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Inflammopharmacology

ORIGINAL ARTICLE

Anti-inflammatory effect of a new piperazine derivative: (4-methylpiperazin-1-yl)(1-phenyl-1*H*-pyrazol-4-yl)methanone

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Abstract
Aims This study investigates the anti-nociceptive and anti-inflammatory effects of new piperazine compound (LQFM182) as well as the toxicity acute in vitro.
Main methods To evaluate the anti-nociceptive activity, the acetic acid-induced abdominal writhing test, tail flick test and formalin-induced pain test were used. The anti-inflammatory activity was evaluated using the models of paw oedema and pleurisy induced by carrageenan and some inflammatory parameters were evaluated, including cell migration, myeloperoxidase enzyme activity and the levels of TNF-α and IL-1β cytokines in pleural exudate. The acute oral systemic toxicity of LQFM182 in mice was evaluated through the neutral red uptake (nru) assay.
Key findings LQFM182 (50, 100 or 200 mg/kg, p.o.) decreased the number of writhings induced by acetic acid in a dose-dependent manner, and an intermediate dose (100 mg/kg, p.o.) reduced the paw licking time of animals in the second phase of the formalin test. Furthermore, LQFM182 (100 mg/kg, p.o.) reduced oedema formation at all hours of the paw oedema induced by carrageenan test and in pleurisy test reduced cell migration from the reduction of polymorphonuclear cells, myeloperoxidase enzyme activity and the levels of pro-inflammatory cytokines IL-1β and TNF-α. Therefore, it was classified in GHS category $300 < LD_{50} < 2000$ mg/kg.
Significance Reduction of the TNF-α and IL-1β levels.

Keywords Anti-inflammatory · *N*-arylheterocycles · IL-1β · TNF-α

Introduction

The inflammatory process is defined as a protective, dynamic and adaptive reaction of the human body against any aggressive agent (Nathan and Ding 2010; Ashley et al. 2012). This process is characterised by the production and release of pro-inflammatory mediators, such as histamine, bradykinin, prostaglandins, leukotrienes and cytokines, which leads to the appearance of cardinal signs of inflammation including pain, heat, redness and tumour; if there is no proper resolution of the inflammatory process, it may result in the loss of function of the affected tissue (Nathan 2002; Medzhitov 2008).

Among the pro-inflammatory mediators is the histamine which belongs to class of vasoactive amines. Histamine is produced and stored primarily in mast cells and basophils from the decarboxylation of L-acid–amine histidine and performs its physiological functions by stimulating histaminergic receptors, which are well-known as H_1R, H_2R,

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H_3R and H_4R (Parsons and Ganellin 2006; Kiss et al. 2008). The H4 receptor, expressed in the membrane of immune cells, is responsible for histamine-induced chemotaxis and, therefore, is a potential target for the design and synthesis of new compounds for treating chronic inflammatory diseases (Zampeli and Tiligada 2009; Kiss and Keseru 2014). Moreover, there are reports of the involvement of H_4 receptors in the differentiation and metastasis of various neoplasic diseases such as breast (Medina et al. 2008; Tiligada et al. 2009) and lung cancer (Cai et al. 2014), eye diseases such as macular degeneration (Kaneko et al. 2014), vestibular disorders (Wersinger et al. 2013) and ulcerogenesis (Adami and Coruzzi 2014; Coruzzi et al. 2011).

Prototypes (JNJ7777120, VUF6002 and VUF6007) have recently been developed which act to antagonise the H_4 receptor, exerting anti-inflammatory and anti-nociceptive activities (Thurmond et al. 2004a, b; Coruzzi et al. 2007; Kiss and Keseru 2014). In the future, these compounds and new analogues could be used as therapeutic as well as pharmacological tools.

In the scope of a research programme aimed at developing new drugs for the treatment of central inflammatory diseases, we describe the synthesis and biological evaluation of LQFM182 (**2**), a new piperazine compound. LQFM182 (**2**) was designed through a ring bioisosterism strategy from the JNJ-7777120 (**1**) lead compound, a histamine H4 antagonist (Fig. 1). We replaced the indole ring present in JNJ-7777120 (**1**) with the 1-(phenyl)-1*H*-pyrazole scaffold present in LQFM182 (**2**), a strategy which has been used with success in drug discovery programmes (Lima and Barreiro 2005).

Materials and methods

Chemistry

Synthesis of (4-methylpiperazin-1-yl)(1-phenyl-1H-pyrazol-4-yl)methanone—LQFM182 (**2**) (Ermondi et al. 1998). To a heated heterogeneous mixture of 1-phenyl-1*H*-pyrazole-4-carboxylic acid (**6**) (188.06 mg, 1 mmol), $SOCl_2$ (4 mL, 55.14 mmol) and a few drops of anhydrous DMF were added to make one solution. The mixture was heated at 80 °C for 3 h. In turn, the excess $SOCl_2$ was then evaporated and *N*-methylpiperazine (**7**) (0.14 mL, 1 mmol), triethylamine (0.17 mL, 1 mmol) and CH_2Cl_2 (4 mL) were added to the residue; the new mixture was kept at room temperature for 2 h. At the end of the reaction, the residue was split between water and CH_2Cl_2. The phases were separated and the aqueous layer was extracted into 3 × 15 mL with CH_2Cl_2 and the combined organic layers were dried (Na_2SO_4), concentrated *in vacuo*, and the crude product was purified by column chromatography (SiO_2, CH_2Cl_2/MeOH = 98:2) to (4-methylpiperazin-1-yl)(1-phenyl-1*H*-pyrazol-4-yl)methanone (**2**) (190 mg, 70%) as a beige solid, m.p. = 97–106 °C, Rf = 0,42 (CH_2Cl_2/MeOH = 95:5). IR_{max} (KBr) cm^{-1}: 3098 (ν C–H), 2990 (ν C–H), 1606 (ν C=O) and 1555 (ν C=C); ^{1}H-NMR (500.13 MHz) $CDCl_3$ (δ): 8.23 (1H, d, J = 0.45 Hz; H-5); 7.82 (1H, d, J = 0.40 Hz, H-3), 7.68 (1H, dddd, J = 7.5; 2.2; 1.6 and 0.7 Hz; H-2′); 7.68 (1H, dddd, J = 7.2; 2.2; 1.2 and 0.9 Hz; H-6′); 7.46 (1H, dddd, J = 7.5; 7.4; 1.9 and 0.9 Hz; H-3′); 7.46 (1H, dddd, J = 7.4; 7.2; 1.9 and 0.7 Hz; H-5′); 7.33 (1H, tdd, J = 7.4; 1.6 and 1.2 Hz; H-4′); 3.82 (4H, t, J = 4.55 Hz; H-2″ and 6″); 2.53 (4H, t, J = 3.70 Hz; H-3″ and 5″); 2.38 (3H, s, H-4″); 2D NMR (HSQC/HMBC-125.76 MHz) $CDCl_3$/TMS (δ): 162.9 (C-6); 139.9 (C-1′); 139.8 (C-3); 129.5 (C-3′ and 5′); 128.8 (C-5); 126.9 (C-4′); 119.4 (C-2′ and 6′); 118.8 (C-4); 54.8 (C-3″ and 5″); 45.9 (C-2″ and 6″); 45.5 (C-4″); MS: $[M+H]^+m/z$ of 271.1551; purity >98% (Supporting Information)

