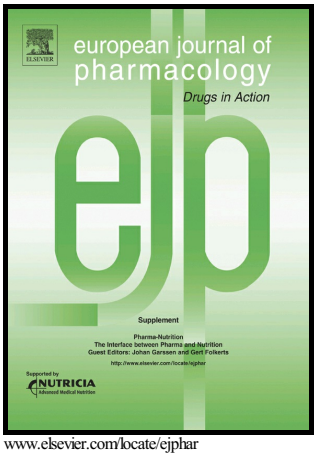

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Design, synthesis and pharmacological evaluation of new anti-inflammatory compounds

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Abstract

Inflammatory diseases and pain are among the main problems that significantly influence the lifestyle of millions of people and existing therapies aren't always effective and can cause several adverse effects. In this context, the molecular modifications or synthesis of compounds continue being the best strategies for the identification of new compounds for the treatment of pain and inflammation. The aim of this study was to evaluate the analgesic and anti-inflammatory activities of new analogues of pyrazole compounds containing subunits N-phenyl-1-H-pirazoles and

1,3,4-oxadiazole-2(3H)-thione, LQFM-146, LQFM-147 and LQFM-148. In the acetic acid-induced abdominal writhing test, treatments with LQFM-146, LQFM-147 or LQFM-148 at doses 89, 178 and 356 μmol/kg p.o. reduced the abdominal writhing in a dose-dependent manner. In the formalin test, these compounds at dose 178 μmol/kg p.o. reduced the licking time only in inflammatory phase of this test, suggesting an antinociceptive effect dependent of the anti-inflammatory effect. The treatment with the three compounds in intermediate dose (178 μmol/kg p.o.) reduced the edema at all tested time points in the carrageenan-induced paw edema test and reduced polymorphonuclears cell migration, activity myeloperoxidase and TNF-α levels in the carrageenan-induced pleurisy test. Our date suggest that the new compounds LQFM-146, LQFM-147 and LQFM-148 possess satisfactory anti-inflammatory and antinociceptive effects that involves the reduction of pro-inflammatory cytokines and inhibition of the myeloperoxidase enzyme.

Keywords: pyrazole compounds; LQFM-146; LQFM-147, LQFM-148; inflammation.

1. Introduction

Inflammation is the response of the organism to an external challenge or tissue injury, giving birth to immunological processes of repair and healing. However, prolonged inflammation ceases to be a beneficial event and contributes to the pathogenesis of many diseases, such as, rheumatoid arthritis and osteoarthritis, are characterized by accumulation of inflammatory cells in the joints and leads to joint damage (Gilroy et al., 2004).

Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used in the treatment of inflammation symptoms and signs, and exert their effects by inhibition of the synthesis of cyclooxygenase (COX), key enzymes in the production prostaglandins, biosynthesized from arachidonic acid. There are two main types of cyclooxygenases enzymes (COX-1 and COX-2). COX-1 is constitutively and responsible for, among others, by a cytoprotective activity, while COX-2 is induced during the inflammatory process, pain and fever (Rainsford, 2007; Jahn et al., 2008; Rao; Knaus, 2008).

The first line of clinical treatment for the inflammatory disorders is NSAIDs via COX pathway. Due the gastrointestinal bleeding and toxicity associated with NSAIDs long term use does the investigation of new analgesic and anti-inflammatory agents a major challenge. Multiple-ligand drugs using hybridization techniques can act on a single or multiple targets with synergistic action and minimize toxicity or adverse reactions (Abdellatif et al., 2009; Hernández et al., 2012; Shenvi et al., 2015).

COX-2 inhibitors are marketed as a new generation of NSAIDs, their mechanism of action exhibit less gastrointestinal toxicity than traditional NSAIDs, but studies have shown that continuous use causes cardiovascular effects and induced asthma frames. Then, the attention has been focused another acid arachidonic metabolic pathway, the lipoxygenases (LOX) pathway, since it possesses important action in the production and maintenance of inflammation. In this context, dual inhibitors on the routes of COX-2 and 5-LOX have shown significant anti-inflammatory activity supported by tests "in vitro" and "in vivo" with a reduction of adverse action (Leval et al., 2002; Burnett; Levy, 2012; Hwang et al., 2013).

Synthetic approaches based on chemical structure of NSAIDs are being conducted in order to improve its safety profile. Thus structural changes in drug flufenamic acid led to the derived (2), which not only kept his inhibiting the synthesis of

prostaglandins (COX), but also featured 5-LOX inhibitor activity (Boschelli et al., 1993; Julemont et al., 2004). This derivative has in its structure the nucleus 1,3,4-oxadiazole-2(3H)-thione, widely described in the literature due to the anti-inflammatory activities, giving for derived low gastric toxicity (Oliveira et al., 2012; Khalilullah et al., 2012).

Sub-units N-phenyl-1-H-pirazoles are described by their anti-inflammatory and analgesic activities, these come gained prominence after the development of the Celecoxibe (1) how COX-2 selective inhibitor (Bansal et al., 2014; Küçükgüzel; Şenkardeş, 2015). In this context, our group has published studies on new pyrazole compounds, 5-(1-(3-fluorophenyl)-1-H-pyrazol-4-yl)-2H-tetrazole (1), shown analgesic activity involving the peripheral opioid receptors and activation of the NO/cGMP/K(ATP) pathway (Florentino et al., 2015a). In addition, we also found that 5-(1-(3-fluorophenyl)-1-H-pyrazol-4-yl)-2H-tetrazole (1) possess anti-inflammatory activity involving the reduction leukocyte migration, the TNF-α and IL-1β levels and decreased of the myeloperoxidase activity. Furthermore, was observed that the inhibition of nitric oxide synthase promoted by L-NAME reduced the anti-inflammatory effect of this compound, shown that the nitric oxide is important to this effect (Florentino et al., 2015b).

Thus, stimulated by perspective in obtaining compounds with anti-inflammatory activity satisfactory, we describe the synthesis and evaluation of analgesic and anti-inflammatory activities of new analogues containing subunits N-phenyl-1-H-pirazoles and 1,3,4-oxadiazole-2(3H)-thione, with potential for clinical development.

In the scope of a research program aimed at drug development for treatment of anti-inflammatory disease, we describe in the present study the synthesis and biological

evaluation of new heterocyclic compounds (9-11). The compounds (9-11) were designed through strategy of hybridization molecular from 5-(1-(3-fluorophenyl)-1-H-pyrazol-4-yl)-2H-tetrazole (1) and derivative (2) (scheme 1). The synthetic route of title compounds 9-11 is shown in Scheme 2.

