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Investigation of anti-inflammatory potential of 5-(3,5-di-*tert*-butyl-4-hydroxybenzylidene)-2-thioxodihydropyrimidine-4,6(1H,5H)-dione compound

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ABSTRACT

The aim of this study was to synthesise the novel di-*tert*-butylphenol compound, 5-(3,5-di-*tert*-butyl-4-hydroxybenzylidene)-2-thioxo-dihydropyrimidine-4,6(1H, 5H)-dione (LQFM218), and evaluate the potential anti-nociceptive and anti-inflammatory activities in acute (mice) models *in vivo*. The compound was tested on acute models of pain such as acetic acid-induced abdominal writhing, formalin-induced nociception and carrageenan-induced mechanical hyperalgesia. The anti-inflammatory activity was observed in paw oedema, carrageenan-induced pleurisy tests and inflammatory mediator quantification. Key findings: oral treatment with the LQFM218 (50, 100 or 200 mg/kg) reduced abdominal writhing (18.8%, 31.6% and 48.3%). The dose intermediate (100 mg/kg) reduced the nociception in the second phase of the formalin test (61.4%), and also showed anti-hyperalgic activity in carrageenan-induced mechanical hyperalgesia (until 42.3%). In acute inflammation models, the treatment of mice LQFM218 (100 mg/kg) reduced the paw oedema all the time (33.8%, 42.6%, 37.4% and 36%) and in pleurisy test reduced: polymorphonuclear cell migration (35.4%), myeloperoxidase activity (52.2%) and the levels of inflammatory mediators such as PGE_2 (23.0%), TNF-α (67.6%) and IL-1β (53.4%). The present study showed that LQFM218 effectively reduced the nociception and inflammation in different models, and its mechanism might be related to the reduction of PGE_2 and pro-inflammatory cytokines. These findings show LQFM218 as a potential anti-inflammatory drug.

1. Introduction

Inflammation is a defence mechanism of organisms against tissue damage, and its main role is to eradicate the aggressive agent and restore tissue homeostasis (Ferreira, 2013; Oliveira Júnior et al., 2016;). This process is marked by the activation of many intracellular pathways, with leukocytes being attracted to the injury site. These cells can release a diverse number of inflammatory mediators that amplify the inflammatory response. Among these mediators are several cytokines (e.g. IL-1, TNF-alpha) and eicosanoids (PGE_2) (Nathan, 2002; Turner et al., 2014).

Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used in the pharmacological management of pain and acute and chronic inflammation; however, the continuous use of these drugs is associated with adverse effects, such as acute kidney failure, cardiovascular toxicity and gastroduodenal lesions (Gonzalez-Gay and Gonzalez-Juanatez, 2017; Lichtenberger et al., 2012; Perkins and Kniss, 1997; Vonkeman and van de Laar, 2010). Thus, it is necessary to discover new anti-inflammatory drugs. The development of dual

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cyclooxygenase/5lipoxygenase (COX/5LOX) inhibitors have been proposed to reduce the adverse effects of NSAIDs.

A relevant source of dual COX/5-LOX inhibitors is the di-*tert*-butylphenol class of anti-inflammatory compounds. These agents have antioxidant and radical scavenging activities, effects which have been proposed to be adjuvant to their anti-inflammatory efficacy and low ulcerogenic damage (Swingle et al., 1985; Janusz et al., 1998).

Tebufelone was previously identified as a promising member of the di-*tert*- butylphenol class. This drug was designed using structure-activity relationship analyses of several anti-inflammatory arachidonic acid analogues and anti-oxidants (Weisman et al., 1994). Some studies have demonstrated the potential anti-inflammatory, analgesic and anti-pyretic effect of tebufelone with the addition of an enhanced safety profile relative to traditional NSAIDs (Vargas et al., 1990).

Compounds of the di-*tert*-butyl phenol class are widely studied due to their pharmacological activities (Leval et al., 2005). *In vivo* studies with compounds of this class have shown antipyretic and analgesic as well as anti-inflammatory effects (Shirota et al., 1987; Lino et al., 2017). In addition, *in vitro* studies have shown a reduction in the activity of the enzymes cyclooxygenase and lipoxygenase and in the production of leukotriene B_4 and PGE_2 (Hidaka et al., 1984; Moore and Swingle, 1982; Bendele et al., 1992; Lino et al., 2017).

In this context, we describe the compound LQFM218 **(3)**, which was designed from the darbufelone and thiobarbituric acid prototypes through the molecular hybridization strategy. The compound LQFM218 **(3)** maintains in its structure, with the 2,6-di-*tert*-butylphenol subunit present in darbufelone along with the thiobarbituric acid subunit. This strategy has been used successfully in drug discovery programs (Lima and Barreiro, 2005). This study aimed to synthesise and evaluate the pharmacological activity of the anti-inflammatory drug prototype, in different of pain and inflammatory models, seeking to characterise the possible mechanisms of action involved.

2. Material and methods

2.1. Drugs and chemicals

The chemicals used in this study were acetic acid (Merck AG, Darmstadt, Germany), carrageenan (Sigma-Aldrich St. Louis, MO, USA), dexamethasone (Decadron®, Ache, Brazil), DMSO (Sigma-Aldrich St. Louis, MO, USA), formaldehyde (Synth, Brazil), heparin (Cristália, SP, Brazil), hydrogen peroxide (Bioshop, GO, Brazil), indomethacin (Indocid®, Merck Sharp & Dohme Farmacêutica-Ltda), May-Grunwald-Giemsa (Doles Reagentes, Brazil), morphine hydrochloride (Dimorf®, Cristalia, SP, Brazil), *o*-dianisidine (Sigma-Aldrich St. Louis, MO, USA), sodium azide (Sigma-Aldrich St. Louis, MO, USA), Türk liquid (Bioshop, GO, Brazil), PGE_2 (Elisa kit, Cayman Chemical), TNFα and IL-1β (Elisa kit, eBioscience, USA), and LQFM218 **(3)**. The latter was dissolved in 10% DMSO in distilled water, while all other drugs were dissolved in distilled water. The doses of LQFM218 **(3)** were based on the doses of another similar compound (LQFM-091) studied by our research group (Lino et al., 2017).