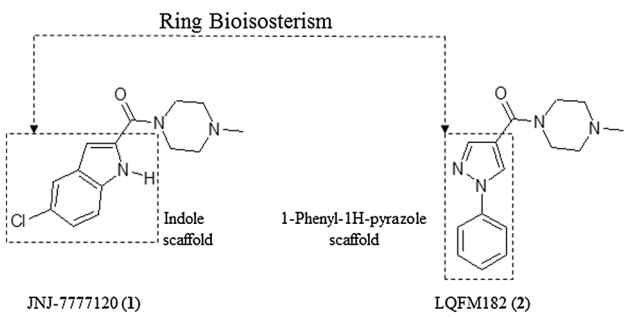

Fig. 1 Structural design of LQFM182 (**2**) from JNJ-7777120 (**1**)

Pharmacology

Animals

Experiments were performed using female Swiss albino mice (25–30 g) from the Central Animal House of the Federal University of Goiás (UFG). Animals were kept in plastic cages at 22 ± 2 °C, with free access to pellet food and water and under a 12-h light/dark cycle, in compliance with the International Guiding Principles for Biomedical Research Involving Animals. The animals were acclimatised for 7 days before the beginning of the experiments. All experimental protocols were developed and approved according to the principles of ethics and animal welfare designated by the Ethics Committee on Animal Use of UFG (Number: 017/13).

Drugs and chemicals

The chemicals used in this study were acetic acid (Merck, USA), DMSO (Sigma Chemical, USA), carrageenan

(Sigma Chemical, USA), dexamethasone (Decadron®, Ache, Brazil), formaldehyde (Synth, Brazil), indomethacin (Indocid®, Merck Sharp & Dohme Farmacêutica-Ltda, SP, Brazil), and morphine hydrochloride (Dimorf®, Cristalia, SP, Brazil). LQFM182 (**2**) was dissolved in 10% DMSO in distilled water, and all other drugs were dissolved in distilled water. The doses of the LQFM182 (**2**) were based on the doses of JNJ7777120 (**1**) used in previous studies (Hsieh et al. 2010).

Anti-nociceptive activity

Acetic acid-induced abdominal writhing test The 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), LQFM182 (**2**) at doses 50, 100 or 200 mg/kg or indomethacin (10 mg/kg, positive control for anti-nociceptive activity) 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 ± SEM of the number of writhings.

Tail flick test The tail flick test was performed as described previously by D'Amour et al. (1941). In this test, the time taken to flick the tail (latency) when it was exposed to a heat source was measured using an analgesimeter (Insight®, Ribeirão Preto, São Paulo, Brazil). The animals were divided into three experimental groups ($n = 8$): vehicle (10% DMSO, 10 mL/kg, p.o.), LQFM182 (**2**) (100 mg/kg, p.o.) or morphine (5 mg/kg, s.c.—positive control for anti-nociceptive activity). The latency baseline was performed 30 min before the treatments (time −30) and immediately after of the treatments (time 0) for each animal. After, the latency to pain reaction was measured at 30, 60, 90, 120 and 150 min. A cut-off of 15 s was set to prevent the risk of burns. The results were expressed as mean ± SEM, in seconds, at the different times.

Formalin test Formalin-induced nociception was performed as described previously by Hunskaar and Hole (1987). Groups of mice ($n = 7$) were treated with vehicle (10% DMSO 10 mL/kg p.o.), LQFM182 (**2**) (100 mg/kg p.o.), 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 phases). Sixty min after the p.o. treatment, or 30 min after s.c. treatment, 20 µL of 3% formalin (in saline) was administered into the plantar surface of the right hind paw. After the phlogistic agent injection, the mice were placed into an acrylic box, and a mirror was placed under this box to enable the unhindered observation of the formalin-injected paw for 30 min. Pain reaction time (licking time) was assessed during two periods: from 0 to 5 min, the first phase, where neurogenic pain is caused by direct stimulation of the nociceptors, and from 15 to 30 min, the second phase, where inflammatory pain is caused by the release of inflammatory mediators. These results were expressed as the mean ± SEM of licking time in seconds.

Anti-inflammatory activity

Carrageenan-induced oedema Carrageenan-induced hind paw oedema in mice was used as the animal model of acute inflammation according to the method of Winter et al. (1962). Initially, the animals ($n = 8$) were treated with vehicle (10% DMSO, 10 mL/kg, p.o.), LQFM182 (**2**) (100 mg/kg, p.o.) or indomethacin (10 mg/kg p.o.—positive control). One hour later, 50 µL of carrageenan (1%) was injected intraplantar into the right hind paw and 50 µL of saline (0.9% NaCl) into the left hind paw (used as the control). The paw volume was measured using a plethysmometer (Model 7141, Ugo Basile, Italy) at 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 in µL as mean ± SEM.

Carrageenan-induced pleurisy The animals ($n = 8$) were treated with vehicle (10% DMSO, 10 mL/kg, p.o.), LQFM182 (**2**) (100 mg/kg, p.o.) or dexamethasone (2 mg/kg, p.o.—positive control). One hour after the treatments, the animals received an injection of 100 µL of 1% carrageenan into the pleural cavity. Four hours later, the pleural exudate was collected with 1 mL of heparinised phosphate-buffered saline and used to count the number of total leukocytes using Türk solution in a Neubauer chamber (Vinegar et al. 1973), to evaluate the myeloperoxidase activity, the levels of TNF-α and IL-1β.

Activity of myeloperoxidase enzyme To measure the myeloperoxidase (MPO) activity, 40 µL of pleural lavage of mice treated with vehicle (10% DMSO, 10 mL/kg, p.o.), LQFM182 (**2**) (100 mg/kg, p.o.) or dexamethasone (2 mg/kg, p.o.—positive control) was transferred to eppendorfs. The reaction was started by adding 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 30 µL of sodium azide 1%. The samples were centrifuged subsequently for 5 min at 1000 rpm. The supernatant was separated and 200 µL was transferred to microplate wells; absorbance was assayed by enzyme-linked immunosorbent assay (ELISA), at a wavelength of 450 nm (Sedgwick 1995; Saleh et al. 1999). The

results were expressed as means ± SEM of enzymatic activity in mU/mL.

Assay of TNF-α and IL-1β levels The pleural exudates of mice treated with vehicle (10% DMSO, 10 mL/kg, p.o.), LQFM182 (**2**) (100 mg/kg, p.o.) or dexamethasone (2 mg/kg, p.o.—positive control) were also used to determine the concentrations of TNF-α and IL-1β using an immunosorbent assay kit (ELISA) (Ebioscience) (Nicoletti et al. 2010; Costa et al. 2013). The results were expressed as means ± SEM in pg/mL.