2. Materials and methods

2.1. General

Reactions were monitored by TLC using commercially available precoated plates (Whatman 60 F254 silica) and developed plates were examined under UV light (254 and 365 nm). ^{1}H and ^{13}C NMR spectra were recorded in the indicated solvent on Bruker Avance III 500 MHz spectrometer. Chemical shifts are quoted in parts per million downfield from TMS and the coupling constants are in Hertz. Infrared spectra were recorded on a Perkin- Elmer Spectrum Bx-II FT-IR System spectrophotometer instrument as films on KBr discs. Melting points were performed using a Marte melting point apparatus, and the results were uncorrected. All assignments of the signals of ^{1}H and ^{13}C NMR spectra are consistent with the chemical structures of the products described. The organic solutions were dried over anhydrous sodium sulfate and organic solvents were removed under reduced pressure in a rotary evaporator. Mass spectra (MS) were obtained with a Q-Exactive (Thermo Scienific, Bremen, Alamanha). The sample preparation for mass spectrometry analysis consisted of diluting 1 mg of each sample in 1 ml of methanol. To perform the analysis in positive mode were added to the samples 1µl of formic acid and to the negative mode, 1µl of ammonium hydroxide. The solution obtained was directly infused at a flow rate of 2µl/min into the ESI source. The ESI (±) source conditions were as follows: a nebulizer gas pressure of 0.5 - 1.0 bar, a capillary voltage of 3.0 kV and a transfer capillary temperature of 250 °C.

2.1.1. Synthesis Steps. Synthesis of 1-(3-fluorophenyl)-1-H-pyrazole-4-carboxylic acid (6) (Kleiderer et.al., 1943)

A mixture of 1-(3-fluorophenyl)-1-H-pyrazole-4-carbaldehyde (5) (0.19 g, 1 mmol) and distilled water (30 ml) was heated at reflux temperature, subsequently was added an aqueous solution of $KMnO_4$ (0.17 g, 1 mmol, in 4 ml water) in small portions over 5 min. The reaction was stirred at reflux temperature until the aldehyde was consumed (checked by TLC). After, was added a solution KOH 10 % and the reaction mixture filtered through a Celite, and then added a solution of HCl 10%. The solid precipitated was filtered and dried to provide 1-(3-fluorophenyl)-1-H-pyrazole-4-carboxylic acid (6) as a white solid in 77% of yield: mp 85°C, Rf=0.56 (hexane/ethyl acetate, 7 : 3). IR_{max} (KBr) cm^{-1}: 3102 (ν O–H), 1666 (ν C=O); 874 e 768 (ν 1,3-C–F). ^{1}H-NMR (500.13 MHz) $CDCl_3$ δ: 9,98 (1H, s, CHO); 8,46 (1H, d, J=0,5 Hz; H-5); 8,17 (1H, d, J= 0,5 Hz, H-3); 7,53 (1H, dddd, J= 10,3; 2,4; 1,9 e 0,7Hz, H-2'); 7,51 (1H, ddd, J= 7,4; 1,9 e 1,5Hz, H-6'); 7,48 (1H, dddd, J=8,1; 7,4; 5,4 e 0,7Hz, H-5'); 7,10 (1H, dddd, J= 8,1; 7,9; 2,4 e 1,5Hz, H-4'). ^{13}C-NMR (125.76 MHz) $CDCl_3$ δ: 183,8 (C-CHO); 164,1 (C-3'); 141,7 (C-3); 140,2 (C-1'); 131,2 (C-5'); 130,3 (C-5); 125,6 (C-4); 114,8 (C-4'); 114,8 (C-6'); 107,5 (C-2'). MS: $[M - H]^-$ m/z of 205.04131 (Fig. 1 – supplementary material).

2.1.2. Synthesis of methyl 1-(3-fluorophenyl)-1-H-pyrazole-4-carboxylate (7) (Brian et.al., 1989)

A mixture of 1-(3-fluorophenyl)-1-H-pyrazole-4-carboxylic acid (6) (0.21 g, 1 mmol), methanol (30 ml), and catalytic amount of H_2SO_4 was heated at reflux temperature for 8 h. After the methanol was distilled at reduced pressure and the residue

was dissolved AcOEt (50 ml) and washed with saturated aqueous solution of $NaHCO_3$ (3 x 10 mL). The organic phase was dried with anhydrous Na_2SO_4 and evaporated under reduced pressure. The residue methyl 1-(3-fluorophenyl)-1-H-pyrazole-4-carboxylate was used in the next step without purification, as a beige solid in 79% of yield: mp 112°C, Rf=0.45 (hexane/ethyl acetate, 8 : 2). IR_{max} (KBr) cm^{-1}: 3130 (ν C–H), 1720(ν C=O); 1245 (ν C–O); 864 e 722 (ν 1,3-C–F). ^{1}H-NMR (500.13 MHz) $CDCl_3$ δ: 3,87 (3H, s, OCH_3), 8,41 (1H, d, J= 0,6 Hz, H-5); 8,09 (1H, d, J= 0,6 Hz; H-3); 7,51 (1H, dddd, J=10,5; 2,4; 1,8; 0,7 Hz, H-2'); 7,48 (1H, ddd, J= 7,4; 1,8; 1,5 Hz, H-6'); 7,44 (1H, dddd, J=8,2; 7,4; 5,4; 0,7 Hz, H-5'); 7,05(1H, dddd, J= 8,2; 7,9; 2,4; 1,5Hz, H-4´). ^{13}C-NMR (125.76 MHz) $CDCl_3$ δ: 162,9 (C-$COOCH_3$); 163,5 (C-3'); 142,4 (C-3); 139,5 (C-1'); 129,9 (C-5); 130,2 (C-5'); 114,2 (C-6'); 116,9 (C-4); 115,5 (C-4'); 107,6 (C-2'); (C-4); 51,6 (C-OCH_3). MS: $[M + H]^+$ m/z of 221.07167 (Fig. 2 - supplementary material).

2.1.3. Synthesis of 1-(3-fluorophenyl)-1-H-pyrazole-4-carbohydrazide (8) (Jha et al., 2010)

A mixture of methyl 1-(3-fluorophenyl)-1-H-pyrazole-4-carboxylate (7) (0.22g, 1 mmol), absolute ethanol (30 ml), and hydrazine hydrate 80 %(0.40 ml, 8 mmol) was heated at reflux temperature for 8 h. Then the solvent was evaporated and the solution was neutralized with dilute hydrochloric acid (10%). The solid obtained was filtered, dried and crystallized from ethanol/water, to provide 1-(3-fluorophenyl)-1-H-pyrazole-4-carbohydrazide (8) as a beige solid in 90% of yield: mp 205°C, Rf=0.62 (CH_2Cl_2/ MeOH, 9 : 1). IR_{max} (KBr) cm^{-1}: 3266 e 3059 (ν N-H), 1607 (ν C=O), 864 e 722 (ν 1,3-C–F). ^{1}H-NMR (500.13 MHz) DMSO–d_6 δ: 4,42 (2H, sl, -NH_2); 9,51 (1H, sl, -COONH); 8,95 (1H, d, J= 0,6 Hz, H-5); 8,15 (1H, d, J=0,6 Hz, H-3); 7,73 (1H, dddd, J=

10,3; 2,3; 2,1 e 0,5Hz, H-2'); 7,70 (1H, ddd, J=8,2; 2,1 e 1,0Hz, H-6'); 7,54 (1H, tdd, J= 8,2; 6,2 e 0,5Hz, H-5'); 7,18 (1H, tdd, J= 8,2; 2,3 e 1,0Hz, H-4'). ^{13}C-NMR (125.76 MHz) DMSO–d_6 δ: 163,3 (C-3'); 161,2 (C-CONH); 141,0 (C-1´); 140,4 (C-3); 132,1 (C-5'); 130,6 (C-5); 119,8 (C-4); 115,6 (C-6'); 113,5 (C-4'); 106,6 (C-2'). MS: $[M + H]^+$ m/z of 221.08307 (Fig. 3 - supplementary material).

