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A pyrroloquinazoline derivative with anti-inflammatory and analgesic activity by dual inhibition of cyclo-oxygenase-2 and 5-lipoxygenase

Inmaculada Rioja [a], M. Carmen Terencio [a], Amalia Ubeda [a], Pedro Molina [b], Alberto Tárraga [b], Antonia Gonzalez-Tejero [b], M. José Alcaraz [a,*]

[a] *Departamento de Farmacología, Facultad de Farmacia, Universidad de Valencia. Av. Vicent Andrés Estellés s/n, 46100 Burjasot, Valencia, Spain*
[b] *Departamento de Química Orgánica, Facultad de Química, Universidad de Murcia, Murcia, Spain*

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Abstract

In a previous study, we reported a new pyrroloquinazoline derivative, 3-(4′-acetoxy-3′,5′-dimethoxy)benzylidene-1,2-dihydropyrrolo[2,1-*b*]quinazoline-9-one (PQ), which inhibited human purified 5-lipoxygenase activity and prostaglandin E_2 release in lipopolysaccharide-stimulated RAW 264.7 cells. In the present work, we show that PQ inhibits cyclo-oxygenase-2 activity in intact cell assays (human monocytes) and purified enzyme preparations (ovine isoenzymes) without affecting cyclo-oxygenase-1 activity. This behaviour was confirmed in vivo by using the zymosan-injected mouse air pouch model, where PQ caused a marked reduction in cell migration and leukotriene B_4 levels at 4 h, as well as inhibition of prostaglandin E_2 levels without affecting cyclo-oxygenase-2 expression at 24 h after zymosan stimulation. In addition, oral administration of this compound significantly reduced carrageenan-induced mouse paw oedema and phenyl-*p*-benzoquinone-induced writhings in mice. These results indicate that oral PQ exerts analgesic and anti-inflammatory effects, which are related to dual inhibition of cyclo-oxygenase-2 and 5-lipoxygenase activities.

Keywords: Pyrroloquinazoline; Eicosanoid; Cyclo-oxygenase-2; Air pouch, mouse; Carrageenan paw oedema; Analgesia

1. Introduction

Eicosanoids derived from the cyclo-oxygenase and lipoxygenase pathways contribute to inflammatory and immune responses. There are two isoforms of cyclo-oxygenase. Cyclo-oxygenase-1 is constitutively expressed in most mammalian tissues and is responsible for the production of prostanoids participating in homeostatic functions, whereas cyclo-oxygenase-2 is induced rapidly upon stimulation of cells with cytokines, endotoxins or mitogens and synthesizes high levels of prostaglandins (Smith and DeWitt, 1996). In animal models of inflammation, cyclo-oxygenase-2 is induced prior to the onset of the inflammatory response followed by the production of high levels of prostaglandin E_2 (Seibert et al., 1994; Masferrer et al., 1994a). Increased cyclo-oxygenase-2 expression is also found in synovial tissues of patients with rheumatoid arthritis (Kang et al., 1996). Although the contribution of cyclo-oxygenase-1 to inflammatory responses has been suggested (Wallace et al., 1998), it is widely accepted that the anti-inflammatory efficacy of non-steroidal anti-inflammatory drugs (NSAIDs) is closely related to inhibitory effects on cyclo-oxygenase-2, whereas inhibition of cyclo-oxygenase-1 accounts for their side effects. This hypothesis has led to the development of selective cyclo-oxygenase-2 inhibitors able to effectively inhibit inflammation, pain and fever and which show a better safety profile in the digestive tract than dual cyclo-oxygenase inhibitors (Vane and Botting, 1998; Hawkey, 1999; Mitchell and Warner, 1999).

The rate-limiting enzyme for the synthesis of leukotrienes is 5-lipoxygenase, which is present in white blood cells, macrophages and mast cells. Leukotriene B_4 potently activates neutrophil migration and chemotaxis in addition to other effects including stimulation of superoxide production and lysosomal enzyme secretion. Increased production of this mediator has been related with pathological conditions such as rheumatoid arthritis, psoriasis and inflammatory bowel disease (Tsuji et al., 1998). The cysteinyl leukotrienes are potent mediators in allergy and inflammation, by modulating smooth muscle tone and vascular permeability, and

* Corresponding author. Tel.: +34-96-386-4292; fax: +34-96-386-4292.
E-mail address: maria.j.alcaraz@uv.es (M.J. Alcaraz).

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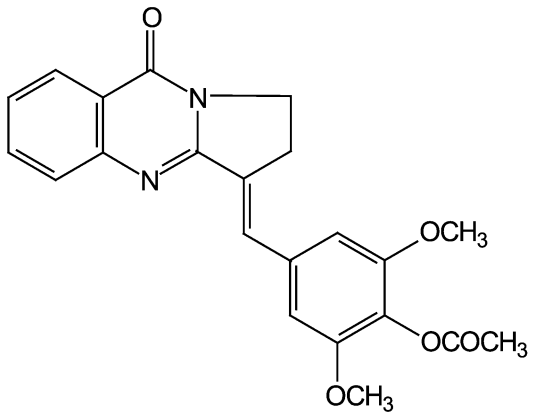

Fig. 1. Structure of PQ.

have an important role in asthma and other respiratory diseases (Lewis et al., 1990; Henderson, 1994; McMillan and Walker, 1992). 5-Lipoxygenase products are also involved in hyperalgesia (Amann et al., 1996; Bennett et al., 1998; Tonussi and Ferreira, 1999), and results obtained with different experimental models suggest that dual cyclo-oxygenase and 5-lipoxygenase inhibitors may be more efficient analgesic agents than selective cyclo-oxygenase inhibitors (Griswold et al., 1991). Interestingly, leukotrienes have been implicated in acute gastric and intestinal damage induced by classical NSAIDs (Rainsford, 1999), whereas 5-lipoxygenase inhibition may play an important role in the prevention of digestive lesions induced by these drugs (Kirchner et al., 1997).

In a previous work, we reported the effect of the alkaloid isaindigotone and a series of pyrroloquinazoline derivatives on leukocyte functions (Molina et al., 2001). The acetylated derivative of isaindigotone, 3-(4′-acetoxy-3′,5′-dimethoxy)-benzylidene-1,2-dihydropyrrolo[2,1-*b*]quinazoline-9-one (PQ) (Fig. 1), had a potent inhibitory effect on human neutrophil 5-lipoxygenase activity ($IC_{50} = 0.6$ μM) and also inhibited the generation of prostaglandin E_2 in lipopolysaccharide-stimulated RAW 264.7 cells ($IC_{50} = 0.2$ μM). The present study was undertaken to determine whether this novel pyrroquinazoline derivative was able to inhibit cyclo-oxygenase-1/cyclo-oxygenase-2 activities in intact cells assays and purified enzyme preparations. We also investigated the anti-inflammatory effect of PQ on experimental inflammation, using the zymosan-injected mouse air pouch and the carrageenan-induced mouse paw oedema models, as well as its analgesic activity against phenyl-*p*-benzoquinone-induced writhing in mice.