Toxicology

*In vitro assay to estimate LD_{50} of LQFM182 (**2**)*

Neutral red uptake (NRU) assay was carried out using Balb/c 3T3 fibroblasts, based on the protocol described by Borenfreund and Puerner (1985) modified by NICEATM (ICCVAM 2006). In brief, the cells were cultured in DMEM, supplemented with 10% (v/v) FBS, 100 IU/mL penicillin, 100 µg/mL streptomycin and routinely grown as a monolayer in tissue culture flasks (75 cm^2) under standard culture conditions at 37 °C and 5% CO_2 in a humid environment. When the cells exceeded 70–80% confluence, they were removed from the culture flasks using trypsinisation (trypsin/EDTA solution, 0.025:0.02%). Cell viability was analysed using a TC20TM automated cell counter (Hercules, CA, USA), according to the manufacturer's instructions. A value of >90% was considered acceptable for conducting the assays.

Thus, 3T3 fibroblasts (0.5×10^5 cells/mL) were seeded in 96-well plates overnight. The cells were then treated with vehicle (DMSO 0.6%) or nine different concentrations of LQFM182 (**2**) (0.02–2.26 mM) in complete medium. The blank wells (with no cells) received complete culture medium with or without the test compounds. After 48 h, the supernatant was discarded and 100 µL of NR (0.25 mg/mL) diluted in DMEM containing SFB (5%, v/v) was added to all wells, followed by incubation. After 3 h, the NR medium was removed, the cells were washed with 100 µL/well of pre-warmed PBS and 100 µL of NR desorb (50 ethanol: 1 acetic acid: 49 ultrapure water) solution was added to all wells. The plates were shaken on a microplate shaker (Orbit P4, Labnet International, Edison, NJ, USA) for 20 min and 35 rpm. Absorbance was measured at 550 nm in a spectrophotometer (Multiskan Spectrum, Thermo Scientific, Waltham, MA, USA). A curve was obtained from the viability data for each concentration LQFM182 (**2**). The concentration that inhibits cell growth by 50% (IC_{50}) was calculated. The lethal dose (LD_{50}) was estimated from the previously established *in house* model equation (Vieira et al. 2011): Log (LD_{50}) = 0.545 × log (IC_{50}) + 0.757. The estimated LD_{50} value obtained was used to classify the probable acute oral systemic toxicity of LQFM182 (**2**) according to the Globally Harmonised Classification and Labelling of Chemicals (GSH) (OECD 2001).

Statistical analysis

The data were analysed statistically by a one-way ANOVA followed by the Newman–Keuls' test as a post hoc or a two-way ANOVA followed by the Bonferroni's test as a post hoc (Sokal and Rohlf 1981). All statistical analysis was carried out using GraphPad Prism version 5.00. Values of $P \leq 0.05$ were considered significant.

Results

Synthesis of (4-methylpiperazin-1-yl)(1-phenyl-1 h-pyrazol-4-yl)methanone—LQFM182 (2)

The synthetic route (Fig. 2) began with a condensation reaction between 1-(phenyl)hydrazine hydrochloride (**3**) and 1,1,3,3-tetramethoxy propane through the work described by Finar and Hurlock (Finar and Hurlock 1957) to provide 1-(phenyl)-1-*H*-pyrazole (**4**) 88% of yields. The chemoselective and regiospecific formylation of 1-(phenyl)-1-*H*-pyrazole (**4**) to 1-(phenyl)-1-*H*-pyrazole-4-carbaldehyde (**5**) was carried out under Duff's conditions, with an 83% yield (De Oliveira et al. 2013). In turn, the 1-(phenyl)-1-*H*-pyrazole-4-carbaldehyde (**5**) was oxidised to compound 1-(phenyl)-1-*H*-pyrazole-4-carboxylic acid (**6**) through the use of $KMnO_4$ in water, with a 98% yield (Shriner and Kleider 1943). Finally, the 1-(phenyl)-1-*H*-pyrazole-4-carboxylic acid (**6**) reacted with $SOCl_2$, using DMF as a catalyser to offer the acyl chloride intermediate which, in turn, reacted with 1-methylpiperazine to provide (4-methylpiperazin-1-yl)(1-phenyl-1*H*-pyrazol-4-yl)methanone—LQFM182 (**2**) at a yield of 70% (Ermondi et al. 1998). As we can see, LQFM182 (**2**) was obtained after four synthetic steps, with a global yield of 50%.

Anti-nociceptive activity

Acetic acid-induced abdominal writhing test

In this test, the oral treatments with LQFM182 (**2**) at doses 50, 100 or 200 mg/kg decreased the number of writhings induced by acetic acid in a dose-dependent manner compared to the control group (vehicle 10% DMSO 10 mL/kg) from 107.1 ± 6.8 (group treated with vehicle) to 85.7 ± 11 (20% less, $P < 0.05$), 48.8 ± 3.3 (54.4% less, $P < 0.001$) and 45.6 ± 5.4 (57.4% less, $P < 0.001$),

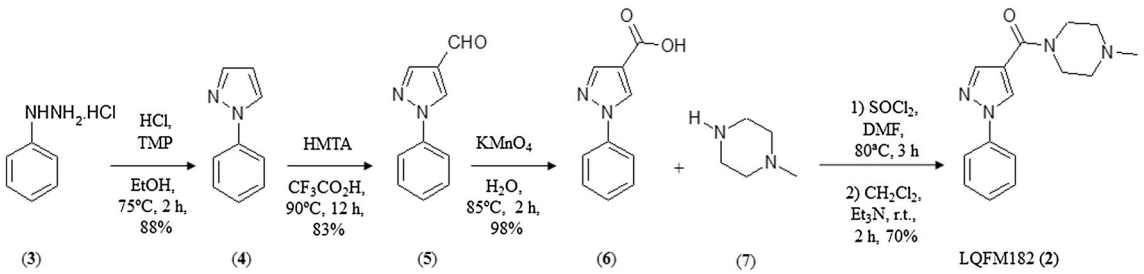

Fig. 2 Synthetic route to preparation of (4-methylpiperazin-1-yl)(1-phenyl-1H-pyrazol-4-yl)methanone (**2**) (LQFM182)

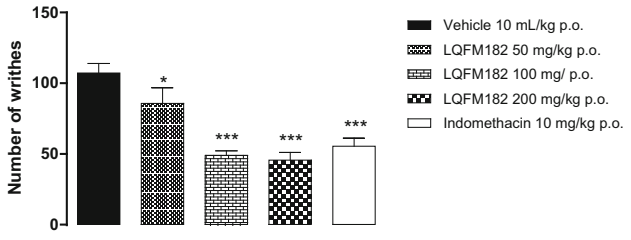

Fig. 3 Effect of LQFM182 (**2**) (50, 100 and 200 mg/kg p.o.) on the number of acetic acid-induced writhings in mice ($n = 8$). Indomethacin (10 mg/kg p.o.) was used as a positive control. *Vertical bars* represent mean ± SEM of number of writhings in 30 min for each experimental group. $^{*}P \leq 0.05$ and $^{***}P \leq 0.001$ (compared with control group) according to ANOVA followed by post hoc Newman–Keuls' test. *Indo* indomethacin