2.1.4. Synthesis of 5-(1-(3-fluorophenyl)-1-H-pyrazol-4-yl)-1,3,4-oxadiazole-2(3H)-thione (9) (Jha et al., 2010)

A mixture of 1-(3-fluorophenyl)-1-H-pyrazole-4-carbohydrazide (8) (0.22 g, 1 mmol), absolute ethanol (30 ml), potassium hydroxide (0.17 g, 3 mmol), and carbon disulphide (0.18 ml, 3 mmol) was heated at reflux temperature for 12 h. After, excess solvent was evaporated under reduced pressure and the residue was dissolved in water and then acidified with dilute hydrochloric acid (10%) to pH=5. The precipitate was filtered off, dried, and crystallized from ethanol/water to provide 5-(1-(3-fluorophenyl)-1-H-pyrazol-4-yl)-1,3,4-oxadiazole-2(3H)-thione (9) as solid color white 71% of yield: mp 217°C, Rf=0.63 (hexane/ ethyl acetate, 6 : 4): IR_{max} (KBr) cm^{-1}: 3120 (ν C–H), 2949 (ν C–N), 1318 (ν C=S), 864 e 722 (ν 1,3-C–F). ^{1}H-NMR (500.13 MHz) DMSO–d_6 δ: 14,65 (1H, sl, H-3''); 9,33 (1H, d, J= 0,6 Hz, H-5); 8,31 (1H, d, J= 0,6 Hz; H-3); 7,85 (1H, dddd, J= 10,3; 2,3; 2,1 e 0,5Hz, H-2'); 7,82 (1H, ddd, J=8,2; 2,1 e 1,0Hz, H-6'); 7,57 (1H, tdd, J= 8,2; 6,2 e 0,5Hz, H-5'); 7,22 (1H, tdd, J= 8,2; 2,3 e 1,0Hz, H-4').^{13}C-NMR (125.76 MHz) DMSO–d_6 δ: 176,8 (C-4''); 161,5 (C-2'); 155,8 (C-1''); 140,1 (C-3); 139,5 (C-1'); 131,6 (C-5'); 128,6 (C-5); 114,8 (C-6'); 113,9 (C-4'); 106,6 (C-2'); 107,9 (C-4). MS: $[M + H]^+$ m/z of 263.03928 (Fig. 4 - supplementary material).

.

2.1.5. Synthesis of 2-(1-(3-fluorophenyl)-1-H-pyrazol-4-yl)-5-(methylthio)-1,3,4-oxadiazole (10) (Brian et.al., 1989)

To a mixture of 5-(1-(3-fluorophenyl)-1-H-pyrazol-4-yl)-1,3,4-oxadiazole-2(3H)-thione (9) (0.26g, 1mmol), potassium carbonate (0.14g, 1mmol) and N,N-dimethylformamide (DMF) (5 ml), was added methyl iodine (0.06 ml, 1 mmol) .The reaction mixture was stirred for 1 h at room temperature and was then allowed to cool and poured into ice. The precipitate was vacuum filtered and dried, to provide 2-(1-(3-fluorophenyl)-1-H-pyrazol-4-yl)-5-(methylthio)-1, 3, 4-oxadiazole (10) as a white solid in 98% of yield; mp 205°C, Rf=0.63 (hexane/ ethyl acetate, 6 : 4): IR_{max} (KBr) cm^{-1}: 3154 (ν C–H), 2235 (ν C–N), 864 e 722 (ν 1,3-C–F); ^{1}H-NMR (500.13 MHz) $CDCl_3$ δ: 2,78 (3H,s, - SCH_3), 8.49 (1H, d, J=0.61 Hz, H-5), 8.18 (1H, d, J=0.61 Hz, H-3), 7,53 (1H, dddd, J= 10,3; 2,3; 2,1 e 0,5Hz, H-2'); 7,51 (1H, ddd, J=8,2; 2,1 e 1,0Hz, H-6'); 7,47 (1H, tdd, J= 8,2; 6,2 e 0,5Hz, H-5'); 7,07 (1H, tdd, J= 8,2; 2,3 e 1,0Hz, H-4'). ^{13}C-NMR (125.76 MHz) $CDCl_3$ δ: 162.4 (C-3′), 153,4 (C-1´´), 148.5 (C-4´´), 141.3 (C-1′), 139.8 (C-3), 130,0 (C-5′), 126.4 (C-5), 113.4 (C-4′), 114.8 (C-6′), 106.1 (C-2′), 108.6 (C-4) 15.8 (C-SCH_3). MS: $[M + H]^+$ m/z of 277.05487 (Fig. 5 - supplementary material).

2.1.6. Synthesis of 2-(1-(3-fluorophenyl)-1-H-pyrazol-4-yl)-5-(methylsulfonyl)-1,3,4-oxadiazole (11) (Xu et al., 2011)

To a solution of 2-(1-(3-fluorophenyl)-1-H-pyrazol-4-yl)-5-(methylthio)-1,3,4-oxadiazole (10) (0.28, 1mmol) and acetic acid (10 ml), was added a solution of $KMnO_4$ 5% (0.47 g, 3 mmol). The reaction mixture was refluxed for 30 minutes. Then, was added a solution of disulfide sodium 10%, and the precipitate product obtained was filtered and dried and crystallized from ethanol, to provide 2-(1-(3-fluorophenyl)-1H-

pyrazol-4-yl)-5-(methylsulfonyl)-1,3,4-oxadiazole (11) as a white solid in 70% of yield; mp 214°C, Rf=0.52 (hexane/ ethyl acetate, 6 : 4): IR_{max} (KBr) cm^{-1}: 3110 (ν C–H), 2235 (ν C–N), 1348 e 1115 (ν , S=O), 864 e 722 (ν 1,3-C–F); ^{1}H-NMR (500.13 MHz) $CDCl_3$ δ: 9,02 (1H, d, J=0.61 Hz, H-5), 8,07 (1H, d, J=0.61 Hz, H-3), 7,78 (1H, dddd, J= 10,3; 2,3; 2,1 e 0,5Hz, H-2'); 7,76 (1H, ddd, J=8,2; 2,1 e 1,0Hz, H-6'); 7,54 (1H, tdd, J= 8,2; 6,2 e 0,5Hz, H-5'); 7,19 (1H, tdd, J= 8,2; 2,3 e 1,0Hz, H-4'). ^{13}C-NMR (125.76 MHz) $CDCl_3$ δ: 163.9 (C-1´´), 164,2 (C-3´), 154,7 (C-4´´), 142,7 (C-3), 139,8 (C-1′), 131,7 (C-5); 132,0 (C-5′), 115.1 (C-6′), 114,0 (C-4´), 109,7 (C-4), 106,9 (C-2´), 39.8 ($C\text{-}CH_3$). MS: $[M - H]^-$ m/z of 291.03598 (Fig. 6 - supplementary material).