2.2. Synthesis of LQFM218 (3) compound

The compound 5-(3,5-di-*tert*-butyl-4-hydroxybenzylidene)-2-thioxodihydropyrimidine-4,6(1H, 5H)-dione (LQFM218) was synthesised by "Laboratório de Química Farmacêutica Medicinal" (LQFM), Faculty of Pharmacy, Federal University of Goias, under the supervision of Prof Dr Ricardo Menegatti.

General: Reactions were monitored by TLC using commercially available pre-coated plates (Whatman 60 F254 silica) and the developed plates were examined under UV light (254 and 365 nm). ^{1}H spectra were recorded in the indicated solvent on a Bruker Avance III 500 MHz spectrometer. Chemical shifts are quoted in parts per million downfield of TMS and the coupling constants are in Hertz. All assignments of the signals of ^{1}H spectra are consistent with the chemical structures of the products described. The organic solutions were dried over anhydrous sodium sulphate and organic solvents were removed under reduced pressure in a rotary evaporator. Mass spectra (MS) were obtained with a microTOF III (Brucker Daltonics Bremen, Germany). The sample preparation for MS analysis consisted of diluting 1 μg of sample in 1 ml of methanol. To perform the analysis in the positive mode, 1 μl of formic acid was added to the sample. The solution obtained was directly infused at a flow rate of 3 μl/min into the ESI source. ESI(+) source conditions were as follows: nebuliser with nitrogen gas, pressure of 0.4 bar and temperature of 200 °C, capillary voltage of −4 kV, transfer capillary temperature of 200 °C, drying gas of 4 L min^{-1}, end plate offset of −500 V, skimmer of 35 V and collision voltage of −1.5 V. Each spectrum was acquired using 2 microscans. The resolving power is: m/Δm50% 16,500.00, where Δm50% is the peak full width at half-maximum peak height. Mass spectra were acquired and processed with Data Analysis software (Brucker Daltonics, Bremen, Germany).

Synthesis of 5-(3,5-di-*tert*-butyl-4-hydroxybenzylidene)-2-thioxodihydropyrimidine-4,6(1H,5H)-dione (3) (Fomenko et al., 2015). In a typical run, a mixture of 2-thiobarbituric acid (**5**) (5.0 mmol), 3, 5-di-*tert*-butyl-4-hydroxybenzaldehyde (**4**) (5.0 mmol) and anhydrous sodium acetate (5 mmol) in 10 ml of glacial acetic acid as solvent and was stirred at 90 °C for 4 h. The reaction mixture was poured out in water and acidified to pH = 5; the precipitate formed was filtered off under vacuum and dried. The crude product was solubilised in 3 ml of DMF and poured out in (H_2O/AcOH = 9:1) to 5-(3,5-di-*tert*-butyl-4-hydroxybenzylidene)-2-thioxodihydropyrimidine-4,6(1H, 5H)-dione (**3**) (1260 mg, 70%) as a yellow solid, M.P. = 198 °C, *Rf* = 0.48 (hexane:ethyl acetate, 70:30): ^{1}H-NMR (500.13 MHz) $CDCl_3$ *δ*: 8.42 (1H, s, H-1'), 7.72 (2H, s, H-2 and 6) 5.84 (1H, s, –OH), 1.48 (18H, s, H-7 and 8); MS: $[M+H]^+$ *m/z* of 361.1642; purity >98%.

2.3. Animals

All experiments were performed with adult male *Swiss* albino mice (weighing 27–32 g) obtained from the Central Animal House of UFG. The animals were kept at a controlled temperature (22 ± 2 °C) and humidity from 50 to 55%, under a 12-h light/dark cycle with food and water available *ad libitum*. Animal studies are reported in compliance with the ARRIVE guidelines (Koster, 1959; McGrath and Lilley, 2015). The animals were acclimatised for 7 days before the start of the experiments. All experiments were performed in accordance with the national and international legislation (guidelines of Brazilian Council of Animal Experimentation and of the U.S. Public Health Service's Policy on Humane care and Use of Laboratory Animals-PHS Policy), under the ethical guidelines established for investigations of experimental pain in conscious animals (Zimmermann, 1983). To determine the size of each group of animals, we respect the principle of the 3 Rs (replacement, reduction, and refinement). The study was approved by the Ethics Committee in Research of the Federal University of Goias (052/18).

2.4. Pharmacological models of nociception and inflammation acute

2.4.1. Acetic acid-induced abdominal writhing test

Acetic acid-induced nociception was achieved as described previously by Koster et al. (1959). Groups of mice (n = 8) were treated by gavage (p.o.) with vehicle (10% DMSO 10 ml/kg), LQFM218 **(3)** at doses of 50, 100 or 200 mg/kg or indomethacin (10 mg/kg, positive control) 60 min before the application of acetic acid solution (1.2% v/v; 10 ml/kg, i.p.). The number of abdominal constrictions (writhing) was counted for each animal over a period of 30 min after acetic acid injection; the results are expressed as the mean ± S.E.M. of the number of writhing.

2.4.2. Formalin-induced nociception test

The formalin-induced nociception was performed as described

previously by Hunskaar and Hole (1987). Groups of mice (n = 8) were treated with vehicle (10% DMSO, 10 ml/kg, p.o.), LQFM218 **(3)** (100 mg/kg, p.o.) or indomethacin (10 mg/kg, p.o. - positive control for anti-nociceptive activity in the second phase) or morphine (5 mg/kg, s.c. - positive control for anti-nociceptive activity in the first and second phase). Sixty min after the p.o. treatment, or 30 min after s.c. treatment, 20 μl of 3% formalin was administered into the plantar surface of the right hind paw. Pain reaction time (licking time) was assessed during two periods: first phase (0–5 min), where neurogenic pain is caused by direct stimulation of the nociceptors, and the second phase (15–30 min), where inflammatory pain is caused by release of inflammatory mediators. These results were expressed as the means ± S.E.M. of licking time in s (Florentino et al., 2015).