2. Materials and methods

2.1. Preparation of human monocytes

Human leukocytes were obtained from the citrated blood of healthy volunteers after sequential centrifugation as previously described (Bustos et al., 1995). The mononuclear cell interphase was obtained by Ficoll-Paque density gradient centrifugation. Cells were resuspended in RPMI 1640 supplemented with 10% foetal bovine serum (10^7 cells/ml) and incubated in Petri dishes. Some plates contained aspirin (300 μM). Monocytes, purified by discarding non-adherent cells after a 2-h incubation, were removed from the Petri dishes using a cell scraper, centrifuged at $400 \times g$ for 10 min, resuspended at a concentration of 2×10^6/ml in a total volume of 200 μl and cultured in 96-well culture plates. Cell viability was greater than 95% according to the Trypan blue exclusion test.

2.2. Cyclo-oxygenase-1/cyclo-oxygenase-2 activity in intact human monocytes

To assess the effects of the compounds on cyclo-oxygenase-2 activity, aspirin-treated human monocytes were incubated with *Escherichia coli* [serotype 0111:B4] lipopolysaccharide (1 μg/ml) for 24 h to induce cyclo-oxygenase-2. Cultured medium was then changed and PQ, 5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulphonyl)phenyl-2(5*H*)-furanone (DFU), indomethacin or vehicle was added for a 15-min preincubation at 37 °C. Arachidonic acid (10 μM) was then added and the cells were incubated for a further 2 h. In parallel experiments, aspirin-untreated monocytes were preincubated for 15 min with test compounds or vehicle. Afterwards, arachidonic acid (10 μM) was added and cells were cultured for 2 h at 37 °C to assess the effects of compounds on cyclo-oxygenase-1 activity. After the incubation period, supernatants were collected for the measurement of prostaglandin E_2 levels by radioimmunoassay (Moroney et al., 1988).

2.3. Cell viability assay

The mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan (Gross and Levi, 1992) was used to exclude a possible cytotoxic effect of PQ and reference compounds. Treatment of human monocytes with lipopolysaccharide at 1 μg/ml for 24 h did not significantly inhibit cell viability.

2.4. Cyclo-oxygenase-1/cyclo-oxygenase-2 ovine enzyme assay

PQ and reference compounds were preincubated for 15 min with cyclo-oxygenase-1 or cyclo-oxygenase-2 purified ovine isoenzymes at 37 °C in 50 mM Tris–HCl pH 8 in the presence of 2 μM hematin and 1 mM L-tryptophan (Brownlie et al., 1993). Arachidonic acid was then added at a final concentration of 0.5 μM, and the samples were further incubated at 37 °C for 30 min. The reaction was stopped by boiling the samples for 5 min, and after centrifugation at $10{,}000 \times g$ at 4 °C for 30 min, prostaglandin E_2 levels in supernatants were determined by radioimmunoassay.

2.5. Phospholipase A_2 assay

Secretory phospholipase A_2 was assayed by using [^{3}H]oleate-labelled membranes of *E. coli* (Franson et al., 1974). Bee venom, porcine pancreatic, and human recombinant synovial enzymes were used as sources of secretory phospholipase A_2. Cytosolic phospholipase A_2 was prepared from the RAW 264.7 macrophage cell line (Cell Collection, Department of Animal Cell Culture, C.S.I.C., Madrid, Spain) and its enzymatic activity was measured as the release of radiolabelled arachidonic acid according to the method of Clark et al. (1990).

2.6. 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 performed 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-injected air pouch, PQ (1, 10, and 100 nmol/pouch) was administered at the same time as zymosan. The reference inhibitors indomethacin, DFU and 6-[[3-fluoro-5-(4-methoxy-3,4,5,6-tetrahydro-2*H*-pyran-4-yl)phenoxy]-methyl]-1-ethylquinol-2-one (ZM230,487) were assayed at 100 nmol/pouch. In the 24-h zymosan-injected air pouch, PQ (20 mg/kg), DFU (20 mg/kg) and dexamethasone (5 mg/kg) were administered orally 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 the 4-h exudates, the supernatants were used to measure leukotriene B_4 and prostaglandin E_2 levels by radioimmunoassay. The supernatants of the 24-h exudates were used to measure prostaglandin E_2 levels and the cell pellets were used to determine cyclo-oxygenase-2 expression by Western blot analysis as described below. Protein was quantified by the Bradford technique, using bovine serum albumin as standard.

2.7. Western blot analysis

Microsomal fractions from leukocytes present in 24-h pouch exudates were used to measure cyclo-oxygenase-2 expression. Equal amounts of protein were loaded on 12.5% polyacrylamide-sodium dodecyl sulphate gels for electrophoresis and then transferred onto polyvinylidene difluoride membranes for 90 min at 125 mA. Membranes were blocked in phosphate-buffered saline (0.02 M, pH 7.0)–Tween 20 (0.1%) containing 3% w/v unfatted milk and incubated with specific anti-cyclo-oxygenase-2 polyclonal antiserum (1/1000). Finally, membranes were incubated with peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) (1/20,000). The immunoreactive bands were visualized using an enhanced chemiluminescence system (ECL, Amersham Iberica, Madrid, Spain).

2.8. Carrageenan paw oedema

The anti-inflammatory activity of PQ was assessed in the carrageenan paw oedema test in mice according to the method of Sugishita et al. (1981). This compound (10, 20 and 40 mg/kg), DFU (20 mg/kg), indomethacin (5 mg/kg), or vehicle (water/ethanol/Tween 80, 90:5:5 v/v/v) was administered orally (0.5 ml) 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 is 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 by radioimmunoassay. Stomachs were homogenized in 2 ml of methanol and the content of prostaglandin E_2 was measured in supernatants after centrifugation.

2.9. Phenyl-p-benzoquinone-induced writhing in mice

Female Swiss mice (20–25 g) were injected intraperitoneally with 0.2 ml of a 0.02% w/v solution of phenyl-*p*-benzoquinone in ethanol/distilled water (5/95 v/v) and then were placed singly in a plastic animal cage measuring 50 × 50 cm. 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 or drugs were administered orally 1 h prior to phenyl-*p*-benzoquinone in a volume of 0.5 ml.

2.10. Hot-plate test

Groups of 8–12 female Swiss mice (20–25 g) were used. The reaction time (seconds) of each mice 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 test compounds or vehicle and removed immediately after they licked the footpad of any paw.