2.2. Pharmacological methods

2.2.1. Animals

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

2.2.2. Drugs and chemicals

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

USA), dexamethasone (Decadron®, Ache, Brazil), formaldehyde (Synth, Brazil), indomethacin (Indocid®, Merck Sharp & Dohme Farmacêutica-Ltda, SP, Brazil), morphine hydrochloride (Dimorf®, Cristalia, SP, Brazil). LQFM-146, LQFM-147 and LQFM-148 were dissolved in 10% DMSO in distilled water, and all other drugs were dissolved just in distilled water. The doses of the three compounds (LQFM-146, LQFM-147 and LQFM-148) were based on doses of 5-(1-(3-fluorophenyl)-1-H-pyrazol-4-yl)-2H-tetrazole (1) used in previous studies (Florentino et al., 2015a, 2015b).

2.2.3. Anti-nociceptive activity

2.2.3.1.Acetic acid-induced abdominal writhing test

The acetic acid-induced nociception was performed 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), LQFM-146, LQFM-147 or LQFM-148 at doses 89, 178 or 356 µmol/kg or indomethacin (28 µmol/kg, positive control for antinociceptive 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, and the results are expressed as the means ± S.E.M. of number of writhing.

2.2.3.2.Formalin test

The formalin-induced nociception was performed as described previously by Hunskaar et al., (1987). Groups of mice (n = 8) were treated with vehicle (10% DMSO 10 ml/kg p.o.), LQFM-146, LQFM-147 or LQFM-148 at dose 178 µmol/kg p.o., indomethacin (28 µmol/kg, p.o. - positive control for antinociceptive activity in the

second phase), or morphine (17.5 µmol/kg, s.c. - positive control for antinociceptive activity in the first and second phases). Following 60 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 unhindered observation of the formalin-injected paw for 30 min. Pain reaction time (licking time) was assessed during two periods: 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 release of inflammatory mediators. These results were expressed as the means ± S.E.M. in seconds.

2.2.4. Anti-inflammatory activity

2.2.4.1.Carrageenan-induced edema

Carrageenan-induced hind paw edema in mice was used as the animal model of acute inflammation according to the method of Passos et al. (2007). Initially, the animals (n = 9) were treated with vehicle (10% DMSO 10 ml/kg p.o.), LQFM-146, LQFM-147 or LQFM-148 at dose 178 µmol/kg p.o., or indomethacin (28 µmol/kg, p.o). One hour after, 50 µl of carrageenan (1%) was injected intraplantar the right hind paw and 50 µl of saline (0.9% NaCl) of 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 hours after the injection of carrageenan.

2.2.4.2.Carrageenan-induced pleurisy

In this test, it was possible to evaluate important inflammation parameters, such as leukocyte migration and protein extravasations. The animals (n = 8) were treated with vehicle (10% DMSO 10 ml/kg p.o.), LQFM-146, LQFM-147 or LQFM-148 at dose 178 µmol/kg p.o., or dexamethasone (5 µmol/kg, p.o. - positive control for anti-inflammatory activity). One hour after the treatments, the animals received an injection of 100 µL of 1% carrageenan into the pleural cavity. Four hours after administration, the pleural exudate was collected with 1 ml of heparinized Phosphate Buffered Saline and used to count the number of total leukocytes using a Türk solution in a Neubauer chamber (Saleh et al., 1999; Vinegar et al., 1973), to evaluate the myeloperoxidase activity and the TNF-α levels.

2.2.4.3.Myeloperoxidase activity

To measure the myeloperoxidase (MPO) activity, 20 µl of pleural lavage of mice treated with vehicle (10% DMSO, 10 ml/kg), LQFM-146, LQFM-147 or LQFM-148 at dose 178 µmol/kg p.o., or dexamethasone (5 µmol/kg, p.o) was transferred to microplates wells. The reaction was started by adding 180 ml of phosphate buffer pH 6.0 containing 0.167 mg/ml of o-dianisidine 2HCl in 0.0005% H_2O_2. The enzyme reaction was stopped after 15 min by adding 1% sodium azide. The samples were centrifuged subsequently for 5 min at 1000 rpm. The supernatant fraction was separated and transferred to 100 ml microplate wells and absorbance was assayed by enzyme-linked immunosorbent assay (ELISA) reading, at wavelength 450 nm. (Lino et al 2012; Saleh et al., 1999).

2.2.4.4.Determination of TNF-α concentration

TNF-α concentration in pleural exudates were evaluated using an immunosorbent assay kit (ELISA) (Ebioscience) as described previously by Nicoletti et al. (2010). Results were expressed as means ± S.E.M. of TNF-α concentration (pg/ml).

2.3. Statistical analysis

The data were analyzed 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' 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.

3. Results

3.1. Synthesis of LQFM-146, LQFM-147 and LQFM-148

As illustrated in scheme 2, the synthetic route began with reaction condensation between 1-(3-fluorophenyl) hydrazine hydrochloride (3) and 1,1,3,3-tetramethoxypropane and proceeded through the classical method described by Finar and Godfrey (1957). The Chemoselective and regiospecific formylation of 1-(3-fluorophenyl)-1-H-pyrazole (4) to 1-(3-fluorophenyl)-1-H-pyrazole-4-carbaldehyde (5) was performed under Duff's conditions (Almeida et.al., 2011). Following the 1-(3-fluorophenyl)-1-H-pyrazole-4-carbaldehyde (5) is oxidized to intermediate 1-(3-fluorophenyl)-1-H-pyrazole-4-carboxylic acid (6) through the use of potassium permanganate in water (Shriner; Kleiderer, 1943). Then, the derived (6) is subject to the Fischer esterification reaction to methyl 1-(3-fluorophenyl)-1-H-pyrazole-4-carboxylate (7) in methanol under acid catalysis at reflux temperature for 8 h in 85.0% yield (Brian et.al., 1989). Subsequently, the ester (7) is treated with hydrazine hydrate 80% at reflux temperature for 8 h in 90% yield, by the mechanism of addition of the carbonyl

followed by elimination, leading to the formation of the new 1-(3-fluorophenyl)-1-H-pyrazole-4-carbohydrazide (8) (Jha et al., 2010).