2.4.3. Carrageenan-induced mechanical hyperalgesia test

Mechanical pressure was applied focally to the plantar surface of the hind paw, which was placed between a pointed probe tip and a flat surface. The pressure was then increased at a constant rate until a nociceptive behavioural response was observed (Deuis et al., 2017). In this experiment, the animals were separated into three groups of male mice (n = 8). Each experimental group was treated with: vehicle (control, 10% DMSO, 10 ml/kg p.o.), LQFM218 **(3)** (100 mg/kg, p.o.) or dexamethasone (1 mg/kg, positive control) 1 h before the injection of 50 μl of 1% (w/v) carrageenan into the right paw. The left paw which received the same volume of 0.9% (w/v) NaCl solution was used as control. The nociceptive behaviour in response to the mechanical stimulus was measured from the exposure of the inflamed and non-inflamed hind paw to an increasing force until the appearance of a nociceptive reaction (vocalisation or paw withdrawal). The stimulation was stopped when mice struggled to withdraw the corresponding paw, and the force value was recorded. Measurements were alternately performed on each paw. The cut-off for each animal was 450 g. The nociceptive threshold was evaluated by differences in pressure (Δ) between the two paws (inflamed and non-inflamed) at 1, 2, 3 and 4 h after the carrageenan injection, using an analgesimeter Raldall Selitto (Insight EFF-440, Brazil). The baseline was performed before the treatments (time 0) for each animal. The results were expressed as means ± S.E.M., in grams (Randall, 1957; Capua et al., 2016).

2.4.4. Carrageenan-induced paw oedema test

Experimental groups of male mice (n = 8) were treated with vehicle (control, 10% DMSO, 10 ml/kg p.o.), LQFM218 **(3)** (100 mg/kg, p.o.) or dexamethasone (1 mg/kg, positive control) 1 h before the injection of 50 μl of 1% (w/v) carrageenan in the right paw. The left paw, which received the same volume of 0.9% (w/v) NaCl solution, was used as a control. Oedema was measured using a plethysmometer (Ugo Basile Co. - Italy) at different times (1, 2, 3 and 4 h) after the injection of carrageenan. The baseline was performed before the treatments (time 0) for each animal. The results were expressed as the difference between the volume of the paw that received carrageenan and the volume that received saline injection, at the specified time intervals (Passos et al., 2007; Nascimento et al., 2016).

2.4.5. Carrageenan-induced pleurisy test

The pleurisy test was performed as described previously by Saleh et al. (1999). Experimental groups of mice (n = 8) were treated with vehicle (10% (v/v) DMSO, 10 ml/kg p.o.), LQFM218 **(3)** (100 mg/kg, p.o.) or dexamethasone (1 mg/kg, positive control) 1 h before the injection of 100 μl of 1% carrageenan into the pleural cavity. The pleural exudate was collected with 1 ml of heparinised PBS 4 h after carrageenan administration. One aliquot was used to determine the total leukocyte content using Türk liquid in a Neubauer's chamber, and the other aliquot was used for the measurement of myeloperoxidase activity and cytokine levels.

2.4.6. Myeloperoxidase (MPO) assay

Measurement of the myeloperoxidase (MPO) activity was determined as described previously by Lin et al., 2004. Briefly, 40 μl of the pleural exudate samples, the animals treated with vehicle (10% (v/v) DMSO, 10 ml/kg p.o.), LQFM218 **(3)** (100 mg/kg, p.o.) or dexamethasone, was added to 360 μl of phosphate buffer pH 6.0 containing 0.167 mg/ml of o-dianisidine 2 HCl and 0.0005% H_2O_2. The enzyme reaction was stopped after 15 min by adding 20 μl of 1% (w/v) sodium azide. The samples were centrifuged subsequently for 5 min at 300 g. The supernatant (100 μl) was transferred to a microplate well, and the absorbance was monitored at a wavelength of 450 nm (Saleh et al., 1999). The results were expressed as means ± S.E.M. of enzymatic activity in mU/ml.

2.4.7. Measurement of cytokines

The pleural exudates of mice treated with vehicle (10% (v/v) DMSO, 10 ml/kg p.o.), LQFM218 **(3)** (100 mg/kg, p.o.) or dexamethasone (1 mg/kg, positive control) were also used to determine the concentrations of TNF-α and IL-1β using an immunosorbent assay kit (ELISA) (Ebioscience). Samples were collected 4 h after the induction of pleurisy and centrifuged at 1200 g for 10 min at 4 °C, and the supernatant was separated and stored (−70 °C) until required for the assay. The results were expressed as mean ± S.E.M. in pg/ml (Fröde et al., 2001; Costa et al., 2013).

2.4.8. Measurement of prostaglandin E2 (PGE2)

The concentration of PGE_2 in pleural exudate samples was determined by ELISA using a commercial kit (Cayman Chemical). Samples were collected 4 h after the induction of pleurisy, with PBS containing 10 μM indomethacin, and were then centrifuged at 1200 g for 4 min at 4 °C, followed by separation and storage (−70 °C) of the supernatant until required for the assay. Then, the basal PGE_2 values were removed from all the samples (Florentino et al., 2016). The results were expressed as mean ± S.E.M. of the PGE_2 levels in the pleural exudate (pg/ml).

2.5. Statistical analysis

All of the data were analysed statistically by one-way ANOVA followed by *Tukey's* test as post-hoc or by two-way ANOVA followed by *Bonferroni's* test as post-hoc. Values of $P \leq 0.05$ were considered significant. All statistical analyses were carried out using Graph Pad Prism version 5.0.

3. Results

3.1. Synthesis of LQFM218 (3)

The structural design of the molecular hybridisation strategy used darbufelone (**1**) and thiobarbiturate (**2**) (Fig. 1A). As illustrated in Fig. 1B, compound (**3**) was obtained through Knoevenagel reaction in 70% yield.

3.2. Evaluation of the effects of LQFM218 in the tests of nociception and inflammation acute

3.2.1. Acetic acid-induced abdominal writhing test

The oral treatments with LQFM218 **(3)** at doses 50, 100 or 200 mg/kg decreased the amount of writhing induced by acetic acid in a dose-dependent manner compared to the negative control group (vehicle 10% DMSO 10 ml/kg) from 89.9 ± 4.2 (group treated with vehicle) to 73.0 ± 2.4 (18.8%, $P \leq 0.01$), 61.5 ± 2.0 (31.6%, $P \leq 0.001$) and 46.5 ± 2.4 (48.3%, $P \leq 0.001$), respectively. The positive control, indomethacin (10 mg/kg), reduced the amount of writhing by 52.9 ± 2.9 (41.2%, $P \leq 0.001$) (Fig. 2A).