2.11. Materials

PQ was synthesized as described previously (Molina et al., 2001). [5,6,8,11,12,14,15(*n*)-^{3}H]prostaglandin E_2 and [5,6,8,9,11,12,14,15(*n*)-^{3}H]leukotriene B_4, were from Amersham Iberica. [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). Cyclo-oxy-

genase-1 and cyclo-oxygenase-2 ovine isoenzymes and cyclo-oxygenase-2 polyclonal antiserum were purchased from Cayman Chem. (MI, USA). DFU was kindly provided by Merck Sharp & Dohme (NJ, USA). Antibody against leukotriene B_4 and the reference compound ZM230,487 were kindly provided by Zeneca Pharmaceuticals (Macclesfield, Cheshire, UK). The other reagents were from Sigma (MO, USA). *E. coli* strain CECT 101 was a gift from Prof. Uruburu, Department of Microbiology, University of Valencia, Spain.

2.12. Statistical analysis

The results are presented as means ± S.E.M.; *n* represents the number of experiments or the number of animals per group. Inhibitory concentration 50% (IC_{50}) values were calculated from at least four significant concentrations ($n=6$) and effective dose 50% (ED_{50}) from at least three doses ($n=6-12$). When appropriate, 95% confidence limits were calculated. 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. Cyclo-oxygenase-1/cyclo-oxygenase-2 activity in human monocytes

Cyclo-oxygenase-2 was induced in aspirin-treated monocytes by 24-h stimulation with lipopolysaccharide. Cultured medium was then changed and after a 15-min preincubation of vehicle or test compounds, arachidonic acid was added and cells were incubated for a further 2 h. In a parallel experiment, cyclo-oxygenase-1 activity was studied in non-induced cells after a 2-h incubation with arachidonic acid. As shown in Table 1, PQ significantly inhibited prostaglandin E_2 production derived from cyclo-oxygenase-2 activity, with an IC_{50} value in the micromolar range. In contrast, only a weak effect was observed at 10 μM in non-induced cells. As expected, the reference compound indomethacin was more potent as cyclo-oxygenase-1 inhibitor, whereas DFU selectively inhibited cyclo-oxygenase-2 activity.

Table 1
Effect of PQ and reference compounds on cyclo-oxygenase-1 and cyclo-oxygenase-2 activities in human monocytes

	Cyclo-oxygenase-1		Cyclo-oxygenase-2	
	Prostaglandin E_2 (ng/ml)[a]	IC_{50}[b]	Prostaglandin E_2 (ng/ml)[a]	IC_{50}[b]
Basal	3.3 ± 0.4[c]	–	7.6 ± 0.5[c]	–
Control	10.0 ± 0.4	–	40.9 ± 2.1	–
PQ	8.3 ± 0.4[d]	>10 μM	17.6 ± 0.8[c]	1.2 (0.7–1.9) μM
DFU	4.7 ± 0.2[c]	1.5 (0.7–3.2) μM	14.8 ± 0.8[c]	3.3 (1.5–5.6) nM
Indomethacin	3.5 ± 0.1[c]	6.2 (1.0–23.4) nM	9.2 ± 1.6[c]	50.3 (20.4–100.0) nM

[a] Compounds were assayed at 10 μM. Data show means ± S.E.M. ($n=9-12$).
[b] Values represent the concentration required to produce 50% inhibition of the response, along with the 95% confidence limits.
[c] $P<0.01$ compared with control values.
[d] $P<0.05$ compared with control values.

Table 2
Effect of PQ and reference compounds on cyclo-oxygenase-1 and cyclo-oxygenase-2 activities in purified ovine isoenzymes

	Cyclo-oxygenase-1		Cyclo-oxygenase-2	
	Prostaglandin E_2 (ng/ml)[a]	IC_{50}[b]	Prostaglandin E_2 (ng/ml)[a]	IC_{50}[b]
Basal	0.5 ± 0.2[c]	–	0.4 ± 0.1[c]	–
Control	35.2 ± 2.9	–	28.5 ± 1.1	–
PQ	34.6 ± 0.8	>50 μM	6.7 ± 1.1[c]	20.5 (14.1–31.6) μM
DFU	30.6 ± 0.5	>50 μM	11.8 ± 0.7[c]	6.4 (4.5–8.0) μM
Indomethacin	0.6 ± 0.1[c]	34.9 (25.1–50.1) nM	5.3 ± 0.4[c]	0.3 (0.2–0.6) μM

[a] Compounds were assayed at 10 μM. Data show means ± S.E.M. ($n=9-12$).
[b] Values represent the concentration required to produce 50% inhibition of the response, along with the 95% confidence limits.
[c] $P<0.01$ compared with control values.

3.2. Cyclo-oxygenase-1/cyclo-oxygenase-2 ovine enzyme assay

To confirm the results obtained in the intact cell assays, we examined the effects of PQ on cyclo-oxygenase activity using ovine isoenzymes and arachidonic acid as substrate. PQ and DFU dose dependently reduced prostaglandin E_2 levels derived from cyclo-oxygenase-2 activity (Table 2). In contrast, cyclo-oxygenase-1 activity was not affected by these compounds even at concentrations over 50 μM. Under the

Table 3
Effect of PQ and reference compounds on secretory PLA_2 ($sPLA_2$) activity belonging to groups I (porcine pancreatic), II (human synovial) or III (bee venom) and on cytosolic PLA_2 ($cPLA_2$) prepared from RAW 264.7 macrophages

	$sPLA_2$ (pmol OA/mg/min)			$cPLA_2$ (pmol AA/mg/min)
	Pancreatic	Human synovial	Bee venom	RAW 264.7
Control	19097.4 ± 992.0	2111.7 ± 151.6	252.8 ± 9.3	11.4 ± 0.9
PQ	18067.8 ± 441.7	1995.2 ± 110.4	241.3 ± 6.0	9.7 ± 0.8
Scalaradial	9888.3 ± 792.9[a]	735.6 ± 47.3[a]	98.9 ± 7.7[a]	N.D.
PTK	N.D.	N.D.	N.D.	2.7 ± 0.2[a]

All compounds were assayed at 10 μM. Data represent means ± S.E.M. ($n=6-8$).
[a] $P<0.01$ compared with control values. OA=[^{3}H]oleic acid; AA=[^{14}C] arachidonic acid. PTK = Palmityl trifluoromethyl ketone.