The preparation of the 5-(1-(3-fluorophenyl)-1-H-pyrazol-4-yl)-1,3,4-oxadiazole-2(3H)-thione (9) was achieved by reaction of compound acyl hydrazide (8) with carbon disulphide under strong basic conditions at reflux temperature for 12 h, followed by acidification with HCl 10%. The reaction proceeds via nucleophilic addition followed by 1,5-cyclisation to give the compound (9) in 71% yield. The S-alkylation of the subunit 1,3,4-oxadiazole-2(3H)-thione of the intermediate (9) was carried out using classical conditions bimolecular nucleophilic SN_2 substitution reactions to corresponding intermediate 2-(1-(3-fluorophenyl)-1H-pyrazol-4-yl)-5-(methylthio)-1,3,4-oxadiazole (10) (Jha et al., 2010; Salman et al., 2015). Treatment of compound (9) with methyl iodide (CH_3I), potassium carbonate and DMF was performed at room temperature for 1 h in 98% yield (Brian et.al., 1989). Finally, the intermediate (10) was oxidized to 2-(1-(3-fluorophenyl)-1-H-pyrazol-4-yl)-5-(methylsulfonyl)-1,3,4-oxadiazole (11) in the presence of potassium permanganate and acetic acid at reflux temperature for 30 minutes in 70% yield. In this stage the methylthio moiety was oxidized the corresponding methylsulfonyl species (11), the advantage of this oxidation is that it uses an inexpensive oxidant without a catalyst, furthermore, the reaction is quickly complete and easy to work up (Xu et al., 2011). The physical characteristics, IR, ^{1}H-NMR, ^{13}C-NMR or mass spectrometry measurements for all the synthesized compounds are reported in the Experimental section.

3.2. Anti-nociceptive activity

3.2.1. Acetic acid-induced abdominal writhing test

In this test the oral treatments with LQFM-146, LQFM-147 or LQFM-148 decreased in a dose-dependent manner the number of writhing induced by acetic acid when compared to the control group (vehicle 10% DMSO 10 ml/kg). LQFM-146 at doses 178 or 356 μmol/kg reduced the number of writhing of 99.75 ± 3.9 (group treated with vehicle) to 75.44 ± 3.6 (24.4 % less, $P < 0.001$) and to 60.22 ± 2.8 (39.6 % less, $P < 0.001$), respectively. LQFM-147 at doses 89, 178 or 356 μmol/kg reduced this number to 83 ± 5.7 (16.8 % less, $P < 0.05$), 66.86 ± 4.8 (33 % less, $P < 0.001$) or 53.83 ± 5.9 (46 % less, $P < 0.001$), respectively. LQFM-148 at doses 89, 178 or 356 μmol/kg reduced this number to 75.17 ± 5.8 (24.6 % less, $P < 0.001$), 64.5 ± 3.8 (35.3 % less, $P < 0.001$) or 53.71 ± 3.2 (46.2 % less, $P < 0.001$), respectively. The positive control of this test, indomethacin (28 μmol/kg), reduced by 42 % (57.88 ± 5.0 number of writhing) (Fig. 1 A-C).

3.2.2. Formalin test

In the formalin test, LQFM-146, LQFM-147 or LQFM-148 in intermediate dose (178 μmol/kg) showed antinociceptive activity when compared to the control group in the second phase of the test, but in the first phase of the test these compounds not showed significant effect (Fig. 2). In the second phase, the vehicle group showed a licking time (s) of 223.6 ± 13.5; LQFM-146 reduced this time to 163.4 ± 14.4 (26.9 % less, $P < 0.01$); LQFM-147 reduced to 126.6 ± 13.2 (43.4% less, $P < 0.001$) and LQFM-148 reduced to 108.8 ± 9.7 (51.4 % less, $P < 0.001$). The group treated with indomethacin (28 μmol/kg, p.o.), anti-inflammatory positive control, experienced a decrease in the licking time (s) only in the second phase, to 79.25 ± 12.7 (a reduction of 64.6 %, $P < 0.001$). The positive control morphine (17.5 μmol/kg, s.c.), decreased the

first phase to 5.37 ± 2.9 (90.3 % less, P < 0.001) and the second phase to 2.0 ± 1.7 (a reduction of 99.1 %, P < 0.001).

3.3. Anti-inflammatory activity

3.3.1. Carrageenan-induced edema

In this study, the treatments with LQFM-146, LQFM-147 or LQFM-148 reduced the paw edema in the test of carrageenan-induced edema. In the first hour, the oral treatments with these compounds at dose 178 µmol/kg reduced the edema to 117.27 ± 3.8 (14.9 % reduction, P < 0.001), 112.5 ± 9.2 (18.4 % reduction, P < 0.001), or 117.27 ± 4.3 µL (reducing by 14.9%, P < 0.001), respectively, compared to the control group (difference between the paws 137.77 ± 3.7 µL). These treatments also reduced the edema to 107 ± 5.6 (21.5 % reduction, P < 0.001), 103.0 ± 3.7 µL (24.4% reduction, P < 0.001) or 110.9 ± 2.5 (18.6% reduction, P < 0.001) respectively, in second hour, reduced to 98.18 ± 4.6 (17.6 % reduction, P < 0.001), 89 ± 5.7 µL (reducing by 25.3 %, P < 0.001) or 105.55 ± 4.7 (11.4 % reduction, P < 0.001), respectively, in the third hour, and to 91,81 ± 2.9 (19.8 % reduction, P < 0.001), 91 ± 5.3 µL (20.5 % reduction, P < 0.001), or 94.54 ± 3.4 (17.4% reduction, P < 0.001) respectively, in the four hour of the test when compared to the control group (difference between the paws 136.25 ± 6.5, 119.09± 3.7, and 114.44 ± 1.8 µL, respectively). The group treated with indomethacin (28 µmol/kg, p.o.), anti-inflammatory positive control, decreased the carrageenan-induced paw edema at the times of 1, 2, 3, and 4 hours after the treatment (Fig. 3A).