3.2.2. Formalin-induced nociception test

In the formalin test, LQFM218 **(3)** in an intermediate dose (100 mg/

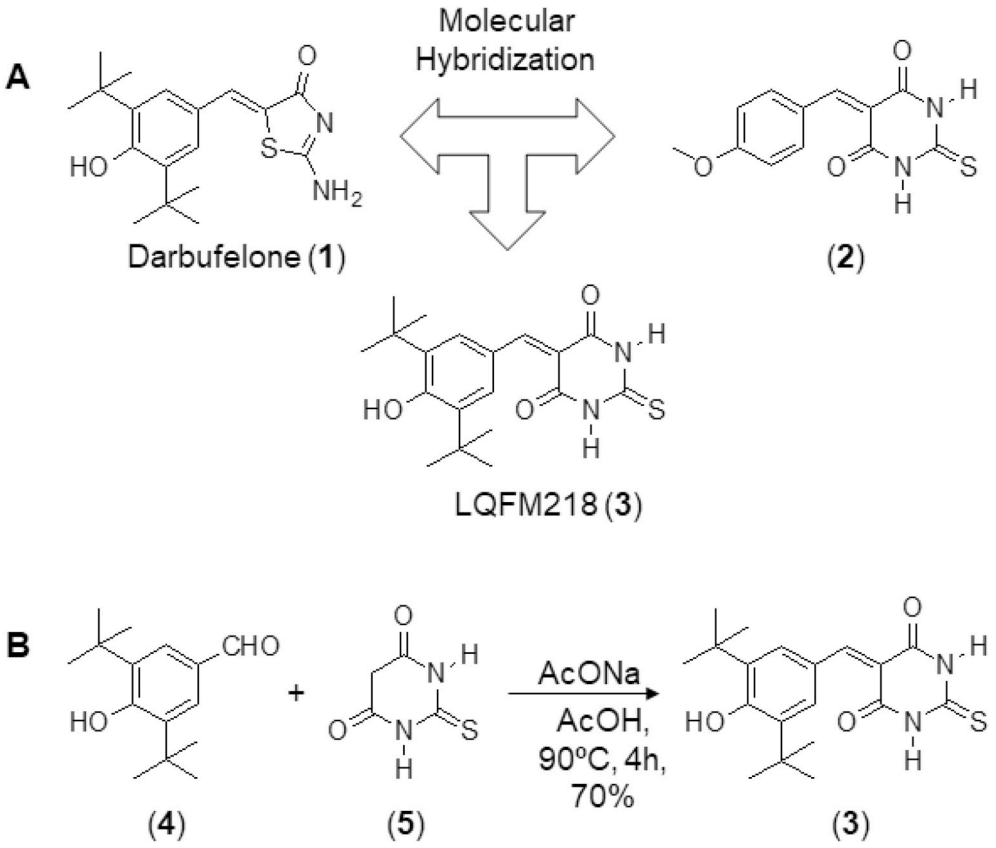

Fig. 1. (A) Structural design of LQFM218 (**3**) from darbufelone (**1**) and thiobarbiturate (**2**); (B) Synthetic route for the preparation of 5-(3,5-di-*tert*-butyl-4-hydroxybenzylidene)-2-thioxodihydropyrimidine-4,6(1H, 5H)-dione (**3**) – LQFM218.

kg, p.o.) showed anti-nociceptive activity when compared to the control group only in the second phase of the test. In the second phase, the vehicle group showed a licking time (s) of 189.8 ± 6.6; LQFM218 **(3)** reduced this time to 73.2 ± 7.7 (61.4%, $P < 0.001$). The group treated with the positive controls indomethacin (10 mg/kg, p.o.) and morphine (5 mg/kg, s.c.) also decreased licking time in the second phase to 79.4 ± 10.5 (58%, $P < 0.001$) and 2.6 ± 1.0 (98.6%, $P < 0.001$), respectively. The positive control morphine also decreased the first phase of 54.4 ± 1.8 (vehicle group) to 2.5 ± 0.8 (95.4%, $P < 0.001$), as shown in Fig. 2B-C.

3.2.3. Carrageenan-induced mechanical hyperalgesia test

The treatment with LQFM218 **(3)** (100 mg/kg) reduced the difference in the nociceptive threshold between the non-inflamed and inflamed paws of the animals in response to mechanical stimulus at all times of the paw pressure test. In the first h of the test, compared with the negative control group (168 ± 2.49 g), the treatments with LQFM218 **(3)** (100 mg/kg) and dexamethasone (1 mg/kg) reduced mechanical hyperalgesia by 31.9% ($P < 0.001$) and 32% ($P < 0.001$), respectively. In the second h of the test, compared with the negative control group (168 ± 4.67 g), the treatments with LQFM218 **(3)** and dexamethasone reduced mechanical hyperalgesia by 36.5% ($P < 0.001$) and 36.5% ($P < 0.001$), respectively. In the third h of the test, compared with the negative control group (162 ± 3.60 g), the treatments with LQFM218 **(3)** or dexamethasone reduced mechanical hyperalgesia by 42.3% ($P < 0.001$) and 42.3% ($P < 0.001$), respectively. In the fourth h of the test, compared with the negative control group (140 ± 2.11 g), the treatments with LQFM218 **(3)** or dexamethasone reduced mechanical hyperalgesia by 35.7% ($P < 0.001$) and 35.7% ($P < 0.001$), respectively (Fig. 3A).

