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A new chloroquinolinyl chalcone derivative as inhibitor of inflammatory and immune response in mice and rats

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Abstract

The synthetic chalcone derivative 1-(2,4-dichlorophenyl)-3-(3-(6,7-dimethoxy-2-chloroquinolinyl))-2-propen-1-one (ClDQ) was evaluated for its anti-inflammatory, analgesic and immunomodulatory efficacy in-vitro and in-vivo. ClDQ concentration-dependently inhibited the production of nitric oxide (NO) (IC50 4.3 μM) and prostaglandin E_2 (PGE_2) (IC50 1.8 μM) in RAW 264.7 macrophages stimulated with lipopolysaccharide. Human mononuclear cell proliferation was significantly inhibited by 10 μM ClDQ. Oral administration of ClDQ (10–30 mg kg^{-1}) in the 24-h zymosan-stimulated mouse air-pouch model produced a dose-dependent reduction of cell migration as well as NO and PGE_2 levels in exudates. ClDQ (20 mg kg^{-1}, p.o.) inhibited ear swelling and leucocyte infiltration in the delayed-type hypersensitivity response to 2,4-dinitrofluorobenzene in mice. In the rat adjuvant-arthritis model, this compound reduced joint inflammation as well as PGE_2 and cytokine levels. In addition, ClDQ displayed analgesic effects in the phenylbenzoquinone-induced abdominal constriction model in mice and in the late phase of the nociceptive response to formalin. Our findings indicated the potential interest of ClDQ in the modulation of some immune and inflammatory conditions.

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Introduction

Macrophages are important in non-specific host resistance to microbial pathogens and serve as central regulators of the specific immune response. Upon activation, they produce prostaglandins, nitric oxide (NO), and cytokines such as interleukin-1β (IL-1β) or tumour necrosis factor-α (TNF-α) together with other chemical mediators (Laskin & Pendino 1995). Prostaglandins are produced via a complex cascade regulated by two principal enzymes, phospholipase A_2 (PLA_2) and cyclo-oxygenase (COX). Constitutive COX-1 is responsible for basal production of prostaglandin E_2 (PGE_2), whereas inducible COX-2 catalyses the synthesis of high levels of this eicosanoid in the inflammatory response (Masferrer et al 1994). Thus, the overproduction of PGE_2 and NO by induction of COX-2 and inducible nitric oxide synthase (NOS-2), respectively, plays a key role in the pathophysiology of arthritis and other inflammatory conditions (Kaur & Halliwell 1994; Vane et al 1994; Kang et al 1996).

Rheumatoid arthritis is a systemic illness characterized by chronic inflammation, bone erosion and proliferation of the synovial tissue. This complex autoimmune disease is regulated by numerous mediators generated by different cell types including macrophages and lymphocytes. Rheumatoid synovial tissues also release cytokines, and express increased levels of COX-2, yielding large quantities of PGE_2 that mediates erosion and destruction of bone and cartilage (Choy & Panayi 2001; Anderson et al 1996).

Activated T lymphocytes contribute to the development and pathogenesis of rheumatoid arthritis and other autoimmune or allergic diseases (Holmdahl et al 1985). Their activation involves cell proliferation through multiple intracellular signalling pathways, interleukin-2 being the main lymphokine involved (Pimentel-Muiños et al 1994).

The chalcone skeleton has been considered the biological precursor of flavonoids. This family of natural products is composed of a large number of compounds possessing a wide array of biological effects including anti-inflammatory, analgesic and

antioxidant activities (Haraguchi et al 1998; Hsieh et al 2000). Our previous reports indicated that a series of synthetic 2-chloroquinolinyl chalcone derivatives exerted acute anti-inflammatory effects through the inhibition of NO and PGE_2 production in activated macrophages (Herencia et al 1998, 1999). This study has described the effect of 1-(2,4-dichlorophenyl)-3-(3-(6,7-dimethoxy-2-chloroquinolinyl))-2-propen-1-one (ClDQ), a new 2-chloroquinolinyl chalcone derivative (Figure 1), on some parameters related to macrophage and lymphocyte activation. In addition, anti-inflammatory, analgesic and immunosuppressive activities in several in-vivo models have been assessed.

