies.

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