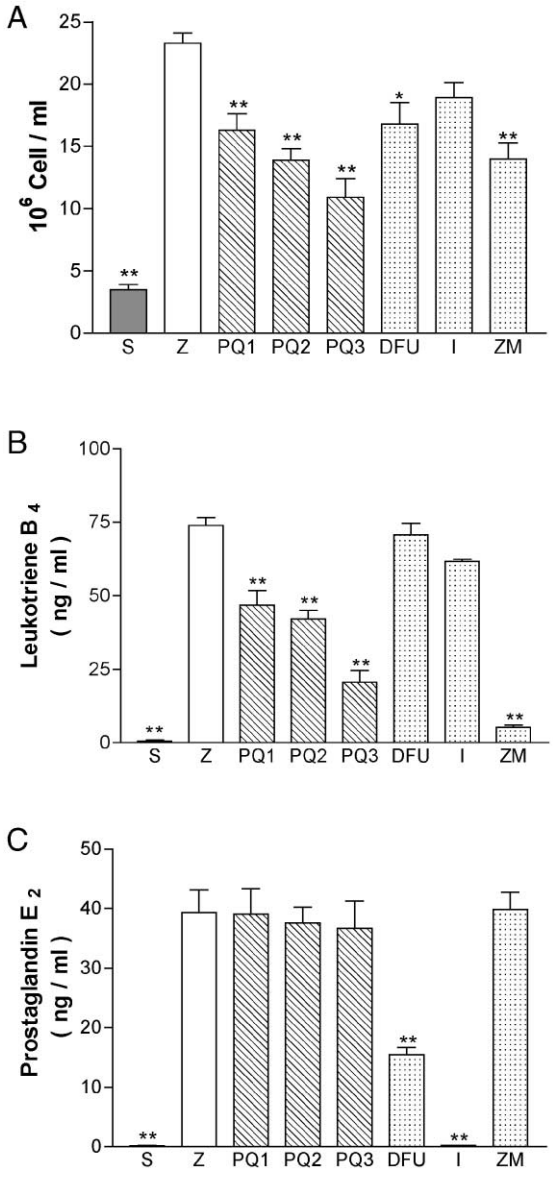

Fig. 2. Effect of PQ and reference compounds on the 4-h zymosan-injected air pouch. Leukocyte influx (A), leukotriene B_4 (B) and prostaglandin E_2 levels (C) measured in exudates. S = saline; Z = zymosan; PQ1, PQ2 and PQ3 = PQ injected at 1, 10 and 100 nmol/pouch, respectively. DFU, I = indomethacin and ZM = ZM230,487 were assayed at 100 nmol/pouch. Data represent means ± S.E.M. ($n = 8–12$ animals). * $P < 0.05$, * * $P < 0.01$.

same conditions, the non-selective inhibitor indomethacin exerted very potent inhibitory effects on cyclo-oxygenase-1.

3.3. Phospholipase A_2 activity

To exclude that the inhibitory effect on prostaglandin E_2 production in intact cells could be related to interference with the release of arachidonic acid, PQ (10 μM) was assayed with different types of phospholipase A_2. As shown in Table 3, 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 I (porcine pancreatic), II (human synovial) or III (bee venom) (data not shown). Cytosolic phospholipase A_2 activity from RAW 264.7 macrophages was not inhibited by PQ at the above concentration (data not shown).

3.4. Mouse air pouch

We used the 4-h zymosan-stimulated mouse air pouch model to assess the in vivo effect of PQ on leukocyte migration, leukotriene B_4 generation and prostaglandin E_2 levels derived from cyclo-oxygenase-1 activity (Posadas et al., 2000). As shown in Fig. 2, the number of leukocytes infiltrated into the pouch exudate collected 4 h after zymosan

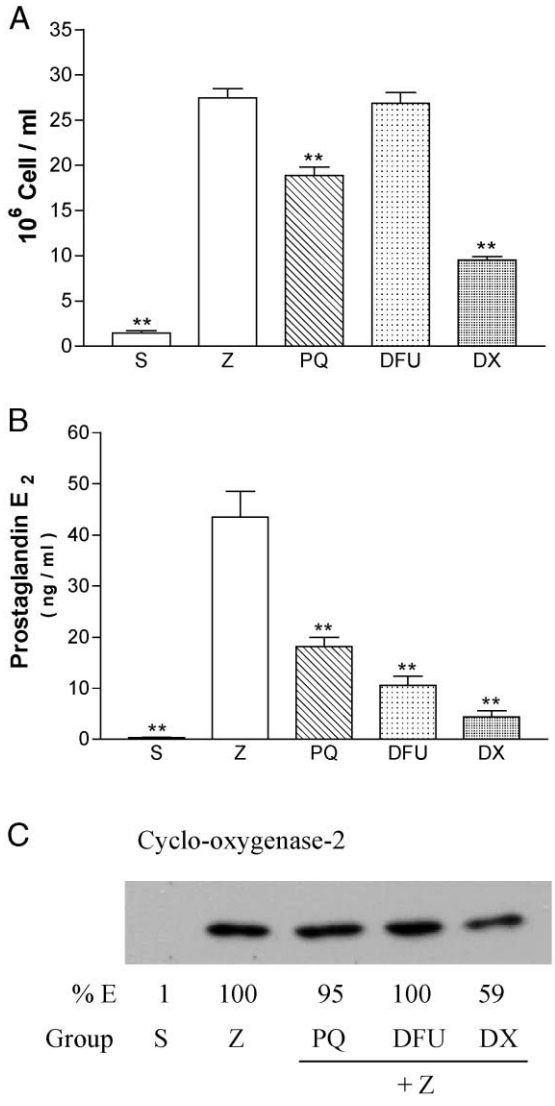

Fig. 3. Effect of PQ (20 mg/kg, p.o.), DFU (20 mg/kg, p.o.) and DX = dexamethasone (5 mg/kg p.o.) on the 24-h zymosan-injected air pouch. Drugs were administered orally 1 h before and 8 h after zymosan injection. Leukocyte influx (A) and prostaglandin E_2 levels (B) measured in exudates. Data represent means ± S.E.M. ($n = 8–12$ animals); * $P < 0.05$, * * $P < 0.01$. (C) Western blot analysis of cyclo-oxygenase-2 protein expression in cells from exudates and densitometric analysis. S = saline, Z = zymosan, DX = dexamethasone. The figure is representative of three experiments.

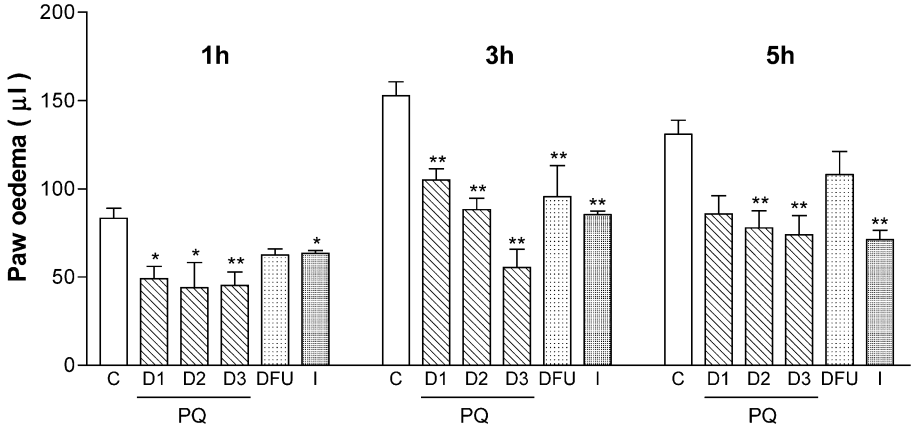

Fig. 4. Effect of PQ, DFU and indomethacin on carrageenan-induced mouse paw oedema, 1, 3 and 5 h after the induction of inflammation. C = vehicle; D1, D2 and D3 = PQ administered orally at 10, 20 and 40 mg/kg, respectively. DFU at 20 mg/kg, p.o.; I = indomethacin at 10 mg/kg, p.o. Data represent means ± S.E.M. ($n = 8–12$ animals). * $P < 0.05$, ** $P < 0.01$.