Materials and Methods

Materials

1-(2,4-Dichlorophenyl)-3-(3-(6,7-dimethoxy-2-chloroquinolinyl))-2-propen-1-one was prepared by Claisen-Schmidt condensation according to previous procedures from the literature (Li et al 1995). [5,6,8,11,12,14,15(n)-^{3}H]PGE_2, [5,6,8,9,11,12,14,15(n)-^{3}H], [5,6,8,9,11,12,14,15(n)-^{3}H] thromboxane B_2 (TXB_2) and [methyl-^{3}H]thymidine were from Amersham Biosciences (Barcelona, Spain). [9,10-^{3}H] Oleic acid and 1-palmitoyl-2-[^{14}C]arachidonyl-*sn*-glycero-3-phosphocholine were purchased from Du Pont (Itisa, Madrid, Spain). N-(2-Cyclohexyloxy-4-nitrophenyl)-methanesulfonamide (NS398), palmityl trifluoromethyl ketone (PTK) and N-(3-(aminomethyl)benzyl)acetamidine dihydrochloride (1400W) were purchased from Cayman Chem. (Ann Arbor, MI). Anti-mouse TNF-α and IL-1β antibodies were from Immunokontact (Frankfurt, Germany). *Mycobacterium butyricum* was obtained from Difco Chem. (MI). All other reagents were from Sigma Chem. (St Louis, MO). *Escherichia coli* strain CECT 101 was a gift from Professor Uruburu, Department of Microbiology, University of Valencia, Spain.

Animals

Female Swiss mice (25–30 g) and female Lewis rats (175–200 g) (Harlan, Barcelona, Spain) were housed according to the guidelines of the Institutional Animal Care and Use Committee. All studies were performed in accordance with European Union regulations for the handling and use of laboratory animals.

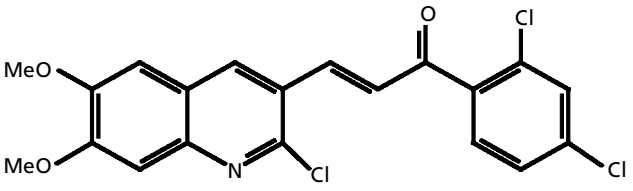

Figure 1 Chemical structure of 1-(2,4-dichlorophenyl)-3-(3-(6,7-dimethoxy-2-chloroquinolinyl))-2-propen-1-one (ClDQ).

Culture of murine macrophage RAW 264.7 cell line

RAW 264.7 macrophages (Cell Collection, Department of Animal Cell Culture, C.S.I.C., Madrid, Spain) were cultured in Dulbecco's Minimum Essential Medium containing 2 mM L-glutamine, 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin and 10% foetal bovine serum. Macrophages were removed from the tissue culture flask using a cell scraper and resuspended at a concentration of 2×10^6 mL^{-1}.

Nitrite and PGE_2 production in RAW 264.7 macrophages (induction phase)

RAW 264.7 macrophages (2×10^6 mL^{-1}) were co-incubated in 96-well culture plates (200 μL) with 1 μg mL^{-1} *Escherichia coli* (serotype 0111:B4) lipopolysaccharide (LPS) at 37 °C for 24 h in the presence of test compounds or vehicle. Nitrite (as index of NO generation) and PGE_2 levels were determined in culture supernatants by a fluorometric method (Misko et al 1993) and by radioimmunoassay (Moroney et al 1988), respectively. Cytotoxicity was assessed by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Gross & Levi 1992).

NOS-2 and COX-2 activity in intact RAW 264.7 macrophages (post-induction phase)

RAW 264.7 macrophages (2×10^6 mL^{-1}) were stimulated with LPS in the absence of test compounds. After 24 h, cells were washed and fresh medium supplemented with L-arginine (0.5 mM) and arachidonic acid (5 μM) was added for 2-h incubation in the presence of test compounds. Supernatants were collected for the measurement of nitrite and PGE_2 accumulation for the last 2 h. Nitrite and PGE_2 levels were assayed as above.

COX-1 activity in human platelet microsomes

Human platelets were sonicated at 4 °C in an ultrasonicator at maximum potency. Microsomes were prepared by centrifugation at 2000 *g* for 5 min at 4 °C followed by centrifugation of the supernatant at 100 000 *g* for 100 min at 4 °C. Microsomes (20 μg 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 haematin and 1 mM L-tryptophan (Brownlie et al 1993). The reaction was stopped by boiling the samples for 5 min, and TXB_2 levels were determined by radioimmunoassay.

Assay of $sPLA_2$

Secretory phospholipase A_2 ($sPLA_2$) was assayed by using a modification of the method of Franson et al (1974). Bee venom enzyme was diluted in 10 μL 100 mM Tris-HCl, 1 mM $CaCl_2$ buffer pH 7.5, and pre-incubated at 37 °C for 5 min with test compound or vehicle in a final volume of 250 μL. Incubation proceeded for 15 min in the

presence of 10 μL [^{3}H]oleic-*E. coli* membranes and was terminated by addition of 100 μL ice-cold solution 0.25 % bovine serum albumin (BSA) in saline to a final concentration of 0.07% w/v. After centrifugation at 2500 *g* for 10 min at 4 °C, the radioactivity in the supernatants was determined by liquid scintillation counting.