3.2.4. Carrageenan-induced paw oedema test

The treatments with LQFM218 **(3)** (100 mg/kg) reduced the paw oedema formation at all times of evaluation. In the first h of the test, compared with the negative control group (113.3 ± 8.7 μl), the treatments with LQFM218 **(3)** or dexamethasone (1 mg/kg) reduced the oedema by 33.8% and 37.3% ($P \leq 0.001$), respectively. In the second h, compared with the negative control group 116.7 ± 8.7 μl, the oedema was reduced by 42.6% and 47.6% ($P \leq 0.001$), respectively. In the third h, compared with the negative control group 102.2 ± 6.19 μl, the oedema was reduced by 37.4% and 50% ($P \leq 0.001$), respectively. In the fourth h, compared with the negative control group 92.2 ± 4.3 μl, the oedema was reduced by 36% and 49.4% ($P \leq 0.001$), respectively (Fig. 3B).

3.2.5. Carrageenan-induced pleurisy test and myeloperoxidase (MPO) assay

The injection of carrageenan into the pleural cavity of mice induced an acute inflammatory response characterized by the accumulation of leukocytes. The oral treatments with LQFM218 **(3)** (100 mg/kg) or dexamethasone (1 mg/kg) reduced the leukocyte count in the pleural cavity by 35% and 65.5% ($P \leq 0.001$), respectively, when compared to the negative control group (5.06 ± 0.22 ml leukocytes × 10^6/ml). These reductions were due to a decrease in polymorphonuclear cells of 35.4 ($P \leq 0.01$) and 78% ($P \leq 0.001$), and mononuclear cells 34.5 ($P \leq 0.05$) and 45.1% ($P \leq 0.01$), respectively, showed in Fig. 4A. In addition, the Fig. 4B shows that treatment with LQFM218 **(3)** and dexamethasone decreased the myeloperoxidase activity by 52.2% ($P \leq 0.01$) and 54.2% ($P \leq 0.001$), compared with that in the negative control group (257.1 ± 32.9 mU/ml).

3.2.6. Measurement of cytokines

The treatment with LQFM218 **(3)** (100 mg/kg) showed a significant

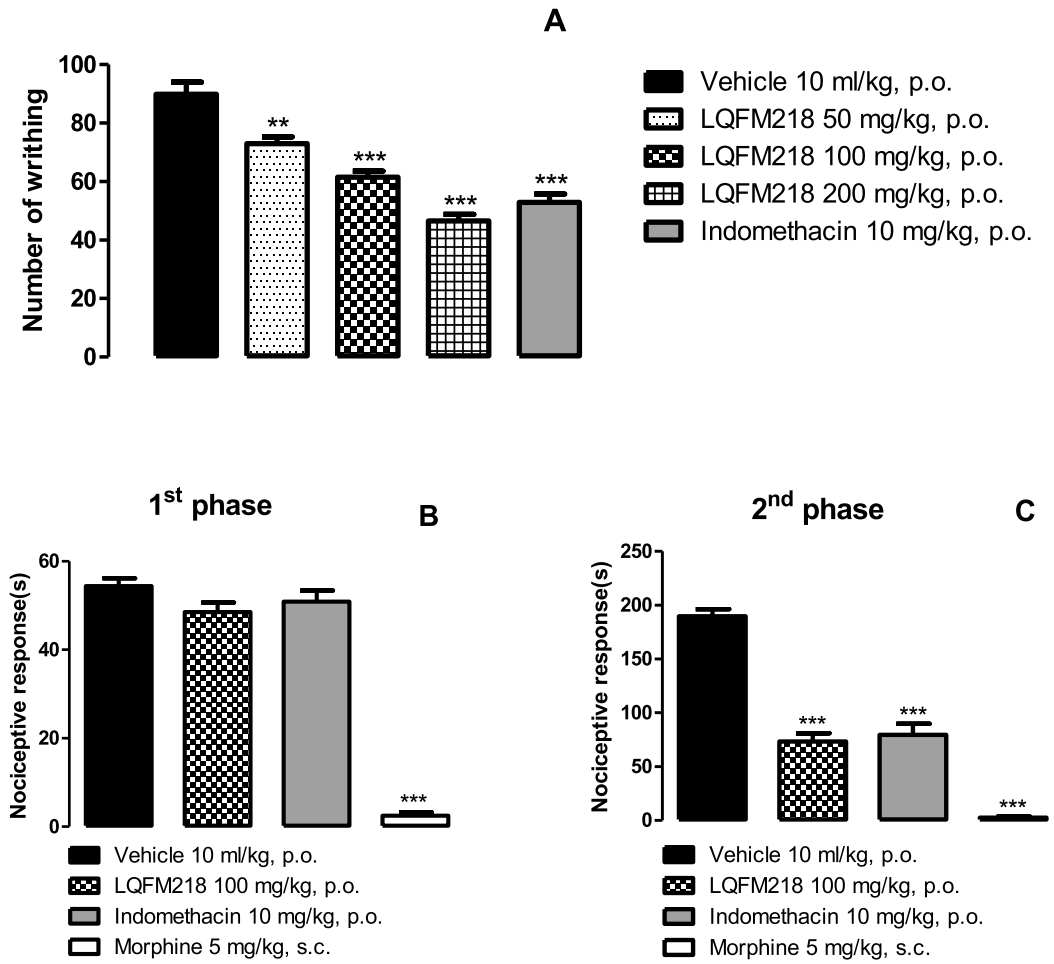

Fig. 2. **A**Effect of LQFM218 **(3)** on acetic acid-induced writhing test in mice (n = 8). Vehicle (control), LQFM218 **(3)** (50, 100 and 200 mg/kg) or Indomethacin (Indo, 10 mg/kg) were administered (p.o.) 1 h before acetic acid injection (1.2%, i.p.). Vertical bars represent mean ± S.E.M. of number of writhing in 30 min for each experimental group. **P ≤ 0.01 and ***P ≤ 0.001 *vs.* control group-vehicle (one-way ANOVA followed by *Tukey's* test).
(B–C). Anti-nociceptive effect of LQFM218 **(3)** on the pain induced by the formalin test in mice (n = 8). (**B**) Represents the first (0–5 min). (**C**) Represents the second phase (15–30 min). Oral treatment with LQFM218 **(3)** (100 mg/kg) reduced the licking time in only the second phase of the test. Indomethacin (10 mg/kg, p.o.) and morphine (5 mg/kg, s.c.) were used as positive controls, during the first (0–5 min) and second phase (15–30 min). Vertical bars represent mean ± S.E.M. of licking time of paw, in s. ***P ≤ 0.001 *vs.* control group-vehicle (one-way ANOVA followed by *Tukey's* test).

reduction in TNF-α levels (% inhibition of 67.6, P ≤ 0.001), when compared with the negative control group 18.2 ± 2.1 pg/ml (Fig. 5A). The same dose also reduced IL-1β levels (% inhibition of 53.6, P ≤ 0.05), when compared with the negative control group (122.1 ± 20.6 pg/ml) (Fig. 5B). As expected, dexamethasone (1 mg/kg) showed the expected profile through the inhibition of pro-inflammatory cytokines (% inhibition on TNF-α of 88.2 and IL-1β of 95.5, P ≤ 0.001).