challenge was significantly reduced by intrapouch administration of PQ at the three doses assayed, with an ED_{50} of 12.8 nmol/pouch. Leukotriene B_4 levels were also strongly reduced at the same doses ($ED_{50} = 7.9$ nmol/pouch), whereas prostaglandin E_2 levels were unaffected. Reference inhibitors were assayed at 100 nmol/pouch. ZM230,487 (selective 5-lipoxygenase inhibitor) reduced leukocyte infiltration and leukotriene levels at the dose assayed. Indomethacin potently inhibited prostaglandin E_2 levels without having a significant effect on cell migration or leukotriene B_4 production. The cyclo-oxygenase-2 inhibitor DFU partially reduced leukocyte migration and prostaglandin E_2 levels but it did not affect leukotriene B_4 generation.

In the 24-h zymosan-stimulated air pouch, maximal expression of cyclo-oxygenase-2 is observed and prostaglandin E_2 levels are increased in exudates (Posadas et al., 2000). We used this time point to assess the effect of PQ on cyclo-oxygenase-2 activity and protein expression. Cell influx was reduced to a minor extent by PQ (33.4%), whereas DFU did not significantly affect cell recruitment (Fig. 3A). Prostaglandin E_2 levels were significantly reduced after oral administration of PQ or DFU at the dose of 20 mg/kg, with per- centages of inhibition of 58.6 and 75.6, respectively (Fig. 3B). Dexamethasone was also assayed as reference compound and at the dose of 5 mg/kg p.o. caused a marked inhibition of prostaglandin E_2 levels (90.5%) and leukocyte infiltration (69.1%).

Western blot analysis (Fig. 3C) for cyclo-oxygenase-2 was carried out on microsomal fractions of leukocytes obtained by centrifugation of exudates from the 24-h zymosan assay. As expected, dexamethasone potently inhibited the expression of this enzyme, whereas in animals treated with PQ or DFU, protein expression was unaffected.

3.5. Carrageenan-induced mouse paw oedema

After oral administration, PQ caused a dose-dependent reduction of carrageenan-induced oedema in mice 1, 3, and 5 h after induction of inflammation (Fig. 4). The greatest effect was observed at 3 h with an ED_{50} of 27.2 mg/kg. At the same time, DFU (20 mg/kg) and indomethacin (10 mg/kg) caused a significant reduction of swelling (37.5% and 44.1%, respectively).

After the last determination of oedema (5 h), the animals were killed and the paws injected with carrageenan were homogenized to determine the levels of prostaglandin E_2. PQ and DFU at a dose of 20 mg/kg produced a moderate but significant reduction of this prostanoid, with a similar percentage of inhibition for both compounds. In contrast, indomethacin strongly reduced prostaglandin E_2 levels at 10 mg/kg (Table 4). In addition, the content of prostaglandin E_2 measured in stomach homogenates was not significantly affected by the administration of PQ or DFU, whereas indomethacin potently reduced the levels of this metabolite (Table 4). It should be noted that indomethacin produced macroscopically visible gastric damage at the dose assayed, in contrast to PQ or DFU.

3.6. Phenyl-p-benzoquinone-induced writhing in mice

Administration of phenyl-*p*-benzoquinone to mice at a concentration of 0.02% of irritant resulted in 52.6 ± 2.1

Table 4
Inhibition by PQ, indomethacin and DFU of prostaglandin E_2 levels in homogenates of inflamed paws or stomachs (control values 128.2 ± 9.6 and 37.9 ± 3.3 ng/ml, respectively)

Dose (mg/kg, p.o.)	Percentage of inhibition of prostaglandin E_2	
	Inflamed paws	Stomachs
PQ (10)	13.1 ± 7.0	11.4 ± 7.1
PQ (20)	33.7 ± 5.7[a]	11.4 ± 7.0
PQ (40)	45.9 ± 3.7[a]	11.3 ± 4.5
DFU (20)	31.8 ± 3.9[a]	18.4 ± 4.9
Indomethacin (10)	93.8 ± 8.9[a]	87.8 ± 9.7[a]

Data represent means ± S.E.M. ($n = 8–12$ animals).
[a] $P < 0.01$ compared with control group.

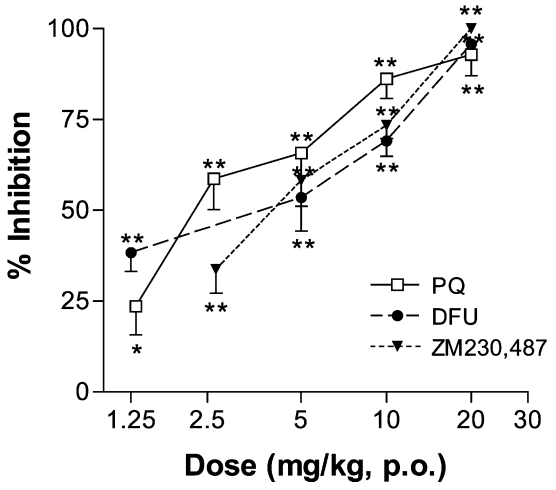

Fig. 5. Dose–response relationship for the inhibition by PQ, DFU and ZM230,487 of the phenyl-*p*-benzoquinone-induced writhing response. Compounds were administered orally 1 h before the intraperitoneal injection of irritant. Control group showed 52.6 ± 2.1 writhes. Results are the means ± S.E.M. for 8–12 mice. * $P < 0.05$; * * $P < 0.01$.

writhes in the control group. As shown in Fig. 5, PQ displayed dose-related inhibitory effects after oral administration with an ED_{50} (95% confidence limits) of 2.6 (1.5–3.5) mg/kg. In this model, the reference compounds DFU, ZM 230,487 and codeine showed ED_{50} values of 3.9 (1.2–7.5), 4.2 (2.8–6.0) and 2.3 (1.1–5.2) mg/kg, respectively, after oral administration.

3.7. Hot-plate test

The reaction time for the control group in this test was 6.9 ± 0.4 s. The responses of animals treated orally with PQ or DFU at the dose of 20 mg/kg did not differ from those of the control group (data not shown). In contrast, administration of codeine at the same dose produced a significant increase in reaction time (14.1 ± 1.2 s, $P < 0.01$).