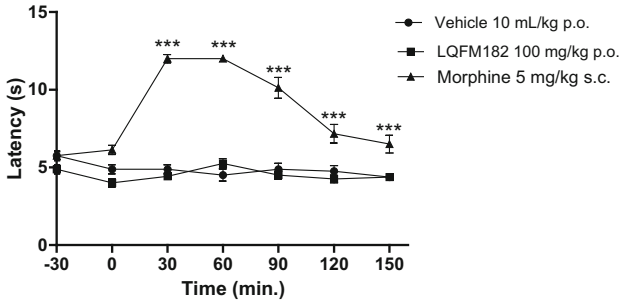

Fig. 4 Effect of LQFM182 (**2**) on the tail-flick test in mice ($n = 8$). The oral treatment with LQFM182 (**2**) (100 mg/kg p.o.) did not change latency to thermal stimulation compared to the control group. Morphine (5 mg/kg s.c.) was used as a positive control. The values are expressed as mean ± SEM of the latency for the nociceptive behaviour, in seconds. $^{***}P \leq 0.001$ (compared with control group) according to two-way ANOVA followed by Bonferroni's post hoc test

respectively. The positive control for this test, indomethacin (10 mg/kg), reduced the number of writhings by 48% (55.43 ± 5.7, $P < 0.001$) (Fig. 3).

Tail flick test

In the thermal nociception tail-flick test, treatment with LQFM182 (**2**) (100 mg/kg, p.o.) manifested no significant anti-nociceptive activity compared to the control group (vehicle). Morphine (5 mg/kg, s.c.—opioid agonist) demonstrated significant anti-nociception at the times of 30, 60, 90, 120 and 150 min after treatment (Fig. 4).

Formalin test

In the formalin test, LQFM182 (**2**) in an intermediate dose (100 mg/kg, p.o.) showed anti-nociceptive activity compared to the control group only in the second phase of the test (Fig. 5). In the second phase, the vehicle group showed a licking time (s) of 179.3 ± 10.7; LQFM182 (**2**) reduced this time to 68.3 ± 14.6 (62% less, $P < 0.001$). The group treated with indomethacin (10 mg/kg, p.o.), the anti-inflammatory positive control, also showed a decreased licking time (s) in the second phase, to 111.2 ± 7.2 (a reduction of 38%, $P < 0.001$), as expected. The positive control morphine (5 mg/kg, s.c.) decreased the first phase of 72.5 ± 3.3 (vehicle group) to 7.29 ± 2.8 (90% less, $P < 0.001$) and the second phase to 8.09 ± 0.8 (a reduction of 95%, $P < 0.001$).

Anti-inflammatory activity

Carrageenan-induced oedema

The oral treatment with LQFM182 (**2**) at a dose of 100 mg/kg reduced paw oedema in all hours of the test. In the first hour, LQFM182 (**2**) reduced the difference between the paws from 142.5 ± 5.6 to 115.7 ± 6.8 µL (19% reduction, $P < 0.01$); in the second hour from 140 ± 6.3 to 88.6 ± 7 µL (37% reduction, $P < 0.001$); in the third hour from 130 ± 3.8 to 85.7 ± 6.1 µL (34% reduction, $P < 0.001$); and finally, in the fourth hour from 126.7 ± 6.9 to 77.1 ± 5.2 µL (39% reduction, $P < 0.001$). The treatment with indomethacin (10 mg/kg, p.o.) reduced the paw oedema in all hours of the test to 92.4 ± 4.6 (35% reduction, $P < 0.001$), 77.2 ± 4.4 µL (45% reduction, $P < 0.001$), 61.3 ± 3.8 (53% reduction, $P < 0.001$) or 61 ± 7.5 µL (52% reduction, $P < 0.001$), respectively, compared to vehicle (Fig. 6).

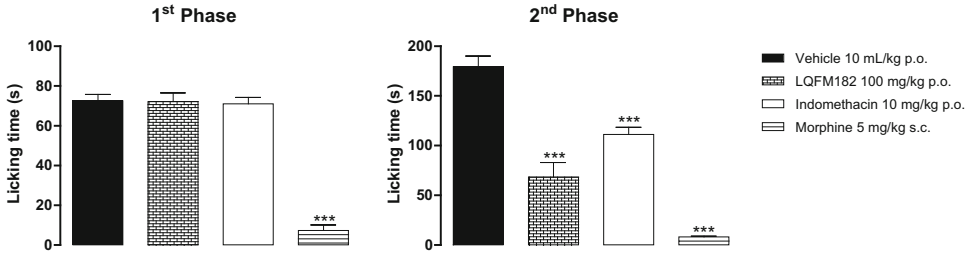

Fig. 5 The anti-nociceptive effect of LQFM182 (**2**) on the pain induced by the formalin test in mice ($n = 8$). Oral treatment with LQFM182 (**2**) (100 mg/kg) reduced the licking time in only the second phase of the test. Indomethacin (10 mg/kg, p.o.) and morphine (5 mg/kg, s.c.) were used as positive controls, during the first (0–5 min) and second phase (15–30 min). *Vertical bars* represent mean ± SEM of licking time of paw, in seconds. $***P \leq 0.001$ compared with control group, according to ANOVA followed by post hoc Newman–Keuls' test

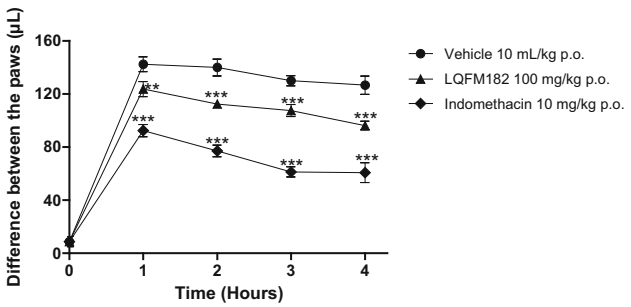

Fig. 6 Effect of LQFM182 (**2**) on the carrageenan-induced oedema test in mice ($n = 8$). The oral treatment with LQFM182 (**2**) at 100 mg/kg reduced the oedema at all hours of the test. Indomethacin (10 mg/kg, p.o.) was used as positive control. The values were expressed as mean ± SEM of the difference between the paws, in µL. $*P \leq 0.05$, $**P \leq 0.01$ or $***P \leq 0.001$, compared to control group, according to two-way ANOVA followed by Bonferroni's post hoc test

Carrageenan-induced pleurisy

Cell migration In the test of carrageenan-induced pleurisy, the oral treatments with LQFM182 (**2**) (100 mg/kg) or dexamethasone (2 mg/kg) reduced the total number of leukocytes to 4.06 ± 0.43 (34.5% reduction, $P < 0.001$) or 2.0 ± 0.56 leukocytes × 10^6/mL (68% reduction, $P < 0.001$), respectively, compared to the control group (6.2 ± 0.77 leukocytes × 10^6/mL). In the differential count of leukocytes, it was observed that the oral treatment with LQFM182 (**2**) at dose 100 mg/kg significantly reduced the migration of the polymorphonuclear leukocytes from 5.2 ± 0.34 (vehicle group) to 3.3 ± 0.43 (36.5% reduction, $P < 0.001$). The positive control dexamethasone also significantly reduced the migration of the polymorphonuclear leukocytes to 0.61 ± 0.2 (88% reduction, $P < 0.001$) (Fig. 7a).