3.3.2. Leukocytes content in the carrageenan-induced pleurisy

In this test, the treatment with the three compounds (LQFM-146, LQFM-147 or LQFM-148 - 178 µmol/kg p.o.) reduced the cell migration, MPO activity and the TNF-

α levels . The number of total leukocytes migrated was reduced to 4.617 ± 0.4 (23.4 % reduction, P < 0.05), 3.494 ± 0.3 (42 % reduction, P < 0.001) or 3.385± 0.3 (43.8 % reduction, P < 0.001) respectively, compared to the control group (6.029 ± 0.5 leukocytes x 10^6/mL). As expected, the positive control dexamethasone also reduced cell migration to 2.375 ± 0.3 (60.6 % reduction, P < 0.001) (Fig. 3B). In the differential count of leukocytes was observed that the oral treatment with LQFM-146, LQFM-147 or LQFM-148 at dose 178 µmol/kg reduced the migration of the polymorphonuclear leukocytes from 3.888 ± 0.2 (vehicle group) to 3.004 ± 0.2 (22.7 % reduction, P < 0.01), 1.548 ± 0.2 (60.2 % reduction, P < 0.001) or 1.485± 0.2 (61.8 % reduction, P < 0.001), respectively. The positive control dexamethasone also reduced the migration of the polymorphonuclear leukocytes to 0.9826± 0.2 (74.7 % reduction, P < 0.001) (Fig. 4A).

3.3.3. Myeloperoxidase activity

The treatments with LQFM-146, LQFM-147 or LQFM-148 at dose 178 µmol/kg reduced the MPO activity from 199.4 ± 20.9 mU/ml (vehicle group) to 155.5 ± 8.3 (22 % reduction, P < 0.05), 136.5 ± 5.6 (31.6 % reduction, P < 0.01) or 143.9 ± 6.7 (27.8 % reduction, P < 0.01) , while dexamethasone (5 µmol/kg, p.o.) decreased it to 117.9 ± 13.5 mU/ml (40.9 % reduction, P < 0.01) (Fig. 4B).

3.3.4. Determination of TNF-α concentration

After intrapleural injection of carrageenan, the oral treatment with LQFM-146, LQFM-147, or LQFM-148, all in dose 178 µmol/kg, reduced the TNF-α concentration in pleural exudates to 40 ± 7.0 (35.8 % reduction, P < 0.01), 21.48 ± 3.6 (65.5 % reduction, P < 0.001) or 27.37± 4.8 pg/ml (56 % reduction, P < 0.001), respectively, when compared to the vehicle group (62.28 ± 5.6 pg/ml). As expected, dexamethasone

(5 μmol/kg, p.o.) also reduced the TNF-α concentration to 14.02 ± 0.4 pg/ml (77.5 % reduction, $P < 0.001$) (Fig. 5).

4. Discussion

The synthesis of compounds or molecular modifications based on chemical structure of NSAIDs are important strategies for the development of new drugs more effective and safe for the treatments of pain and inflammation. In this context was synthesized and evaluated the compounds LQFM-146, LQFM-147 and LQFM-148, new analogues containing subunits N-phenyl-1-H-pirazoles and 1,3,4-oxadiazole-2(3H)-thione, which present potential analgesic and/or anti-inflammatory effects.

In order to evaluate possible anti-nociceptive and/or anti-inflammatory activity of news compounds, it was first performed the acetic acid induced writhing test that is a visceral pain model widely used to evaluate antinociceptive activity. The administration of the acetic acid induces acute peripheral inflammatory activity from the liberation of a variety of chemical mediators such as bradykinin, substance P, prostaglandins and cytokines (Malvar et al., 2014; Ribeiro et al., 2000; Zakaria et al., 2006). The acetic acid induced writhing test has a good sensitivity to evaluate agents with anti-nociceptive activity and also agents with others pharmacological proprieties and, therefore it isn't a test with good specificity (Le Bars et al., 2001). In this model it is observed the abdomen contraction and extension followed by rotating one or both hind legs of mice. In this test the oral treatments with LQFM-146, LQFM-147 or LQFM-148 decreased in a dose-dependent manner the number of writhing induced by acetic acid suggesting a nociceptive activity for the three compounds tested. Thereby, in the next tests it was used only the intermediate dose for all compounds with the perspective of reduce the number of animals as recommended by the CEUA.

Due to the low specificity of the writhing test and to characterize the analgesic effect detected, it was performed the formalin test, a test more specific for substances with analgesic activity, which allows to differentiate two types of pain: the neurogenic pain which corresponds to the first phase of test and involves the direct stimulation of the nociceptors by formalin and nociceptive mediators preforms such as serotonin, substance P, kinins and histamine (Dubuisson et al, 1977; Hunskaar et al, 1987; Shibata et al., 1989; Correa et al., 1993; Munron, 2007); and the inflammatory pain which corresponds to the second stage of the test and involves the stimulation of nociceptors and release of many pro-inflammatory mediators for example, the prostaglandins (Fujimaki et al., 1992; Shibata et al., 1989; Omote et al., 1998). It was observed that LQFM-146, LQFM-147 or LQFM-148 in intermediate dose (178 µmol/kg) showed antinociceptive activity only in the second phase of the test, suggesting that these compounds have anti-inflammatory activity.

To better evaluate the anti-inflammatory effect of these compounds was performed the carrageenan-induced edema test. The carrageenan is inflammatory agent which, when injected into the paw of animals promotes the development of the edema, characterized by multiple inflammatory mediators and exudate of inflammation (Liew and McInnes, 2002).The results show that the treatment of animals with LQFM-146, LQFM-147 or LQFM-148 at dose 178 µmol/kg reduced the edema formation at all hours of the test, showing that these compounds has anti-edema activity similar to the positive control indomethacin and confirming the action anti-inflammatory suggested in the second phase of formalin test .

In addition, was performed the carrageenan-induced pleurisy to confirm the anti-inflammatory effect of LQFM-146, LQFM-147 and LQFM-148. In this model, the inflammation in pleural cavity caused by carrageenan injection is characterized by the

accumulation of exudate and cell migration (Saleh et al., 1999; Murai et al., 2003). From the analysis of the pleural exudate collected, the results observed suggest that LQFM-146, LQFM-147 and LQFM-148 at dose 178 µmol/kg reduced the cell migration from the reduction of polymorphonuclears cells migration similar to the positive control dexamethasone.

It is known that MPO is an enzyme present predominantly polymorphonuclears leukocytes actives and the level of MPO activity is directly proportional to the neutrophil concentration in inflamed tissue (Eddouks et al., 2012; Desmarchelier et al., 1997). These results confirm the anti-inflammatory effect of the LQFM-146, LQFM-147 and LQFM-148 and suggest that this effect also may involve a reduction and/or blockade in the release of inflammatory mediators that promote leucocyte chemotaxis, such as, cytokines (IL-1and TNF-α) (Frode et al., 2001; Mazzon and Cuzzocrea, 2007), chemokines (Kobayashi, 2008; Sanz and Kubes, 2012), leukotrienes (LTB4) (Afonso et al, et al.,2012; Samuelsson, 1983).

TNF-α is an important pro-inflammatory cytokine mainly involved in cellular chemotaxis (Frode et al., 2001 and Mazzon and Cuzzocrea, 2007) and inflammatory hypernociception (Chichorro et al., 2004 and Cunha et al., 2005). The results showed that LQFM-146, LQFM-147 and LQFM-148 also are able of reduce the levels of the cytokine TNF-α similar to the positive control dexamethasone. These activities can explain the anti-inflammatory effect seen in the different models used. However, it is necessary an evaluation of the real interaction these compounds with different enzymes involved in the inflammatory process and the quantification of other cytokines may contribute to better understanding of the mechanisms involved in the anti-inflammatory effect of these compounds.