Assay of $cPLA_2$

Cytosolic phospholipase A_2 ($cPLA_2$) was determined from cytosolic fraction of sonicated RAW 264.7 macrophages. Enzymatic activity was measured as the release of radiolabelled arachidonic acid according to the method of Clark et al (1990). The substrate consisted of 5 μL micelles (10^4 cpm) containing 1-palmitoyl-2-[^{14}C]arachidonyl-*sn*-glycero-3-phosphocholine.

Isolation and proliferative response of human lymphocytes

Peripheral blood mononuclear cells were isolated from buffy coats obtained from healthy blood donors by Ficoll-Paque density gradient centrifugation (Boyum 1976). The mononuclear cells interphase was carefully aspirated and washed twice in saline. Cells were resuspended in RPMI-1640 media supplemented with 2 mM L-glutamine, 15 mM $NaHCO_3$, 10 mM HEPES, 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin with 10% foetal bovine serum. The cell suspension was placed in culture Petri dishes and after 1-h incubation non-adherent cells were collected and cultured in 96-well tissue culture plates in a volume of 200 μL/well (1×10^6 cells mL^{-1}) in the presence of 12 μg mL^{-1} phytohaemagglutinin. Compounds or vehicle (1% methanol) were added before mitogen stimulus. Cells were cultured for 72 h at 37 °C in a humidified atmosphere containing 5% CO_2 and then pulsed for 18 h with [methyl-^{3}H]thymidine (0.08 μCi/well). Cells were harvested and thymidine incorporation measured with a Microbeta trilux counter (Wallac, Turku, Finland). The cytotoxicity of ClDQ was assessed using the lactate dehydrogenase release assay (Babson & Phillips 1965).

2,4-Dinitro-1-fluorobenzene-induced delayed type hypersensitivity (DNFB-DTH)

The sensitization phase was induced by topical application of 20 μL 0.2% (v/v) DNFB in acetone onto the shaved abdomen of Swiss female mice on days 0 and 1 (total 80 μg). Five days after the initial sensitization, animals were challenged with 0.2% DNFB (10 μL) on both sides of right and left ears (Góngora et al 2000). ClDQ (20 mg kg^{-1}) or dexamethasone (2 mg kg^{-1}) was administered orally in propylene glycol/distilled water (1:1 v/v) for three consecutive days (72, 48 and 24 h) before DNFB challenge. The ear swelling reaction was assessed 24 h after challenge by measuring ear thickness with a micrometer. The oedema was calculated for each ear as the difference in thickness before and 24 h after challenge. Animals were killed by cervical dislocation and ear sections were homogenized with 750 μL hexadecyltrimethylammonium bromide (0.5%) in phosphate-buffered saline (PBS) pH 5.4. After centrifugation at 10 000 *g* for 15 min at 4 °C, myeloperoxidase activity was measured in supernatants (Suzuki et al 1983).

24 h-Zymosan stimulated mouse air-pouch

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 sterile saline (untreated animals) or 1% w/v zymosan in saline was injected into the air-pouch. ClDQ (10, 20 and 30 mg kg^{-1}), dexamethasone (3 mg kg^{-1}) or vehicle (propylene glycol/distilled water (1:1, v/v)) was orally administered 1 h before and 8 h after zymosan injection. After 24 h, the animals were killed by cervical dislocation and the exudate in the pouch was collected. Leucocytes present in exudates were measured using a Coulter counter. After centrifugation of exudates, the supernatants were used to measure nitrite and PGE_2 levels as above.

Adjuvant arthritis

Adjuvant arthritis was elicited in female Lewis rats (175–200 g) by injection of 0.1 mL *Mycobacterium butyricum* (10 mg mL^{-1}) in mineral oil into the base of the tail (Taurog et al 1988). Paw volumes were measured at the beginning of the experiment by using a water displacement plethysmometer (Ugo Basile, Comerio, Italy). Animals were housed in propylene cages with food and water freely available. The light cycle was automatically controlled (on 0700 h; off 1900 h) and the room temperature thermostatically regulated to 21 ± 1 °C. The magnitude of the inflammatory response was evaluated by measuring the volume of both paws at day 20. Animals with paw oedema volumes 1.1 mL larger than normal paws were then randomized into treatment groups. One millilitre of ClDQ (20 mg kg^{-1}), dexamethasone (2 mg kg^{-1}) or vehicle (propylene glycol, distilled water: 1:1, v/v) was administered orally once daily and the oedema in paws was measured on days 20–28. Serum was collected on the last day of the experiment (day 28) for the determination of TXB_2. After death, paws from arthritic treated groups were amputated above the ankle and homogenized in 2.5 mL saline. After centrifugation at 10 000 *g* for 15 min at 4 °C, supernatants were used for the determination of PGE_2 (radioimmunoassay) and TNF-α and IL-1β levels (time resolved fluoroimmunoassay) (Pennanen et al 1995). Stomachs were homogenized in 2.0 mL methanol and samples of supernatants were used to determine the PGE_2 content.