3.2.7. *Measurement of prostaglandin E_2 (PGE_2)*

PGE_2, a major product of the enzymatic reaction catalysed by COX-2, was also analysed. Compared with the negative control group (21.560 ± 177.3 pg/ml), oral treatment with LQFM218 **(3)** 100 mg/kg showed a significant reduction in PGE_2 levels, by 23% (P ≤ 0.001). Dexamethasone showed the expected profile and reduced the PGE_2 levels by 24% (P ≤ 0.001) (Fig. 5C).

4. Discussion

Compounds that belong to the di-*tert*-butyl-phenol class have anti-inflammatory and analgesic effects via different mechanisms of action (Bishayee and Khuba-Bukhsh, 2013; Dee et al., 1991; Clive and Stoff, 1984; Lino et al., 2017; Marques et al., 1986). Thus, this work aimed to evaluate such activities in a new compound of this class, 5- (3, 5-di-*tert*-butyl-4-hydroxybenzylidene)-2-thioxo-dihydropyrimidine-4.6 (1H, 5H)-dione (LQFM218) **(3)**, using acute models of pain and inflammation, and suggest a possible mechanism of action according to the results identified.

Initially, to evaluate the anti-inflammatory and/or analgesic activity of the compound LQFM218 **(3)**, the acetic acid-induced abdominal writhing test was performed. This test allows the anti-nociceptive activity of different anti-inflammatory and analgesic substances that act at central and/or peripheral mechanisms to be evaluated (Peraza et al., 2007). The oral treatments with LQFM218 **(3)** at doses of 50, 100 and 200 mg/kg reduced the amount of writhing induced by acetic acid for all doses tested. A similar effect was observed in compounds that belong to the same class, suggesting a potential analgesic effect to new compounds of this class (Lee and Us, 2010; Lino et al., 2017; Scherz et al., 1996). The LQFM218 (3) doses (50, 100 and 200 mg/kg) were chosen considering the treatment schedule used in other study performed by our group (Lino et al., 2017). In the next tests, only the intermediate dose of LQFM218 **(3)** was used to reduce the number of animals, as recommended by the Ethics Committee in Research and which also showed a maximal effect.

Despite the high degree of sensitivity of the abdominal writhing test, this test presents low specificity; therefore, the formalin test was employed to confirm and characterise this effect (Le Bars, 2001). The

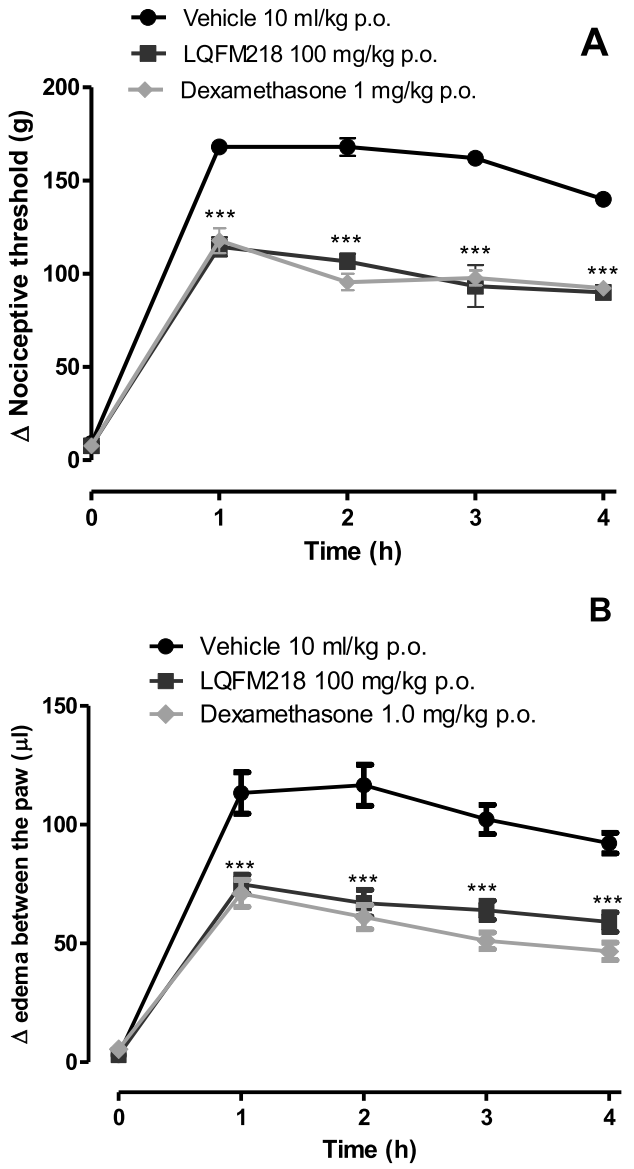

Fig. 3. (A–B). Anti-hyperalgesic effect of LQFM218 **(3)** (100 mg/kg, p.o.) or dexamethasone (Dexa, 1 mg/kg, p.o. — positive control) on the carrageenan-induced mechanical hyperalgesia test **(A)** and on the carrageenan-induced oedema test **(B)**, in mice (n = 8). The values were expressed as mean ± S.E.M. of the difference between the non-inflamed and inflamed paws, in grams (g). ***P ≤ 0.001 *vs.* control group-vehicle (two-way ANOVA followed by *Bonferroni's* test).