4. Discussion

In this study, we have presented evidence that the pyrroloquinazoline derivative PQ inhibits cyclo-oxygenase-2 activity in vitro in experiments using human intact cells and ovine isozymes. These results confirm previous observations for lipopolysaccharide-stimulated RAW 264.7 cells (Molina et al., 2001; Rioja et al., 2000). As a reference, we used in our experiments DFU, a potent and highly selective cyclo-oxygenase-2 inhibitor (Riendeau et al., 1997). It should be noted that all compounds tested showed a higher potency in intact cells than against purified enzymes, which is in agreement with previous reports of cyclo-oxygenase inhibitory studies using both types of assays (Mitchell et al., 1994). PQ did not modify arachidonic acid release by a direct action on phospholipase A_2 activity.

We have recently described the time course of inflammatory mediator production in the zymosan-injected mouse air pouch as well as the participation of cyclo-oxygenase-2 metabolites in the late phase response in this model (Posadas et al., 2000). According to our previous experiments, we used in the present work the 4-h zymosan-injected mouse air pouch to determine the effects of PQ on 5-lipoxygenase and cyclo-oxygenase-1 activities, whereas the 24-h zymosan-injected mouse air pouch was used to assess the effects of this compound on cyclo-oxygenase-2. In this model, the pyrroloquinazoline derivative exhibited an inhibitory behaviour well correlated with its in vitro effects, since prostaglandin E_2 levels measured in the exudates were inhibited by PQ and the reference compound DFU, without affecting the expression of cyclo-oxygenase-2 in the migrating cells. In contrast, PQ did not modify prostaglandin E_2 levels in the 4-h zymosan-stimulated air pouch exudates, indicating that this compound did not inhibit cyclo-oxygenase-1 activity in vivo. Indomethacin, a non-selective cyclo-oxygenase inhibitor, potently reduced the levels of this eicosanoid at 4 h after challenge, whereas DFU inhibited prostaglandin E_2 levels to a minor extent. In addition, PQ reduced neutrophil migration and leukotriene B_4 levels in the 4-h zymosan-stimulated air pouch. These effects are well correlated with the potent inhibition of 5-lipoxygenase activity in vitro (Molina et al., 2001). Thus, PQ could prevent or slow the progression of neutrophil-mediated tissue injury in addition to its cyclo-oxygenase-2 inhibitory effect.

PQ also exerted an anti-oedematous effect on mouse paw oedema induced by carrageenan after oral administration. In this model, indomethacin abolished prostaglandin E_2 overproduction in paw homogenates, whereas PQ or DFU exerted a smaller effect, likely due to the selective inhibition of cyclo-oxygenase-2 activity.

Cyclo-oxygenase-1 is the major cyclo-oxygenase isozyme present in the gastrointestinal tract of many species (Kargman et al., 1996), and selective cyclo-oxygenase-2 inhibitors do not inhibit stomach prostaglandin E_2 production in contrast to non-selective NSAIDs (Masferrer et al., 1994b). Our results indicate that oral anti-inflammatory doses of PQ have no detectable ulcerogenic effects nor affect prostaglandin E_2 levels in stomach homogenates. This behaviour is similar to that of the cyclo-oxygenase-2 inhibitor DFU. In contrast, indomethacin induced gastrointestinal lesions after a single acute dose of 10 mg/kg and potently reduced prostaglandin E_2 levels in the stomach.

It has been suggested that inhibition of prostaglandin production by NSAIDs would result in an increased formation of leukotrienes and neutrophil infiltration into the digestive tract. As a result, inhibition of 5-lipoxygenase could prevent the gastric side effects of cyclo-oxygenase inhibitors (Kirchner et al., 1997) and treatment with dual cyclo-oxygenase and 5-lipoxygenase inhibitors would have greater gastrointestinal safety, with protective effects against neutrophil adhesion and gastrointestinal inflammation induced by NSAIDs (Argentieri et al., 1994; Kirchner et al.,

1997; Janusz et al., 1998). Therefore, the dual cyclo-oxygenase-2/5-lipoxygenase inhibitory activity of PQ may be a favourable feature and make PQ a non-gastrolesive anti-inflammatory drug.

Selective cyclo-oxygenase-2 inhibitors have demonstrated analgesic efficacy in animal models (Lin et al., 1995) and humans (Morrison et al., 1999). After oral administration, PQ displayed a dose-related inhibitory effect in the phenyl-*p*-benzoquinone-induced writhing test, being 10 times more potent as analgesic than as anti-inflammatory agent. Prostaglandins derived from cyclo-oxygenase-1 activity in peritoneal cells would mediate abdominal pain after irritant administration (Kusuhara et al., 1998), although both selective and non-selective cyclo-oxygenase inhibitors are effective against writhing and it is likely that prostaglandins produced by cyclo-oxygenase-1 and cyclo-oxygenase-2 are involved in this response. Cyclo-oxygenase-2 is the predominant constitutive isoform in brain and spinal cord and it can be induced after peripheral induction of inflammation, leading to a high production of prostaglandins (Beiche et al., 1996; Hoffmann, 2000). Thus, cyclo-oxygenase-2 could participate in both a peripheral and a central component of inflammatory pain (Smith et al., 1998; Ballou et al., 2000), and the continuous production of prostaglandin E_2 by this isozyme could play a key role in the maintenance of inflammatory hyperalgesia (Zhang et al., 1997). Nevertheless, PQ was inactive in the hot-plate test, a model in which the response is not inhibited by NSAIDs and which is likely not related to cyclo-oxygenase-2 activity (Yamamoto and Nozaki-Taguchi, 1996).

It is generally accepted that 5-lipoxygenase products induce the recruitment and activation of leukocytes, leading to tissue damage and hyperalgesia (Levine et al., 1984; Amann et al., 1996; Bennett et al., 1998). In fact, the 5-lipoxygenase inhibitor ZM230,487 reduced the phenyl-*p*-benzoquinone writhing in the present work and previous reports have shown that dual inhibition of cyclo-oxygenase and 5-lipoxygenase can result in higher analgesic and anti-inflammatory activities (Griswold et al., 1991; Mylari et al., 1990). In particular, dual inhibitors of cyclo-oxygenase-2 and 5-lipoxygenase have been selected as antiarthritic drug candidates (Inagaki et al., 2000).

In summary, the present study demonstrates that PQ exerts anti-inflammatory and analgesic effects which are mainly related to dual inhibition of cyclo-oxygenase-2 and 5-lipoxygenase activities, thus controlling the overproduction of mediators and cell infiltration that leads to tissue damage in the inflamed area. Development of this class of compounds could provide new drugs for the modulation of different inflammatory pathologies, with reduced gastric toxicity.

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References

Amann, R., Schuligoi, R., Lanz, I., Peskar, B.A., 1996. Effect of a 5-lipoxygenase inhibitor on nerve growth factor-induced thermal hyperalgesia in the rat. Eur. J. Pharmacol. 306, 89–91.