Phenyl-*p*-benzoquinone-induced abdominal constrictions

Swiss mice were injected intraperitoneally with 0.2 mL 0.02% (w/v) solution phenyl-*p*-benzoquinone in ethanol/distilled water (5:95 v/v). The irritant induced a series of abdominal constrictions and hind limb extensions, which

were counted for a 20 min period commencing immediately after irritant injection. Vehicle (propylene glycol, distilled water: 1:1, v/v) or drugs were administered orally 1 h before phenyl-*p*-benzoquinone in a volume of 0.5 mL.

Formalin-induced pain

Female Swiss mice (25–30 g) received a subplantar injection of formalin (2.5% v/v in PBS pH 7.3, 20 μL) in one hindpaw and the duration of paw licking was monitored in the periods 0–5 min (early phase) and 15–30 min (late phase) (Hunskaar & Hole 1987). Vehicle (propylene glycol, distilled water: 1:1, v/v), ClDQ (20 mg kg^{-1}) or indometacin (5 mg kg^{-1}) was administered orally 1 h before formalin injection.

Hot-plate test

Groups of eight to twelve female Swiss mice were used. The reaction time (s) of each animal was measured in a hot-plate apparatus (Socrel, Milano, Italy) maintained at 55 ± 0.5 °C. Animals were placed on the heated surface 1 h after oral administration of products and removed immediately after they licked the footpad of any paw. The time spent on the hot plate (reaction time) was recorded.

Statistical analysis

The results are presented as means ± s.e.m.; n represents the number of experiments. Inhibitory concentration 50% (IC50) values and their 95% confidence limits were calculated from at least four concentrations (n = 6). The level of statistical significance was determined by analysis of variance followed by Dunnett's *t*-test for multiple comparisons.

Results

Production of nitrite and PGE_2 in 24 h LPS-stimulated RAW 264.7 macrophages

Co-incubation with ClDQ (10 μM) and LPS for 24 h strongly reduced nitrite and PGE_2 production in RAW 264.7 macrophages (Table 1). The IC50 values were 4.3 (2.8–6.9) μM and 1.8 (1.3–2.2) μM for nitrite and PGE_2, respectively. The reference compound 1400W (selective inhibitor of NOS-2) reduced nitrite levels, with an IC50 of 2.3 (1.3–3.2) μM. NS398 (COX-2 inhibitor) showed a high inhibitory potency on PGE_2 production, with an IC50 of 3.0 (1.1–5.0) nM. None of these compounds affected cellular viability, as assessed by the mitochondrial reduction of MTT after 24 h, indicating that they were not cytotoxic (data not shown).

NOS-2 and COX-2 activity in intact RAW 264.7 macrophages

The following experiments were designed to determine if the inhibition of nitrite and PGE_2 production in macrophages was due to direct inhibition of enzyme activities. Twenty-four-hour LPS-treated cells were washed and test products were added at 10 μM, followed by 2-h incubation in fresh culture medium supplemented with L-arginine and arachidonic acid. No significant reduction of either nitrite or PGE_2 levels was observed for ClDQ after this 2-h period (Table 1). Nevertheless 1400W and NS398 caused a very significant reduction of nitrite and PGE_2 production, respectively.

Synthesis of TXB_2 by human platelet microsomes

As shown in Table 2, synthesis of TXB_2 by COX-1 present in microsomes from human platelets was significantly inhibited by the reference compound, indometacin (79%), whereas ClDQ was inactive.

$sPLA_2$ and $cPLA_2$ activities

ClDQ did not modify the amount of [^{3}H]oleic acid released from *E. coli* membranes by $sPLA_2$ activity belonging to group III (bee venom). A lack of effect was also observed on $cPLA_2$ activity obtained from RAW 264.7 cell line (Table 2). In contrast, the reference compounds, scalaradial ($sPLA_2$ inhibitor) and palmityl trifluoromethyl ketone ($cPLA_2$ inhibitor) strongly inhibited these enzyme activities.

Table 1 Effect of ClDQ and reference compounds on nitrite and PGE_2 production in RAW 264.7 cells.