Activity of myeloperoxidase enzyme The oral treatment with LQFM182 (**2**) (100 mg/kg) or dexamethasone (2 mg/kg) also reduced the activity of myeloperoxidase enzyme to 60.5 ± 6.4 (43% reduction, $P < 0.001$) or 29.8 ± 15.6 mU/mL (72% reduction, $P < 0.001$), respectively, compared to the vehicle-treated group (enzymatic activity 106.7 ± 18 mU/mL) (Fig. 7b).

Assay of TNF-α and IL-1β levels The levels of TNF-α was reduced by oral treatment with LQFM182 (**2**) (100 mg/kg) or with dexamethasone (2 mg/kg) to 2.6 ± 0.5 (87% reduction, $P < 0.001$) or 3.5 ± 1.8 (83% reduction, $P < 0.05$), respectively, compared to the vehicle treated group (TNF-α: 20.2 ± 4.1 pg/mL) (Fig. 7c). As can be observed in Fig. 7d, these some treatments significantly reduced the levels of IL-1β interleukin to 428.3 ± 17.4 (26% reduction, $P < 0.001$) or 85.2 ± 9.7 pg/mL (85.3% reduction, $P < 0.001$), respectively, compared to the vehicle group (IL-1β: 579 ± 13.3 pg/mL).

In vitro assay to estimate LD_{50} value

The 3T3 cells were exposed to different concentrations of LQFM 182 (**2**) (0.02–2.26 mM) for 48 h and cell viability was assessed by the NRU assay. This compound promoted cell death in a concentration-dependent manner. The value of IC_{50} obtained was 0.832 mM (Fig. 8). From this IC_{50} value, the LD_{50} was estimated, using previously established *in house* model equation (Vieira et al. 2011), to be 1397 mg/kg. Thus, this compound was classified according to GHS as category 4, $LD_{50} > 300 < 2000$ mg/kg.

Discussion

The present study reported the synthesis and preliminary pharmacological profile of a new heterocyclic compound (**2**) designed through ring the bioisosterism strategy from JNJ-7777120 (**1**), the lead compound.

In order to evaluate the possible anti-nociceptive and/or anti-inflammatory activity of LQFM182 (**2**), the acetic

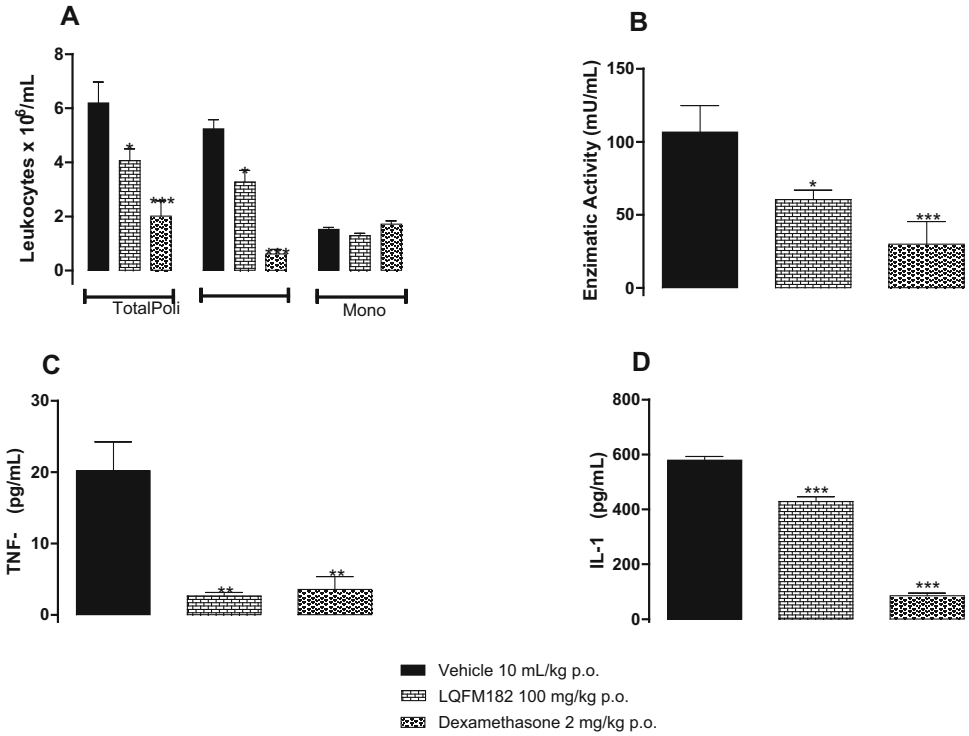

Fig. 7 Effect of LQFM182 (**2**) in carrageenan-induced pleurisy in mice ($n = 8$). The *figure* shows the effect of LQFM182 (**2**) (100 mg/kg, p.o.) or dexamethasone (2 mg/kg p.o.) on **a** cell migration, **b** myeloperoxidase activity, **c** TNF-α levels and **d** IL-1β levels. The *bars* represent the mean ± SEM of number of migrated leukocytes/mL × 106 to pleural cavity, the mean ± SEM of enzymatic activity MPO in mU/mL and the mean ± SEM of TNF-α and IL-1β levels in pg/mL in the pleural exudate. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, compared to control group, according to ANOVA followed by Newman–Keuls' test

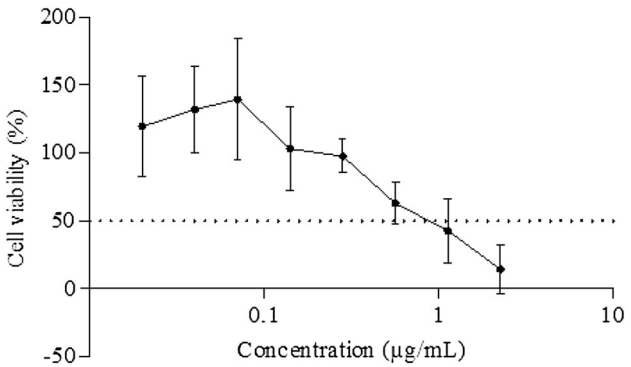

Fig. 8 Cytotoxicity evaluation of LQFM182 (**2**) (0.02–2.26 mM) in 3T3 cells by neutral red uptake (NRU) assay. The 3T3 fibroblasts (0.5×10^5 cells/mL) were seeded in 96-well plates overnight. The cells were then treated with vehicle (DMSO 0.6%) or LQFM182 (**2**) in complete medium. After 48 h of exposure, the cell viability was evaluated using neutral red dye