5. Conclusions

In summary, these results shown that, three new hybrid molecules have been found to exhibit similar effects in pain and acute inflammatory models. Anti-inflammatory activity showed is essential for antinociceptive effect observed with three compounds. These compounds were able to reduce the pain, edema, leukocytes migration, as well as, the activity of myeloperoxidase and TNF-alpha levels. Taken together, these results suggest that these new compounds will be useful in developing new anti-inflammatory drugs.

Conflict of interest statement

The authors declare that they have no conflicts of interest.

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Scheme 1 – Structural design of the derivate 9 (LQFM-148), 10 (LQFM-146) and 11 (LQFM-147).

Scheme 2- Reagents and conditions: (a) 1,1,3,3-Tetramethoxypropane (TMP), HCl, EtOH, reflux 2h, 79% ; (b) Hexamethylenetetramine, trifluoroacetic acid (TFA), reflux 12 hrs, 73% ; (c) Potassium permanganate ($KMnO_4$), H_2O, reflux 4h, , 77%; (d) MeOH,

H_2SO_4, reflux 8 h, 79%; (e) $NHNH_2.H_2O$ 80 %, EtOH, reflux 8 h, 90%; (f) CS_2, KOH, EtOH, reflux 12 h, 71%.(g) N,N-dimethylformamide, K_2CO_3, CH_3I, a.t- r.t 1h, 98%.(h) $KMnO_4$ 5%, CH_3COOH, reflux 1h, 90%.

Fig. 1: Effect of LQFM-146 (A), LQFM-147 (B) or LQFM-148 (C) (89, 178 and 356 μmol/kg p.o.) on the number of acetic acid induced writhing in mice (n = 8). Vehicle (Veh; 10% DMSO 10 ml/kg p.o.). Indomethacin (Indo, 28 μmol/kg p.o.) was used as positive control. Vertical bars represent mean ± S.E.M. of number of writhings in 30 min for each experimental group. * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$ according to ANOVA followed by post-hoc Newman-Keuls' test. Abbreviations: Indo: Indomethacin.

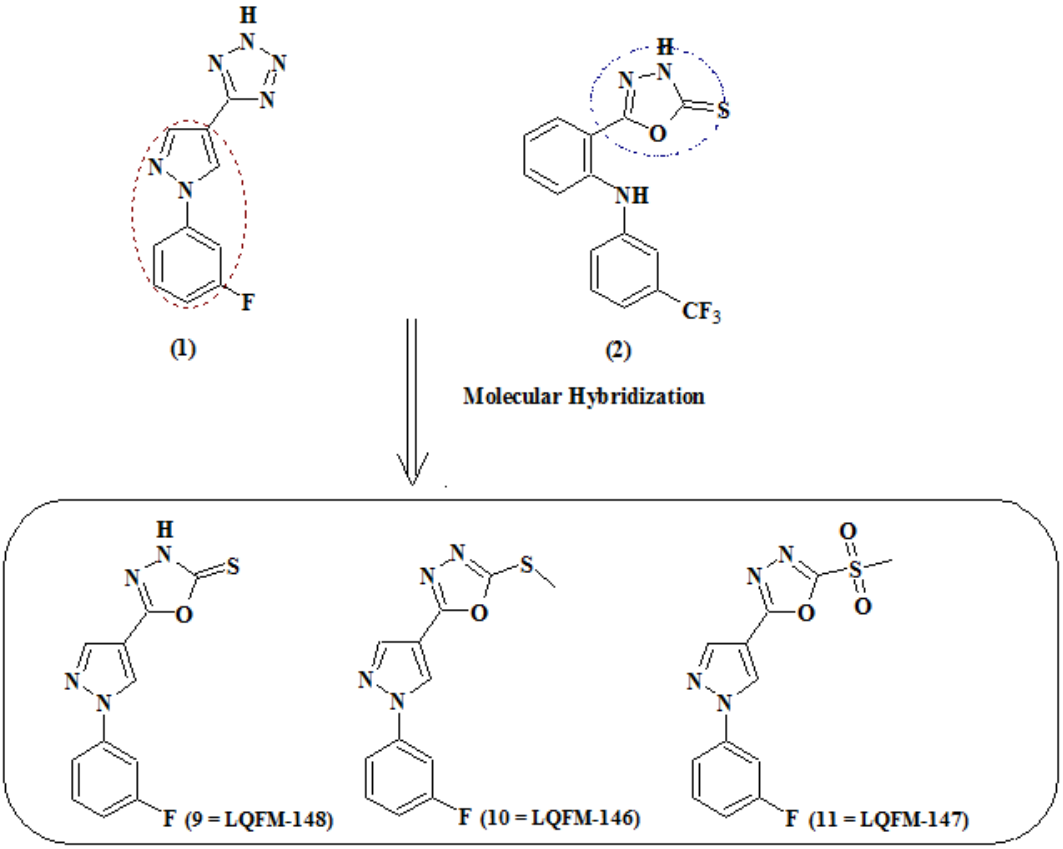
Fig. 2: Anti-nociceptive effect of LQFM-146, LQFM-147 or LQFM-148 (178 μmol/kg, p.o.) in the first and second phase of formalin test in mice (n = 8). Indomethacin (28 μmol/kg, p.o.) and morphine (17.5 μmol/kg, s.c.) on the licking time (s) were used as positive controls, during the first (0–5 min) and second phase (15–30 min). Vertical bars represent mean ± S.E.M. of pain reaction time, in seconds. **$P \leq 0.01$ and ***$P \leq 0.001$ compared with control group, according to ANOVA followed by post-hoc Newman–Keuls' test. Abbreviations: Indo: Indomethacin.

Fig. 3: (A) Effect of LQFM-146, LQFM-147 or LQFM-148 (178 μmol/kg, p.o.) and dexamethasone (5 μmol/kg, p.o. — positive control) on the carrageenan-induced edema test, in mice (n =9). These treatments reduced the edema at all hours after the treatment, when compared with the control group. The values were expressed as mean ± S.E.M. of the difference between the paws, in μL. *$P \leq 0.05$, ***$P \leq 0.001$, compared with control group, according to two-way ANOVA followed by Bonferroni's post-hoc test. (B) Effect of LQFM-146, LQFM-147 or LQFM-148 (178 μmol/kg, p.o.) and dexamethasone (5 μmol/kg, p.o.—positive control) on the total number of leukocytes in the pleural exudates, in mice (n=8). Vertical bars represent mean ± S.E.M. of the number of leukocytes migrated. *$P \leq 0.05$ and ***$P \leq 0.001$, compared with the control group, according to ANOVA followed by post-hoc Newman–Keuls'test. Abbreviations: Dexa: Dexamethasone.