	Induction phase (24-h treatment)		Post-induction phase (2-h treatment)	
	Nitrite (ng mL^{-1})	PGE_2 (ng mL^{-1})	Nitrite (ng mL^{-1})	PGE_2 (ng mL^{-1})
Basal	48.8 ± 4.5**	5.2 ± 0.1**	8.4 ± 0.2**	3.4 ± 0.1**
Control	426.2 ± 15.1	50.0 ± 3.8	115.8 ± 10.2	40.2 ± 3.8
ClDQ	127.9 ± 2.4**	10.4 ± 0.8**	89.1 ± 11.4	32.4 ± 7.9
1400W	51.1 ± 0.1**	N.D.	10.8 ± 0.1**	N.D.
NS398	N.D.	7.2 ± 0.2**	N.D.	8.1 ± 0.2**

In the induction phase, cells were co-incubated with LPS and test compounds for 24 h. In the post-induction phase, cells were stimulated with LPS for 24 h and after washing, test compounds were added and incubated for 2 h in the presence of L-arginine (0.5 μM) and arachidonic acid (5 μM). Data are the mean ± s.e.m. (n = 6–9). **$P < 0.01$ compared with the control group. N.D., not determined. Compounds were assayed at 10 μM.

Table 2 Effect of ClDQ and reference compounds on COX-1, $sPLA_2$ and $cPLA_2$ activities.

	COX-1 TXB_2 ($ng\,mL^{-1}$)	$sPLA_2$ (pmol oleic acid (mg protein)$^{-1}$ min^{-1})	$cPLA_2$ (pmol AA (mg protein)$^{-1}$ min^{-1})
Basal	43.0 ± 1.2**	105.3 ± 2.6**	4.5 ± 0.1**
Control	142.5 ± 8.9	367.1 ± 10.4	12.9 ± 1.4
ClDQ	140.7 ± 3.9	366.8 ± 5.4	10.3 ± 0.4
Indometacin	88.3 ± 3.3**	N.D.	N.D.
Scalaradial	N.D.	114.3 ± 1.2**	N.D.
PTK	N.D.	N.D.	3.4 ± 0.7**

Data are expressed as mean ± s.e.m. (n = 6–9). **$P < 0.01$ compared with the control group. Compounds were assayed at 10 μM. N.D., not determined; PTK, palmityl trifluoromethyl ketone; AA, arachidonic acid.

Immunosuppresive activity on mitogen-stimulated lymphocytes

ClDQ significantly inhibited (43% at 10 μM) the phytohaemagglutinin-induced [^{3}H]thymidine incorporation into human lymphocytes (Table 3). Inhibition of lymphocyte proliferation was not due to cytotoxic effects of ClDQ, since viability, assessed by the lactate dehydrogenase assay, was not decreased after co-incubation of cells with this compound.

DNFB-DTH response

We selected this animal model to assess the possible immunosuppressive effect of ClDQ in-vivo. As shown in Table 3, administration of ClDQ (20 $mg\,kg^{-1}$) to mice for three consecutive days before DNFB challenge significantly inhibited ear swelling at 24 h. In addition, myeloperoxidase activity, determined in ear homogenates as leucocyte infiltration index, was strongly reduced. The reference compound dexamethasone (2 $mg\,kg^{-1}$, p.o.) suppressed both parameters also.

Mouse air-pouch

After 24 h following zymosan injection, nitrite and PGE_2 levels were greatly increased in the mouse pouch exudates. Oral treatment of mice with ClDQ resulted in a dose-dependent reduction of both metabolites in the exudates (Figure 2a and b). Cell accumulation was reduced at the doses assayed also (Figure 2c). The reference compound dexamethasone (3 $mg\,kg^{-1}$, p.o.) significantly reduced cell migration (60% inhibition) as well as nitrite and PGE_2 levels (86% and 76%, respectively).

Adjuvant-induced arthritis

As can be seen in Figure 3, oral administration of ClDQ (20 $mg\,kg^{-1}$) on days 20–28 after adjuvant injection significantly reduced paw oedema as did dexamethasone (2 $mg\,kg^{-1}$). Paw swelling was reduced in the ClDQ-treated animals by 58% relative to the paw volume of vehicle-treated animals. The levels of inflammatory mediators (TNF-α, IL-1β, and PGE_2) in paw homogenates were significantly inhibited by this compound, whereas TXB_2 in serum and PGE_2 in stomach were unaffected (Table 4). It should be noted that rats treated with ClDQ exhibited an important protection on weight loss when compared with the respective control group or with the reference dexamethasone group, even though these results were not statistically significant (data not shown).

Phenyl-*p*-benzoquinone-induced abdominal constriction in mice

Administration of phenyl-*p*-benzoquinone (0.02%) to mice resulted in 36.8 ± 2.2 abdominal constrictions in the

Table 3 Immunomodulatory effect of ClDQ in human lymphocytes and in DNFB-DTH response in mice.