acid-induced abdominal writhing test was performed. The administration of acetic acid in the peritoneal cavity induces acute inflammatory processes from the action of acid and the liberation of chemical mediators such as bradykinin, substance P, prostaglandins and cytokines (Ribeiro et al. 2000; Zakaria et al. 2006; Malvar et al. 2014). After the application of acetic acid, abdominal contraction and extension was observed, followed by one or both hind legs of the mice being rotated. In this test, the oral treatments with LQFM182 (**2**) at doses 50, 100 or 200 mg/kg decreased in a dose-dependent manner the number of writhing induced by acetic acid suggesting a nociceptive activity for the new compound. Similar results have been described with other piperazine derivatives, which have showed potential analgesic effect for the treatment of pain (Chen et al. 2011a, b; Chae et al. 2012). In the next tests, only the intermediate dose of LQFM182 (**2**) was used to reduce the number of animals as recommended by the CEUA.

To investigate the possible involvement of central mechanism in the anti-nociceptive effect of LQFM182 (**2**), the tail-flick was conducted. This test uses the thermal stimulus to evaluate an anti-nociceptive activity mediated by spinal mechanism (D'amour and Smith 1941; Barrot 2012). LQFM182 (**2**) at dose used (100 mg/kg, p.o.) did not increase the latency to thermal stimulus in either test, suggesting that the anti-nociceptive effect of new compound in this study is due to peripheral mechanisms.

In addition, this likely peripheral effect was confirmed in the formalin test that identified two distinct phases of pain. The first phase is characterised by the direct stimulation of nociceptive fibres by formalin action and the release of preformed mediators such as serotonin, kinins and histamine, whereas the second phase is associated with the formation and release of inflammatory mediators such as cytokines and eicosanoids (Hunskaar and Hole 1987; Shibata et al. 1989; Rosland et al. 1990; Tjolsen et al. 1992). It was observed that LQFM182 (**2**) (100 mg/kg) showed anti-nociceptive activity only in the second phase of the test. Shibata et al. (1989) have shown that central

acting analgesics such as narcotics can reduce both phases of test, whereas anti-inflammatory drugs primarily suppress the late phase of nociception. Therefore, ours results suggest that LQFM182 (**2**) has anti-inflammatory activity. Similar result has been seen with non-steroidal anti-inflammatory drugs (Ortiz 2012; Zhao et al. 2017).

Previous research has reported an important role of piperazine derivatives in the control of pain and inflammation in different experimental models in vivo, showing a reduction of pain, oedema and pro-inflammatory cytokines (Rordorf-Adam et al. 1994; Thurmond et al. 2004a, b; Jiang et al. 2008; Chen et al. 2011a, b; Silva et al. 2015).

To confirm the anti-inflammatory effect of LQFM182 (**2**), the carrageenan-induced paw oedema test was performed first. Carrageenan promotes the development of an anti-inflammatory process with the consequent formation of oedema by the action of multiple inflammatory mediators, such as histamine and prostaglandins (Winter et al. 1962; Di Rosa et al. 1971; Liew and McInnes 2002; Morris 2003). The results show that the treatment of animals with LQFM182 (**2**) at a dose of 100 mg/kg reduced the oedema formation in all hours of the test, confirming the anti-inflammatory effect observed in the formalin test.

In addition, the carrageenan-induced pleurisy test was performed. In this test, it is possible to evaluate and quantitate several inflammatory parameters, such as cell migration, myeloperoxidase activity and cytokine levels from the pleural exudate collected (Vinegar et al. 1973; Higgs et al. 1980; Mikami and Miyasaka 1983). From the analysis of the pleural exudate, the results showed that LQFM182 (**2**) reduced the cell migration from the reduction of polymorphonuclear cell migration. Moreover, LQFM182 (**2**) reduced the activity of the myeloperoxidase enzyme, which is predominantly present in active neutrophils, confirming the previous result (Desmarchelier et al. 1997; Eddouks et al. 2012).

In the pleurisy test, it was also observed that LQFM182 (**2**) reduced the levels of pro-inflammatory cytokine IL-1β, as well as promoting a considerable reduction in the TNF-α levels similar to the positive control dexamethasone. TNF-α is an important pro-inflammatory cytokine mainly involved in cellular chemotaxis (Frode et al. 2001; Mazzon and Cuzzocrea 2007) and inflammatory hyper-nociception (Chichorro et al. 2004; Cunha et al. 2005). According to several studies, increased levels of TNF-α are directly related to the increased expression of NF-kappa β (factor nuclear kappa β) (Schütze et al. 1995; Gupta et al. 2005; Hoesel and Schmid 2013) which takes part in the expression of numerous cytokines and adhesion molecules which are critical elements involved in the regulation of immune responses (Liang et al. 2004; Hoesel and Schmid 2013). These activities can explain the anti-inflammatory effect seen in the different models used. However, it is necessary to evaluate the real association of LQFM182 (**2**) and the expression of NF-kappa β to better understand the mechanisms involved in the anti-inflammatory effect of this compound.

In addition, in the attempt to evaluate the toxicity of LQFM182 (**2**), an in vitro assay was performed to estimate the oral acute LD_{50} value of this compound. The value found was 1397 mg/kg, which allowed classified the compound according to GHS as category 4. This category includes substances with a low oral acute toxicity.

Conclusion

We can conclude that LQFM182 (**2**) showed satisfactory anti-inflammatory activity which has been shown to correlate with a decrease in TNF-α levels. In addition, in acute toxicity assays, the prototype was shown to be safe for oral administration.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

References

Adami M, Coruzzi G (2014) The histamine H4 receptor: a novel target for safe anti-inflammatory drugs? Gastro Open J 1(1):7–12. doi:10.17140/GOJ-1-103

Ashley NT, Weil ZM, Nelson RJ (2012) Inflammation: mechanisms, costs, and natural variation. Annu Rev Ecol Evol Syst 43:385–406. doi:10.1146/annurev-ecolsys-040212-092530

Barrot M (2012) Tests and models of nociception and pain in rodents. Neuroscience 211:39–50. doi:10.1016/j.neuroscience.2011.12.041

Borenfreund E, Puerner J (1985) A simple quantitative procedure using monolayer cultures for cytotoxicity assays (HTD/NR-90). J Tissue Cult Methods 9(1):7–9. doi:10.1007/BF01666038

Cai W, Hu J, Li T et al (2014) Activation of histamine H4 receptors decreases epithelial-to-mesenchymal transition progress by inhibiting transforming growth factor-β1 signalling pathway in non-small cell lung cancer. Eur J Cancer 50(6):1195–1206. doi:10.1016/j.ejca.2013.12.025

Chae E, Yi H, Choi Y et al (2012) Synthesis and pharmacological evaluation of carbamic acid 1-phenyl-3-(4-phenyl-piperazine-1-yl)-propyl ester derivatives as new analgesic agents. Bioorg Med Chem Lett 22(7):2434–2439. doi:10.1016/j.bmcl.2012.02.023