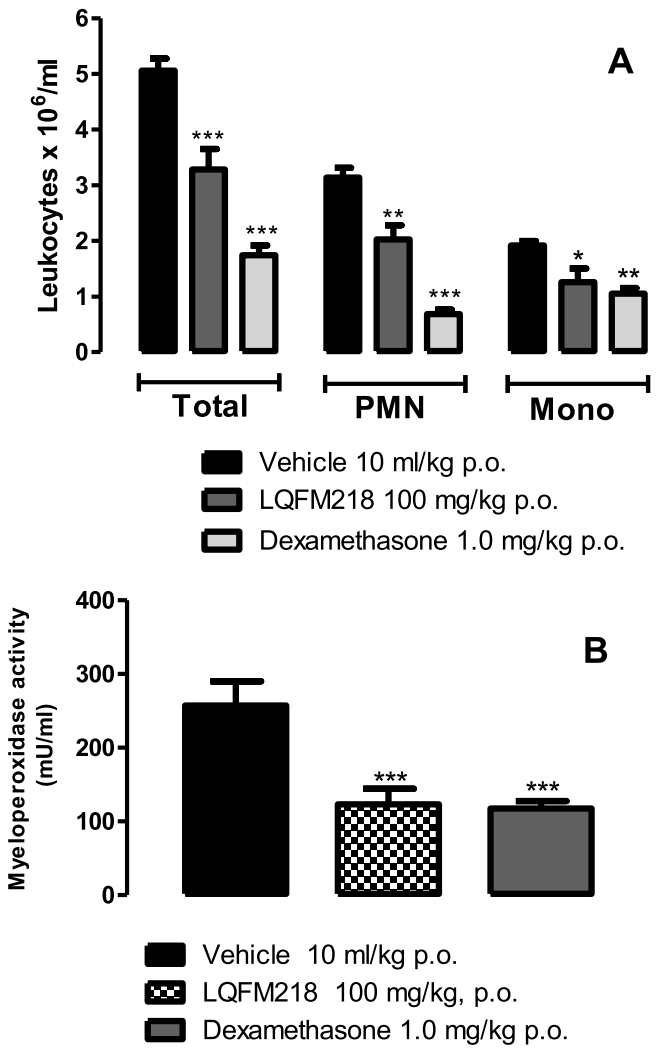

Fig. 4. (A–B). Anti-inflammatory effect of LQFM218 **(3)** (100 mg/kg, p.o.) or dexamethasone (1 mg/kg, p.o. - positive control) on the carrageenan-induced pleurisy test, in mice. **(A)** Represents leukocyte count in the pleural cavity. **(B)** Myeloperoxidase enzyme activity (mU/ml). Values are expressed as mean ± S.E.M. of 8 mice. ***p ≤ 0.001 *vs.* control group-vehicle (one-way ANOVA followed by *Tukey's* test).

injection of formalin into the hind paw enables the reproduction of a biphasic nociceptive response. The first phase (neurogenic pain) is evaluated in the period from 0 to 5 min, while the second phase (inflammatory pain) is in the period from 15 to 30 min (Hunskaar and Hole, 1987). The first phase is subject to the influence of opioid analgesic agents and some downward path agonists, while the second phase of the test is influenced by steroidal and non-steroidal anti-inflammatory and opioid drugs (Abbott et al., 1995; Corrêa and Calixto, 1993; Dubuisson and Dennis, 1977; Le Bars, 2001; Omote et al., 1998; Shibata et al., 1989). It was observed that the compound LQFM218 **(3)** at a dose of 100 mg/kg reduced the time of reactivity to pain only in the second phase of the formalin test, suggesting an anti-nociceptive effect mediated by an anti-inflammatory response.

The Randal-Sellito test was performed to confirm this hypothesis, based on the principle that the inflammation generated by the application of an inflammatory agent, such as carrageenan, reduces the nociceptive threshold, and that anti-inflammatory agents and analgesics are able to modulate this action (Anseloni et al., 2003; Chipkin et al., 1983). The intraplantar injection of carrageenan induces an intense inflammatory process, promoting the sensitisation of the primary nociceptive fibres in the inflamed site and the excitability of neurons in the dorsal horn of the spinal cord. (Handy and Moore, 1998). The results obtained in the mechanical hyperalgesia model showed that compound LQFM218 **(3)** 100 mg/kg exhibits anti-hyperalgic activity, since it reduced the mechanical hyperalgesia from the second h to the fourth h, corroborating the results in the second phase of the formalin and abdominal writhing tests.

To characterise the anti-inflammatory effect of LQFM218 **(3)** observed in previous tests, the carrageenan-induced paw oedema test was performed to evaluate the activity of this compound on the formation of oedema. The initial stage of oedema formation is characterised by vasoconstriction followed by accentuated vasodilation and increased vascular permeability through the action of some mediators such as histamine and bradykinin, which promote the extravasation of plasma proteins (Mollnes et al., 2002). The results showed that oral administration of the compound LQFM218 **(3)**, at intermediate dose, promoted a reduction in paw oedema from the first until the fourth h of