	Human lymphocytes [^{3}H]Thymidine incorporation (cpm)	DNFB-DTH response in mice	
		Δ Ear thickness (μm)	Myeloperoxidase ($U\,mL^{-1}$)
Control	14476 ± 374	260.0 ± 11.0	457.1 ± 12.2
ClDQ	8264 ± 439**	197.2 ± 17.5**	210.3 ± 30.5**
Dexamethasone	2895 ± 189**	150.0 ± 9.3**	100.4 ± 10.6**

Data are expressed as mean ± s.e.m. (n = 6–9). **$P < 0.01$ compared with the control group. In the human lymphocyte assay, compounds were assayed at 10 μM. In the DNFB-DTH response, vehicle, ClDQ (20 $mg\,kg^{-1}$) or dexamethasone (2 $mg\,kg^{-1}$) were administered orally 72, 48 and 24 h, respectively, before DNFB challenge.

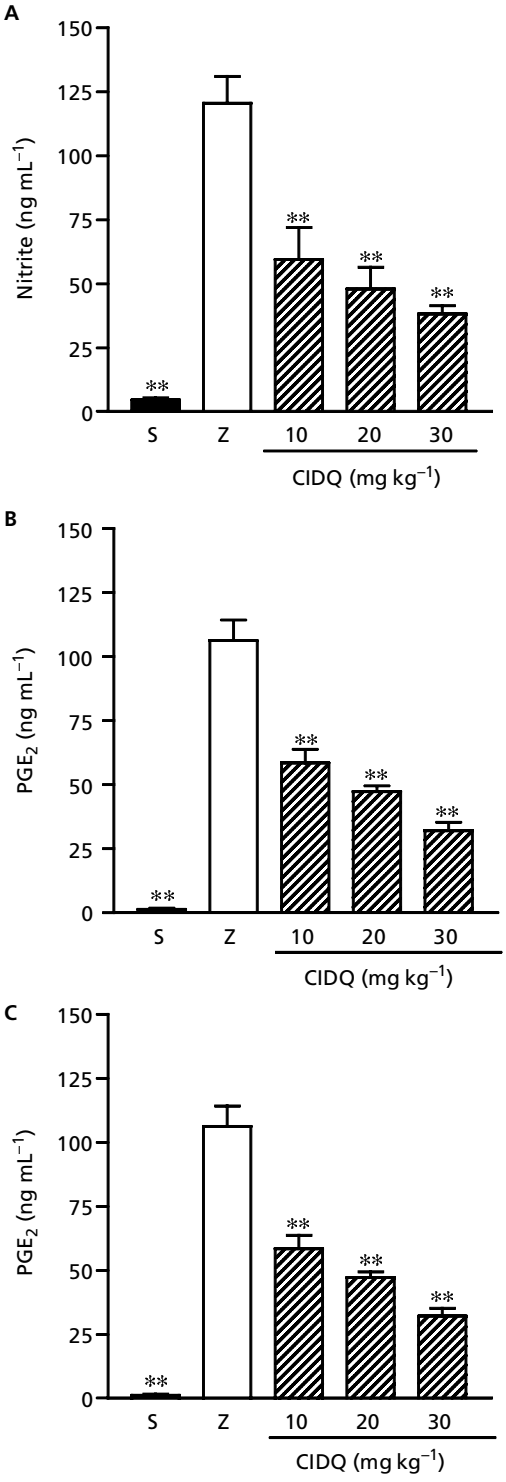

Figure 2 Effect of ClDQ in the 24-h zymosan-injected air-pouch. ClDQ was administered orally 1 h before and 8 h after zymosan injection. Nitrite (A), PGE_2 (B), and leucocyte influx (C), were measured in exudates. S, saline, Z, zymosan. Data represent mean ± s.e.m. (n = 6–12 animals), $^{**}P < 0.01$.

control group. As shown in Table 5, ClDQ displayed a clear inhibitory effect (57.8%) at the dose assayed