Chen Y, Wang G, Xu X et al (2011a) Design, synthesis and biological activity evaluation of arylpiperazine derivatives for the treatment of neuropathic pain. Molecules 16(7):5785–5806. doi:10.3390/molecules16075785

Chen Y, Wang G, Xu X et al (2011b) Design, synthesis and biological activity evaluation of arylpiperazine derivatives for the treatment of neuropathic pain. Molecules 16(7):5785–5806. doi:10.3390/molecules16075785

Chichorro JG, Lorenzetti BB, Zampronio AR (2004) Involvement of bradykinin, cytokines, sympathetic amines and prostaglandins in formalin-induced orofacial nociception in rats. Br J Pharmacol 141(7):1175–1184. doi:10.1038/sj.bjp.0705724

Coruzzi G, Adami M, Guiata E et al (2007) Antiinflammatory and antinociceptive effects of the selective histamine H4-receptor antagonists JNJ7777120 and VUF6002 in a rat model of carrageenan-induced acute inflammation. Eur J Pharmacol 563(1–3):240–244. doi:10.1016/j.ejphar.2007.02.026

Coruzzi G, Adami M, Pozzoli C et al (2011) Selective histamine H3 and H4 receptor agonists exert opposite effects against the gastric lesions induced by HCl in the rat stomach. Eur J Pharmacol 669(1–3):121–127. doi:10.1016/j.ejphar.2011.07.038

Costa EA, Lino RC, Gomes MN et al (2013) Anti-inflammatory and antinociceptive activities of LQFM002—a 4-nerolidylcatechol derivative. Life Sci 92(3):237–244. doi:10.1016/j.lfs.2012.12.003

Cunha TM, Verri WA Jr, Silva JS et al (2005) A cascade of cytokines mediates mechanical inflammatory hypernociception in mice. Proc Natl Acad Sci USA 102(5):1755–1760. doi:10.1073/pnas.0409225102

D'amour FE, Smith FL (1941) A method for determining loss of pain sensation. J Pharm Exp Ther 72:74–79

De Oliveira CHA, Mairink LM, Pazini F et al (2013) Chemoselective and regiospecific formylation of 1-penyl-1H-pyrazoles through the duff reaction. Synth Commun 43:1633–1639. doi:10.1080/00397911.2012.657764

Desmarchelier C, Barros S, Repetto M et al (1997) 4-Nerolidylcatechol from *Pothomorphe* Spp. Scavanges Peroxyl radicals and Inhibits Fe (II)-dependent DNA damage. Planta Med 63(6):561–563. doi:10.1055/s-2006-957767

Di Rosa M, Giroud JP, Willoughby DA (1971) Studies on the mediators of the acute inflammatory response induced in rats in different sites by carrageenan and turpentine. J Pathol 104(1):15–29. doi:10.1002/path.1711040103

Eddouks M, Chattopadhyay D, Zeggwagh NA (2012) Animal models as tools to investigate antidiabetic and anti-inflammatory plants. Evid Based Complement Alternat Med 2012:14. doi:10.1155/2012/142087

Ermondi G, Boschi D, Di Stilo A et al (1998) Pyrazole analogues of prazosin. II Farmaco 53(7):519–524. doi:10.1016/S0014-827X(98)00052-4

Finar I, Hurlock R (1957) The preparation of some trinitrophenylpyrazoles. J Chem Soc. doi:10.1039/JR9570003024

Frode TS, Souza GE, Calixto JB (2001) The modulatory role played by TNF-alpha and IL-1 beta in the inflammatory responses induced by carrageenan in the mouse model of pleurisy. Cytokine 13(3):162–168. doi:10.1006/cyto.2000.0816

Gupta S, Bi R, Kim C et al (2005) Role of NF-kappaB signaling pathway in increased tumor necrosis factor-alpha-induced apoptosis of lymphocytes in aged humans. Cell Death Differ 12(2):177–183. doi:10.1038/sj.cdd.4401557

Higgs GA, Eakins KE, Mugridge KG et al (1980) The effects of non-steroid anti-inflammatory drugs on leukocyte migration in carrageenin-induced inflammation. Eur J Pharmacol 66(1):81–86. doi:10.1016/0014-2999(80)90297-6

Hoesel B, Schmid JA (2013) The complexity of NF-κB signaling in inflammation and cancer. Mol Cancer 12:86. doi:10.1186/1476-4598-12-86

Hsieh GC, Chandran P, Salyers AK et al (2010) H4 receptor antagonism exhibits anti-nociceptive effects in inflammatory and neuropathic pain models in rats. Pharmacol Biochem Behav 95(1):41–50. doi:10.1016/j.pbb.2009.12.004

Hunskaar S, Hole K (1987) The formalin test in mice:dissociation between inflammatory and non-inflammatory pain. Pain 30:103–114. doi:10.1016/0304-3959(87)90088-1

ICCVAM (2006) Test method evaluation report (TIMER): in vitro cytotoxicity test methods for estimating starting doses for acute oral systemic toxicity test. NIH publication no. 07-4519

Jiang W, Lim HD, Zhang M et al (2008) Cloning and pharmacological characterization of the dog histamine H4 receptor. Eur J Pharmacol 592(1–3):26–32. doi:10.1016/j.ejphar.2008.06.095

Kaneko H, Ye F, Ijima R et al (2014) Histamine H4 receptor as a new therapeutic target for choroidal neovascularization in age-related macular degeneration. Br J Pharmacol 171(15):3754–3763. doi:10.1111/bph.12737

Kiss R, Keseru GM (2014) Novel histamine H4 receptor ligands and their potential therapeutic applications: an update. Expert Opin Ther Pat 24(11):1185–1197. doi:10.1517/13543776.2014.959494

Kiss R, Kiss B, Konczol A et al (2008) Discovery of novel human histamine H4 receptor ligands by large-scale structure-based virtual screening. J Med Chem 51(11):3145–3153. doi:10.1021/jm7014777

Koster R, Anderson M, Beer ED (1959) Acetic acid for analgesic screening. Fed Proc J 18:412–418

Liang Y, Zhou Y, Shen P (2004) NF-kappaB and its regulation on the immune system. Cell Mol Immunol 1(5):343–350

Liew FY, McInnes IB (2002) The role of innate mediators in inflammatory response. Mol Immunol 38(12–13):887–890. doi:10.1016/S0161-5890(02)00014-7

Lima LM, Barreiro EJ (2005) Bioisosterism: a useful strategy for molecular modification and drug design. Curr Med Chem 12(1):23–49. doi:10.2174/0929867053363540

Malvar DC, Ferreira RT, Castro RA et al (2014) Antinociceptive, anti-inflammatory and antipyretic effects of 1.5-diphenyl-1H-Pyrazole-3-carbohydrazide, a new heterocyclic pyrazole derivative. Life Sci 95(2):81–88. doi:10.1016/j.lfs.2013.12.005