Argentieri, D.C., Ritchie, D.M., Ferro, M.P., Kirchner, T., Wachter, M.P., Anderson, D.W., Rosenthale, M.E., Capetola, R.J., 1994. Tepoxalin: a dual cyclooxygenase/5-lipoxygenase inhibitor of arachidonic acid metabolism with potent anti-inflammatory activity and a favorable gastrointestinal profile. J. Pharmacol. Exp. Ther. 271, 1399–1408.

Ballou, L.R., Botting, R.M., Goorha, S., Zhang, J., Vane, J.R., 2000. Nociception in cyclooxygenase isozyme-deficient mice. Proc. Natl. Acad. Sci. U.S.A. 97, 10272–10276.

Beiche, F., Scheuerer, S., Brune, K., Geisslinger, G., Goppelt-Struebe, M., 1996. Up-regulation of cyclooxygenase-2 mRNA in the rat spinal cord following peripheral inflammation. FEBS Lett. 390, 165–169.

Bennett, G., al-Rashed, S., Hoult, J.R., Brain, S.D., 1998. Nerve growth factor induced hyperalgesia in the rat hind paw is dependent on circulating neutrophils. Pain 77, 315–322.

Brownlie, R.P., Brownrigg, N.J., Butcher, H.M., Garcia, R., Jessup, R., Lee, V.J., Tunstall, S., Wayne, M.G., 1993. ZD1542, a potent thromboxane A_2 synthase inhibitor and receptor antagonist in vitro. Br. J. Pharmacol. 110, 1600–1606.

Bustos, G., Ferrándiz, M.L., Sanz, M.J., Payá, M., Alcaraz, M.J., 1995. A study of the novel anti-inflammatory agent florifenine. Topical anti-inflammatory activity and influence on arachidonic acid metabolism and neutrophil functions. Naunyn-Schmiedeberg's Arch. Pharmacol. 351, 298–304.

Clark, J.D., Milona, N., Knopf, J.L., 1990. Purification of a 110-kilodalton cytosolic phospholipase A_2 from the human monocytic cell line U937. Proc. Natl. Acad. Sci. U.S.A. 87, 7708–7712.

Edwards, J.C.W., Sedgwick, A.D., Willoughby, D.A., 1981. The formation of a structure with the features of synovial lining by subcutaneous injection of air: an in vivo tissue culture system. J. Pathol. 134, 147–156.

Franson, R., Patriarca, P., Elsbach, P., 1974. Phospholipid metabolism by phagocytic cells. Phospholipases A_2 associated with rabbit polymorphonuclear leukocyte granules. J. Lipid Res. 15, 380–388.

Griswold, D.E., Marshall, P., Martin, L., Webb, E.F., Zabko-Potapovich, B., 1991. Analgetic activity of SK&F 105809, a dual inhibitor of arachidonic acid metabolism. Agents Actions Suppl. 32, 113–117.

Gross, S.S., Levi, R., 1992. Tetrahydrobiopterin synthesis. An absolute requirement for cytokine-induced nitric oxide generation by vascular smooth muscle. J. Biol. Chem. 267, 25722–25729.

Hawkey, C.J., 1999. COX-2 inhibitors. Lancet 353, 307–314.

Henderson, W.R., 1994. The role of leukotrienes in inflammation. Ann. Intern. Med. 121, 684–697.

Hoffmann, C., 2000. COX-2 in brain and spinal cord implications for therapeutic use. Curr. Med. Chem. 7, 1113–1120.

Inagaki, M., Tsuri, T., Jyoyama, H., Ono, T., Yamada, K., Kobayashi, M., Hori, Y., Arimura, A., Yasui, K., Ohno, K., Kakudo, S., Koizumi, K., Suzuki, R., Kawai, S., Kato, M., Matsumoto, S., 2000. Novel antiarthritic agents with 1,2-isothiazolidine-1,1-dioxide (gamma-sultam) skeleton: cytokine suppressive dual inhibitors of cyclooxygenase-2 and 5-lipoxygenase. J. Med. Chem. 43, 2040–2048.

Janusz, J.M., Young, P.A., Ridgeway, J.M., Scherz, M.W., Enzweiler, K., Wu, L.I., Gan, L., Darolia, R., Matthews, R.S., Hennes, D., Kellstein, D.E., Green, S.A., Tulich, J.L., Rosario-Jansen, T., Magrisso, I.J., Wehmeyer, K.R., Kuhlenbeck, D.L., Eichhold, T.H., Dobson, R.L., Sirko, S.P., Farmer, R.W., 1998. New cyclooxygenase-2/5-lipoxygenase inhibitors: 1. 7-*tert*-buty1-2,3-dihydro-3,3-dimethylbenzofuran derivatives as gastrointestinal safe antiinflammatory and analgesic agents: discovery and variation of the 5-keto substituent. J. Med. Chem. 41, 1112–1123.

Kang, R.Y., Freire Moar, J., Sigal, E., Chu, C.Q., 1996. Expression of cyclooxygenase-2 in human and an animal model of rheumatoid arthritis. Br. J. Rheumatol. 35, 711–718.

Kargman, S., Charleson, S., Cartwright, M., Frank, J., Riendeau, D., Man-

cini, J., Evans, J., O'Neill, G., 1996. Characterization of Prostaglandin G/H Synthase 1 and 2 in rat, dog, monkey, and human gastrointestinal tracts. Gastroenterology 111, 445–454.

Kirchner, T., Aparicio, B., Argentieri, D.C., Lau, C.Y., Ritchie, D.M., 1997. Effects of tepoxalin, a dual inhibitor of cyclooxygenase/5 lipoxygenase, on events associated with nsaid induced gastrointestinal inflammation. Prostaglandins Leukot. Essent. 56, 417–423.

Kusuhara, H., Matsuyuki, H., Okumoto, T., 1998. Involvement of prostaglandins produced by cyclooxygenase-1 in murine visceronociception induced by phenylquinone. Prostaglandins Lipid Mediat. 55, 43–49.

Levine, J.D., Lau, W., Kwiat, G., Goetzl, E.J., 1984. Leukotriene B_4 produces hyperalgesia that is dependent on polymorphonuclear leukocytes. Science 225, 743–745.

Lewis, R.A., Austen, K.F., Soberman, R.J., 1990. Leukotrienes and other products of the 5-lipoxygenase pathway. Biochemistry and relation to pathobiology in human diseases. N. Engl. J. Med. 323, 645–655.

Lin, Y.Z., Yao, S.Y., Veach, R.A., Torgerson, T.R., Hawiger, J., 1995. Inhibition of nuclear translocation of transcription factor NF-kappa B by a synthetic peptide containing a cell membrane-permeable motif and nuclear localization sequence. J. Biol. Chem. 270, 14255–14258.

Masferrer, J.L., Reddy, S.T., Zweifel, B.S., Seibert, K., Needleman, P., Gilbert, R.S., Herschman, H.R., 1994a. In vivo glucocorticoids regulate cyclooxygenase-2 but not cyclooxygenase-1 in peritoneal macrophages. J. Pharmacol. Exp. Ther. 270, 1340–1344.