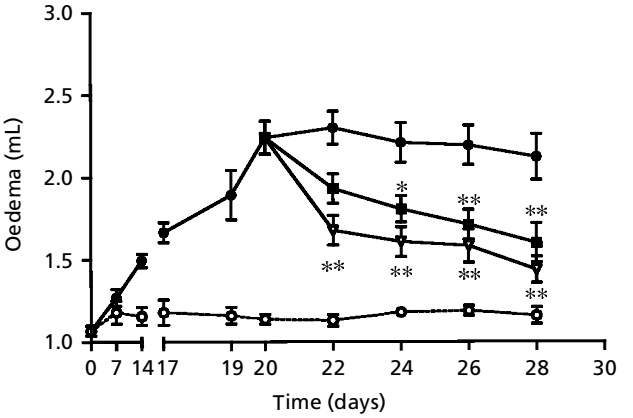

Figure 3 Effect of ClDQ (20 mg kg^{-1}, p.o.) and dexamethasone (2 mg kg^{-1}, p.o.) on the development of adjuvant-induced arthritis in female Lewis rats. Animals were injected with *Mycobacterium butyricum* on day 0 and arthritic rats were randomized into treatment groups on day 20. Compounds were administered once daily on days 20–28 and the oedema in both paws was measured every day to assess the magnitude of the inflammatory response. Non-arthritic group animals, ○; control rats, ●; ClDQ, ■; dexamethasone, ▽. Data are mean ± s.e.m. (n = 6–12 animals). $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the vehicle-treated arthritic rats (control).

(20 mg kg^{-1}, p.o.). The reference compound indometacin showed a significant inhibition after oral administration at 5 mg kg^{-1}.

Formalin-induced pain

Formalin induces a nociceptive response in the mouse paw involving an early phase lasting the first few minutes and a late phase lasting from 20 to 30 min after the injection. Treatment with ClDQ (20 mg kg^{-1}, p.o.) and indometacin (5 mg kg^{-1}, p.o.) reduced the duration of licking during the late phase response following injection of formalin, whereas the early phase was unaffected by both compounds (Table 5).

Hot-plate test

In this test, reaction time was measured as the time taken for mice to lick their paws on a hot plate at 55 °C. Responses of animals treated orally with 20 mg kg^{-1} ClDQ (9.0 ± 0.3 s) did not differ from those of the control group (8.2 ± 1.1 s). In contrast, administration of codeine at the same dose produced a significant increase in reaction time (14.1 ± 1.4 s).

Discussion

The induction of NOS-2 and COX-2 by LPS and other inflammatory stimuli greatly increases the synthesis of NO and prostaglandins, which contribute to the pathophysiology of various inflammatory processes (Seibert et al 1994; Vane et al 1994; Kang et al 1996). In this study we have shown that ClDQ inhibited the production of NO and

Table 4 Effect of ClDQ and dexamethasone on eicosanoid and cytokine level in rat adjuvant-induced arthritis.

	Paws			Stomach	Serum
	PGE_2 ($ng\,mL^{-1}$)	IL-1β ($pg\,mL^{-1}$)	TNF-α ($ng\,mL^{-1}$)	PGE_2 ($ng\,mL^{-1}$)	TXB_2 ($ng\,mL^{-1}$)
Normal	28.8 ± 2.1**	—	—	5.8 ± 4.5**	94.7 ± 8.3**
Control	86.2 ± 9.7	309.6 ± 53.1	23.6 ± 1.2	43.4 ± 3.5	370.7 ± 19.3
ClDQ	40.6 ± 7.4**	161.6 ± 8.6**	20.5 ± 0.9*	41.8 ± 2.5	351.8 ± 30.3
Dexamethasone	34.8 ± 2.1**	150.6 ± 14.3**	13.1 ± 0.1**	25.4 ± 3.4**	105.6 ± 7.6**

Results are the mean ± s.e.m. (n = 6 animals per group). *$P < 0.05$, **$P < 0.01$ compared with the arthritic group. The samples were collected on the last day of the experiment (day 28). Vehicle, ClDQ ($20\,mg\,kg^{-1}$) or dexamethasone ($2\,mg\,kg^{-1}$) were administered orally once daily on days 20–28. Normal is the non-adjuvant injected group of animals.

Table 5 Antinociceptive effect of ClDQ and indometacin in phenyl-*p*-benzoquinone (PBQ)-induced pain and formalin-induced licking in mice.

	PBQ induced pain (writhings)	Formalin-induced licking	
		Early phase (s)	Late phase (s)
Control	36.8 ± 2.0	90.8 ± 6.4	140.6 ± 11.4
ClDQ	15.3 ± 2.8**	80.0 ± 8.2	84.8 ± 7.3**
Indometacin	4.5 ± 0.9**	85.3 ± 7.9	80.0 ± 6.3**

Data are expressed as mean ± s.e.m. (n = 6–9 animals). **$P < 0.01$ compared with the control group. Vehicle, ClDQ ($20\,mg\,kg^{-1}$) or indometacin ($5\,mg\,kg^{-1}$) were administered orally 1 h before PBQ and formalin injection.