Mazzon E, Cuzzocrea S (2007) Role of TNF-α in lung tight junction alteration in mouse model of acute lung inflammation. Respir Res 8:75. doi:10.1186/1465-9921-8-75

Medina V, Croci M, Crescenti E et al (2008) The role of histamine in human mammary carcinogenesis: H3 and H4 receptors as potential therapeutic targets for breast cancer treatment. Cancer Biol Ther 7(1):28–35. doi:10.4161/cbt.7.1.5123

Medzhitov R (2008) Origin and physiological roles of inflammation. Nature 454(7203):428–435. doi:10.1038/nature07201

Mikami T, Miyasaka K (1983) Effects of several anti-inflammatory drugs on the various parameters involved in the inflammatory response in rat carrageenin-induced pleurisy. Eur J Pharmacol 95(1–2):1–12. doi:10.1016/0014-2999(83)90261-3

Morris CJ (2003) Carrageenan-induced paw edema in the rat and mouse. Methods Mol Biol 225:115–121. doi:10.1385/1-59259-374-7:115

Nathan C (2002) Points of control in inflammation. Nature 420(6917):846–852. doi:10.1038/nature01320

Nathan C, Ding D (2010) Nonresolving inflammation. Cell 140(6):871–882. doi:10.1016/j.cell.2010.02.029

Nicoletti F, Auci DL, Mangano K et al (2010) 5-androstenediol ameliorates pleurisy, septic shock, and experimental autoimmune encephalomyelitis in mice. Autoimmune Dis 2010:757432. doi:10.4061/2010/757432

OECD (Organisation for Economic Cooperation and Development) (2001) Acute oral toxicity: acute toxic class method. Guideline for the testing of chemicals, n.423, http://ntp.niehs.nih.gov/iccvam/suppdocs/feddocs/oecd/oecd_gl423.pdf. Accessed 20 Nov 2014

Ortiz MI (2012) Metformin and phenformin block the peripheral antinociception induced by diclofenac and indomethacin on the formalin test. Life Sci 90(1–2):8–12. doi:10.1016/j.lfs.2011.10.009

Parsons ME, Ganellin CR (2006) Histamine and its receptors. Br J Pharmacol 147:127–135. doi:10.1038/sj.bjp.0706440

Ribeiro RA, Vale ML, Thomazzi SM et al (2000) Involvement of resident macrophages and mast cells in the writhing nociceptive response induced by zymosan and acetic acid in mice. Eur J Pharmacol 387(1):111–118. doi:10.1016/S0014-2999(99)00790-6

Rordorf-Adam C, Geiger T, Henn R et al (1994) CGP 47969A: a novel inhibitor of the synthesis of inflammatory cytokines. Agents Actions 43(1–2):53–59

Rosland JH, Tjolsen A, Maehle B et al (1990) The formalin test in mice: effect of formalin concentration. Pain 42(2):235–242. doi:10.1016/0304-3959(90)91167-H

Saleh TS, Calixto JB, Medeiros YS (1999) Effects of antiinflammatory drugs upon nitrate and myeloperoxidase levels in the mouse pleurisy induced by carrageenan. Peptides 20(8):949–956. doi:10.1016/S0196-9781(99)00086-8

Schütze S, Wiegmann K, Machleidt T et al (1995) TNF-induced activation of NF-kappa B. Immunobiology 193(2–4):193–203. doi:10.1016/S0171-2985(11)80543-7

Sedgwick AD (1995) Initiation of inflammatory response and its prevention. In: Bonta IL, Bray MA (eds) EUA: handbook of Inflammation. Elsevier, New York, p 253

Shibata M, Ohkubo T, Takahashi H et al (1989) Modified formalin test: characteristic biphasic pain response. Pain 38(3):347–352. doi:10.1016/0304-3959(89)90222-4

Shriner RL, Kleider E (1943) Piperonylic acid. Org Synth 10:82–83. doi:10.15227/orgsyn.010.0082

Silva DPB, Florentino IF, Oliveira LP et al (2015) Anti-nociceptive and anti-inflammatory activities of 4-[(1-phenyl-1H-pyrazol-4-yl) methyl] 1-piperazine carboxylic acid ethyl ester: a new piperazine derivative. Pharmacol Biochem Behav 137:86–92. doi:10.1016/j.pbb.2015.08.008

Sokal RR, Rohlf FJ (1981) Biometry: the principles and practice of statistics in biological research. EUA: W.H. Freeman and Company., New York

Thurmond RL, Desai PJ, Dunford PJ et al (2004a) A potent and selective histamine H4 receptor antagonist with anti-inflammatory properties. J Pharm Exp Ther 309(1):404–413. doi:10.1124/jpet.103.061754

Thurmond RL, Desai PJ, Dunford PJ et al (2004b) A potent and selective histamine H4 receptor antagonist with anti-inflammatory properties. J Pharmacol Exp Ther 309(1):404–413. doi:10.1124/jpet.103.061754

Tiligada E, Zampeli E, Sander K et al (2009) Histamine H3 and H4 receptors as novel drug targets. Expert Opin Investig Drugs 18(10):1519–1531. doi:10.1517/14728220903188438

Tjolsen A, Berge OG, Hunskaar S et al (1992) The formalin test: an evaluation of the method. Pain 51(1):5–17. doi:10.1016/0304-3959(92)90003-T

Vieira MS, Oliveira V, Lima EM et al (2011) In vitro basal cytotoxicity assay applied to estimate acute oral systemic toxicity of grandisin and its major metabolite. Exp Toxicol Pathol 63(5):505–510. doi:10.1016/j.etp.2010.03.012

Vinegar R, Truax JF, Selph JL (1973) Some quantitative temporal characteristics of carrageenan-induced pleurisy in the rat. Proc Soc Exp Biol Med 143(3):711–714

Wersinger E, Gaboyard-Niay S, Travo C et al (2013) Symptomatic treatment of vestibular deficits: therapeutic potential of histamine H4 receptors. J Vestib Res 23(3):153–159. doi:10.3233/VES-130493

Winter CA, Risley EA, Nuss GW (1962) Carrageenin-induced edema in hind paw of the rat as an assay for antiinflammatory drugs. Proc Soc Exp Biol Med 111:544–547. doi:10.3181/00379727-111-27849

Zakaria ZA, Abdul Ghani ZD, Nor RM et al (2006) Antinociceptive and anti-inflammatory activities of *Dicranopteris linearis* leaves chloroform extract in experimental animals. Yakugaku Zasshi 126(11):1197–1203. doi:10.1248/yakushi.126.1197

Zampeli E, Tiligada E (2009) The role of histamine H4 receptor in immune and inflammatory disorders. Br J Pharmacol 157(1):24–33. doi:10.1111/j.1476-5381.2009.00151.x

Zhao YQ, Wang HY, Yin JB et al (2017) The analgesic effects of celecoxib on the formalin-induced short- and long-term inflammatory pain. Pain Phys 20(4):E575–E584