Masferrer, J.L., Zweifel, B.S., Manning, P.T., Hauser, S.D., Leahy, K.M., Smith, W.G., Isakson, P.C., Seibert, K., 1994b. Selective inhibition of inducible cyclooxygenase 2 in vivo is antiinflammatory and nonulcerogenic. Proc. Natl. Acad. Sci. U.S.A. 91, 3228–3232.

McMillan, R.M., Walker, E.R.H., 1992. Designing therapeutically effective 5-lipoxygenase inhibitors. Trends Pharmacol. Sci. 13, 323–330.

Mitchell, J.A., Warner, T.D., 1999. Cyclo-oxygenase-2: pharmacology, physiology, biochemistry and relevance to NSAID therapy. Br. J. Pharmacol. 128, 1121–1132.

Mitchell, J.A., Akarasereenont, P., Thiemermann, C., Flower, R.J., Vane, J.R., 1994. Selectivity of nonsteroidal antiinflammatory drugs as inhibitors of constitutive and inducible cyclooxygenase. Proc. Natl. Acad. Sci. U.S.A. 90, 11693–11697.

Molina, P., Tárraga, A., Gonzalez-Tejero, A., Rioja, I., Ubeda, A., Terencio, M.C., Alcaraz, M.J., 2001. Inhibition of leukocyte functions by the alkaloid isaindigotone from *Isatis indigotica* and some new synthetic derivatives. J. Nat. Prod. 64, 1297–1300.

Moroney, M.A., Alcaraz, M.J., Forder, R.A., Carey, F., Hoult, J.R.S., 1988. Selectivity of neutrophil 5-lipoxygenase and cyclo-oxygenase inhibition by an anti-inflammatory flavonoid glycoside and related aglycone flavonoids. J. Pharm. Pharmacol. 40, 787–792.

Morrison, B.W., Christensen, S., Yuan, W., Brown, J., Amlani, S., Seidenberg, B., 1999. Analgesic efficacy of the cyclooxygenase-2-specific inhibitor rofecoxib in post-dental surgery pain: a randomized, controlled trial. Clin. Ther. 21, 943–953.

Mylari, B.L., Carty, T.J., Moore, P.F., Zembrowski, W.J., 1990. 1,2-Dihydro-1-oxopyrrolo[3,2,1-kl]phenothiazine-2-carboxamides and congeners, dual cyclooxygenase/5-lipoxygenase inhibitors with antiinflammatory activity. J. Med. Chem. 33, 2019–2024.

Posadas, I., Terencio, M.C., Guillén, I., Ferrándiz, M.L., Coloma, J., Payá, M., Alcaraz, M.J., 2000. Co-regulation between cyclo-oxygenase-2 and inducible nitric oxide synthase expression in the time-course of murine inflammation. Naunyn-Schmiedeberg's Arch. Pharmacol. 361, 98–106.

Rainsford, K.D., 1999. Inhibition by leukotriene inhibitors, and calcium and platelet-activating factor antagonists, of acute gastric and intestinal damage in arthritic rats and in cholinomimetic-treated mice. J. Pharm. Pharmacol. 51, 331–339.

Riendeau, D., Percival, M.D., Boyce, S., Brideau, C., Charleson, S., Cromlish, W., Ethier, D., Evans, J., Falgueyret, J.-P., Ford-Hutchinson, A.W., Gordon, R., Greig, G., Gresser, M., Guay, J., Kargman, S., Léger, S., Mancini, J.A., O'Neill, G., Ouellet, M., Rodger, I.W., Thérien, M., Wang, Z., Webb, J.K., Wong, E., Xu, L., Young, R.N., Zamboni, R., Prasit, P., Chan, C.-C., 1997. Biochemical and pharmacological profile of a tetrasubstituted furanone as a highly selective COX-2 inhibitor. Br. J. Pharmacol. 121, 105–117.

Rioja, I., Terencio, M.C., Ubeda, A., Alcaraz, M.J., Tarraga, A., Gonzalez-Tejero, A., 2000. Effects of some pyrrolo[2,1-*b*]quinazoline-3-one derivatives on macrophage functions. Methods Find. Exp. Clin. Pharmacol. 22, 512.

Seibert, K., Zhang, Y., Leahy, K., Hauser, S., Masferrer, J., Perkins, W., Lee, L., Isakson, P., 1994. Pharmacological and biochemical demonstration of the role of cyclooxygenase 2 in inflammation and pain. Proc. Natl. Acad. Sci. U.S.A. 91, 12013–12017.

Smith, W.L., DeWitt, D.L., 1996. Prostaglandin endoperoxide H synthases-1 and -2. Adv. Immunol. 62, 167–215.

Smith, C.J., Zhang, Y., Koboldt, C.M., Muhammad, J., Zweifel, B.S., Shaffer, A., Talley, J.J., Masferrer, J.L., Seibert, K., Isakson, P.C., 1998. Pharmacological analysis of cyclooxygenase-1 in inflammation. Proc. Natl. Acad. Sci. U.S.A. 95, 13313–13318.

Sugishita, E., Amagaya, S., Ogihara, Y., 1981. Anti-inflammatory testing methods: comparative evaluation of mice and rats. J. Pharmacobio-Dyn. 4, 565–575.

Tonussi, C.R., Ferreira, S.H., 1999. Tumour necrosis factor-alpha mediates carrageenin-induced knee-joint incapacitation and also triggers overt nociception in previously inflamed rat knee-joints. Pain 82, 81–87.

Tsuji, F., Miyake, Y., Horiuchi, M., Mita, S., 1998. Involvement of leukotriene B_4 in murine dermatitis models. Biochem. Pharmacol. 55, 297–304.

Vane, J.R., Botting, R.M., 1998. Mechanism of action of nonsteroidal antiinflammatory drugs. Am. J. Med. 104, 2S–8S.

Wallace, J.L., Bak, A., McKnight, W., Asfaha, S., Sharkey, K.A., MacNaughton, W.K., 1998. Cyclooxygenase 1 contributes to inflammatory responses in rats and mice: implications for gastrointestinal toxicity. Gastroenterology 115, 101–109.

Yamamoto, T., Nozaki-Taguchi, N., 1996. Analysis of the effects of cyclooxygenase (COX)-1 and COX-2 in spinal nociceptive transmission using indomethacin, a non-selective COX inhibitor, and NS-398, a COX-2 selective inhibitor. Brain Res. 739, 104–110.

Zhang, Y., Shaffer, A., Portanova, J., Seibert, K., Isakson, P.C., 1997. Inhibition of cyclooxygenase-2 rapidly reverses inflammatory hyperalgesia and prostaglandin E_2 production. J. Pharmacol. Exp. Ther. 283, 1069–1075.