PGE_2 in RAW 264.7 macrophages stimulated by LPS. The inhibition was concentration-dependent without any evidence of a cytotoxic effect. Nevertheless, this compound was ineffective when NOS-2 and COX-2 were already expressed, suggesting that ClDQ had no inhibitory effect on NOS-2 and COX-2 activities. On the other hand, this compound, at μM concentrations, did not modify the arachidonic acid pathway by a direct action on the activity of enzymes such as PLA_2 or COX-1, which suggested that ClDQ did not reduce PGE_2 generation by inhibition of these enzymes.

We used the 24-h zymosan-stimulated mouse air-pouch model of inflammation to assess the in-vivo effect of ClDQ on NO and PGE_2 overproduction derived from the induction of NOS-2 and COX-2 (Posadas et al 2000). After oral administration, this compound exhibited an inhibitory profile that was well correlated with its in-vitro effects on macrophages, since nitrite and PGE_2 levels measured in exudates from 24-h zymosan-stimulated air-pouches were dose-dependently reduced. In addition, neutrophil migration in pouch exudates was inhibited at all the doses assayed. Thus, ClDQ could prevent or slow the progression of neutrophil-mediated tissue injury in addition to its inhibitory effect on NO and PGE_2 levels.

Immunosuppressive agents limit the functions of active cells including macrophages and lymphocytes. In this study, ClDQ significantly suppressed the proliferative response of human lymphocytes to phytohaemagglutinin. This effect could be correlated to the in-vivo results obtained in the DTH response to DNFB in mice. In this animal model, ear swelling is primarily the result of in-vivo functions of antigen-specific $CD4^+$ T-cell response (Grabbe & Schwarz 1996; Wang et al 1996). When ClDQ was orally administered before the challenge phase, DNFB-induced ear swelling in mice was clearly inhibited, which indicated that this compound was capable of inhibiting T-cell-dependent immune reactions. Interestingly, ClDQ strongly inhibited myeloperoxidase activity measured in ear homogenates as an index of cell migration. This result correlated with the reduction of leucocyte infiltration shown in the 24-h zymosan-stimulated mouse air-pouch, which indicated that ClDQ decreased leucocyte infiltration in experimental inflammation models.

T-cells have been reported to play an important role in the development and pathogenesis of rheumatoid arthritis and other experimental models of inflammatory diseases (Holmdahl et al 1985). In the rat adjuvant arthritis model, rats develop a chronic swelling in multiple joints, with influx of inflammatory cells, erosion of joint cartilage and bone destruction and remodelling. Our data indicated that ClDQ was effective, by the oral route, in the treatment of experimental chronic inflammation. In addition, inhibition of joint inflammation by ClDQ was accompanied by reduction of PGE_2 as well as IL-1β and TNF-α levels. It is noteworthy that ClDQ did not affect PGE_2 levels in stomach homogenates nor TXB_2 in serum of arthritic

rats. It is widely accepted that COX-1 is the major COX isoenzyme present in the gastrointestinal tract of many species and its inhibition can produce gastric side effects (Masferrer et al 1994; Kargman et al 1996). The lack of effect of ClDQ on PGE_2 levels in stomach after repeated oral doses in arthritic rats could be a favourable feature to become a non-gastrolesive anti-inflammatory drug.

ClDQ demonstrated analgesic effects in the phenyl-*p*-benzoquinone-induced abdominal constriction test and in the late phase of formalin-induced pain. It is known that inhibitors of PGE_2 and leukotriene generation can be efficient analgesic agents (Griswold et al 1991; Rioja et al 2002). In this way, the antinociceptive effects of ClDQ could be related to the inhibition of neutrophil infiltration shown in-vivo by this compound and the consequent reduction of these pro-inflammatory mediators. Nevertheless, ClDQ was inactive in the hot-plate test, a central model that is not inhibited by non-steroidal anti-inflammatory drugs (Yamamoto & Nozaki-Taguchi 1996).

Although the precise pharmacological mechanism of ClDQ remains undetermined, previous reports have shown that chloroquinolinyl chalcones inhibited NOS-2 or COX-2 expression in-vitro and in-vivo (Herencia et al 1999, 2001). In addition, other chalcone derivatives block the activation of nuclear factor-κB (Madan et al 2000; Huang et al 2001; Cheng et al 2001), which promotes the expression of genes encoding inducible enzymes such as NOS-2 or COX-2, and different pro-inflammatory mediators (Pahl 1999). Further investigations are required to determine if the anti-inflammatory effects of ClDQ are related to a similar mechanism.

Conclusion

Our data indicated that ClDQ exerted analgesic and anti-inflammatory effects after oral administration in acute and chronic animal models. These effects may have been in part related to the inhibition of leucocyte infiltration and the reduction of pro-inflammatory mediators including cytokines, NO and PGE_2. In addition, the immunosuppressive profile presented by this compound may offer a therapeutic potential for the treatment of T-cell-dependent chronic inflammatory pathologies.

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