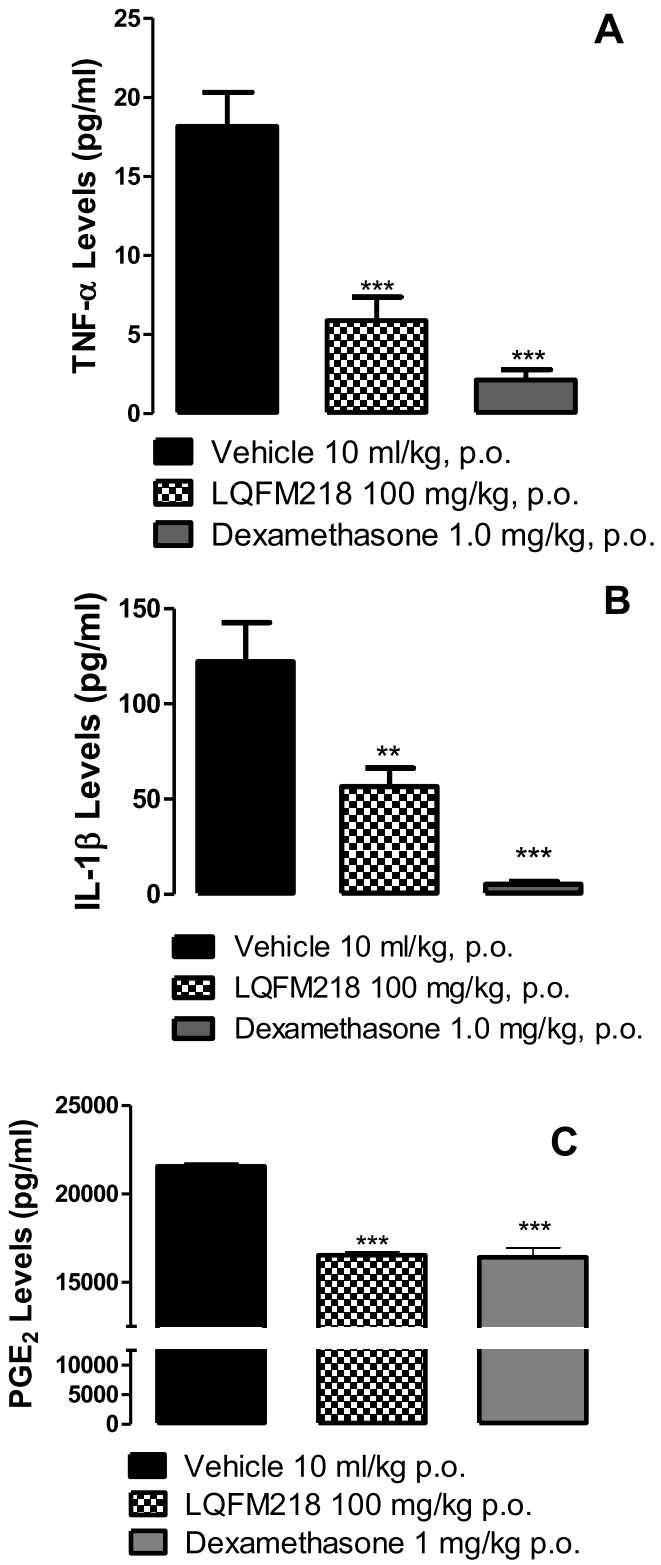

Fig. 5. (A–C). Effect of LQFM218 **(3)** (100 mg/kg, p.o.) or dexamethasone (1 mg/kg, p.o. - positive control) on cytokines levels. **(A)** Represents tumour necrosis factor-alpha (TNF-a) levels. **(B)** Represents interleukin 1β (IL-1β) levels. **(C)** Represents PGE_2 levels. Vertical bars represent mean ± S.E.M. of 8 mice. **P ≤ 0.01 and ***P ≤ 0.001 *vs.* control group-vehicle (One-way ANOVA followed by *Tukey's* test).

testing. These results demonstrate the activity anti-oedematogenic activity of the compound and that this compound may be a good anti-inflammatory drug prototype.

After observing this response with the compound LQFM218 **(3)**, the anti-inflammatory activity and the possible mechanisms of action involved were tested through the carrageenan-induced pleurisy test, where the cell migration profile was evaluated through the collection of the pleural exudate, along with the activity of the myeloperoxidase enzyme, pro-inflammatory cytokines TNF-α and IL-1β levels and PGE_2 levels. The treatment with LQFM218 **(3)** (100 mg/kg) reduced the migration of total leukocytes, promoting a reduction of both polymorphonuclear and mononuclear cells. In addition, we observed no reduction in myeloperoxidase activity. As expected, similar effects were found with the positive control dexamethasone.

Several studies related to blocking the activity of this cytokine form the basis of standard therapy for several diseases, such as autoimmune diseases and lymphomas, since both cytokines are crucial during the inflammatory process, as they are related to effects such as increased vascular permeability, induction of the expression of adhesion molecules and cytokines, and chemotaxis (Commins et al., 2010; Simon and Van Der Meer, 2007). In addition, taking into account the multiple roles of PGE_2, inhibition of the PGE_2 synthesis pathway is of relevance to several inflammation-driven diseases such as arthritis. The beneficial effects with NSAID therapy are mainly a result of the COX pathway, via inhibition of the metabolic transformation of arachidonic acid into prostanoids, especially PGE_2 (Nassar et al., 2005). The results found with LQFM218 **(3)** are promising for the development of new anti-inflammatory drugs and suggest the reduction of these inflammatory mediators as a possible mechanism of action for this new compound.

Rheumatoid arthritis is a chronic and systemic inflammatory process, characterised by inflammation of the joints and bone erosion, leading to destruction and complications such as site oedema, inability to move, chronic pain and loss of function (Marques et al., 2016; Pugner et al., 2000). Dendritic cells migrate to lymph nodes, stimulating T lymphocytes to induce the release of inflammatory cytokines such as TNF-α and IL-1β, which are responsible for endothelial changes that enhance the inflammatory response by increasing the number of inflammatory cells in the joint (Martins et al., 2006). We highlight the importance of the effect of LQFM218 **(3)** on these cytokine levels and how this compound can positively contribute to the treatment of inflammatory diseases.

In the future, it is necessary to perform more studies focused on revealing a safe toxicological and molecular target in the action mechanism which support the results found in this work, such as inhibition of the enzymes COX, 5-LOX or PLA_2. Furthermore, the potential effects should be studied in models of chronic inflammation, as well as the effects of chronic administration of this compound. In general, the present data are in agreement with previous studies showing the di-*tert*-butyl phenol class as good anti-inflammatory drugs (Shirota et al., 1987; Weisman et al., 1994; Lino et al., 2017).

5. Conclusion

This study reports the synthesis and characterisation of the antinociceptive and anti-inflammatory activities of the LQFM218 **(3)** compound, in acute models of pain and inflammation. The anti-inflammatory activity was characterised by the reduction of leukocyte migration in the pleural exudate, associated with a reduction of the myeloperoxidase activity and inflammatory pro-inflammatory cytokines and PGE_2 levels. Taken together, the present findings show LQFM218 **(3)** as a potential anti-inflammatory drug. However, more experiments should be performed to better characterise the involvement of the studied pathway in the action mechanism of the anti-inflammatory activity.

Declaration of competing interest

The authors declare that they have no conflicts of interest.

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