Fig. 4: (A) Effect of LQFM-146, LQFM-147 or LQFM-148 (178 μmol/kg, p.o.) and dexamethasone (5 μmol/kg, p.o.—positive control) on cell migration in the pleural exudates (n=8). Vertical bars represent mean ± S.E.M. of the number of leukocytes migrated according differential counts *P ≤ 0.05 and ***P ≤ 0.001, compared with the control group, according to ANOVA followed by post-hoc Newman–Keuls'test. Abbreviations: Dexa: Dexamethasone. (B) Effects of LQFM-146, LQFM-147 or LQFM-148 (178 μmol/kg, p.o.) and dexamethasone (5 μmol/kg, p.o.—positive control) on the activity of myeloperoxidase enzyme (MPO) in the pleural exudates (n=8). Vertical bars represent mean ± S.E.M. of the enzymatic activity of the MPO at mU/ml in the pleurisy test. *P ≤ 0.05 and **P ≤ 0.01, compared with the control group, according to ANOVA followed by post-hoc Newman–Keuls'test. Abbreviations: Dexa: Dexamethasone.

Fig. 5: Effect of LQFM-146, LQFM-147 or LQFM-148 (178 μmol/kg, p.o.) or dexamethasone (5 μmol/kg, p.o.—positive control) on TNF-α concentration in the pleural exudates. The pleural exudates were collected 4h after the carrageenan injection, in the pleurisy. Vertical bars represent mean ± S.E.M. of the TNF-α concentration at pg/ml in the pleurisy test (n=8). **P ≤ 0.01 and ***P ≤ 0.001, compared with the control group, according to ANOVA followed by post-hoc Newman–Keuls'test. Abbreviations: Dexa: Dexamethasone.

Scheme 1.



Scheme 2.

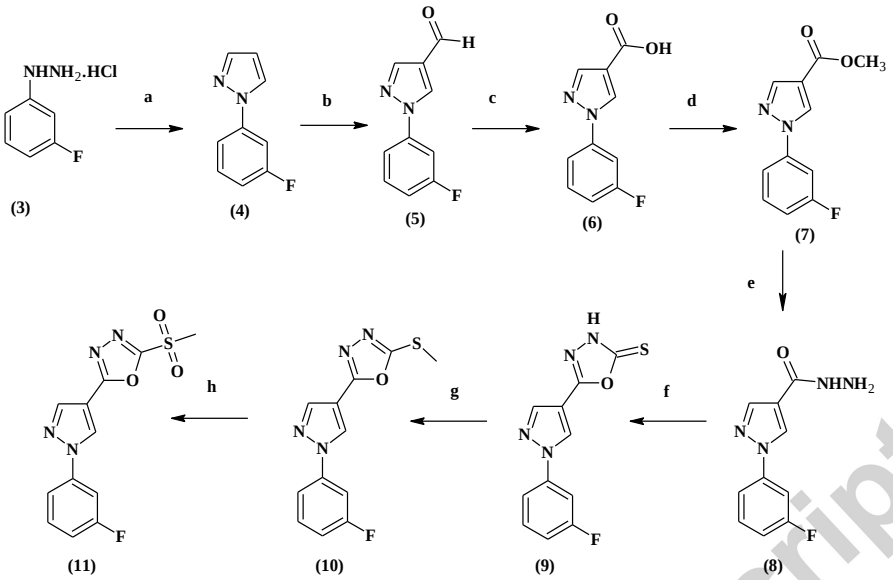


Fig. 1

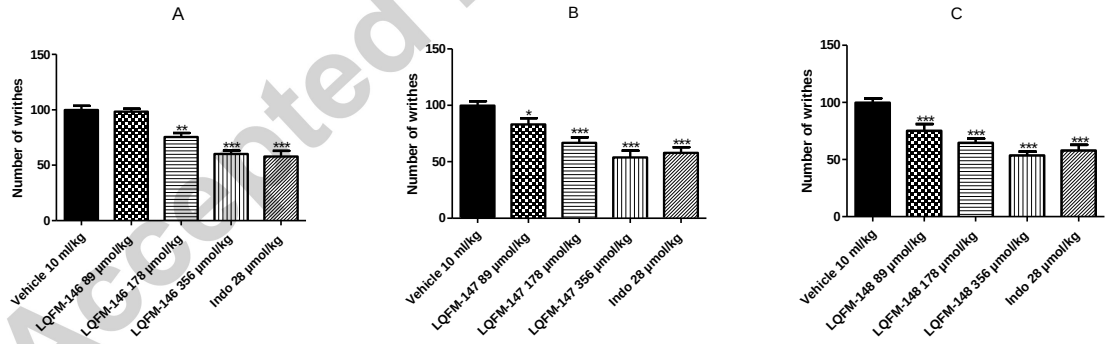


Fig. 2

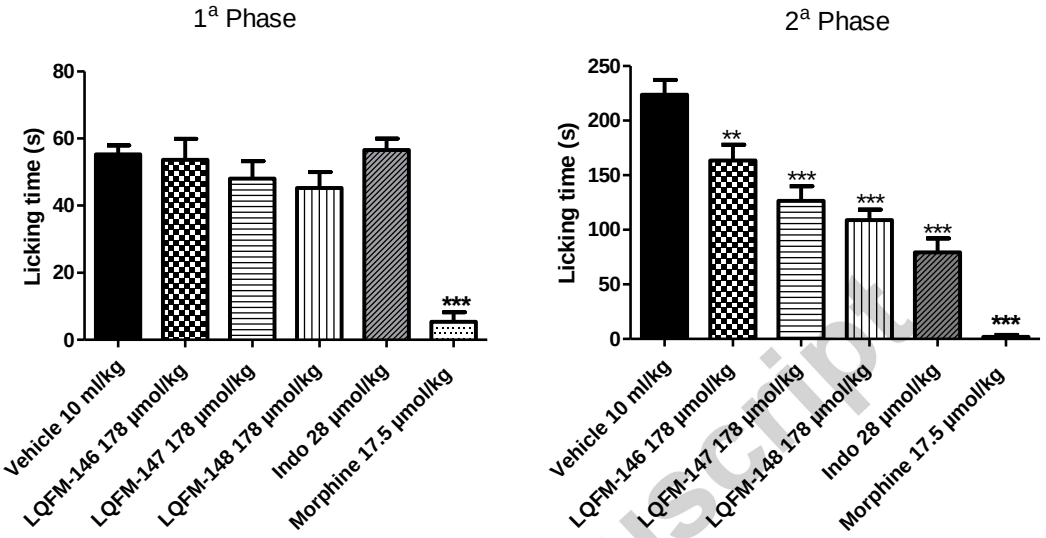


Fig. 3

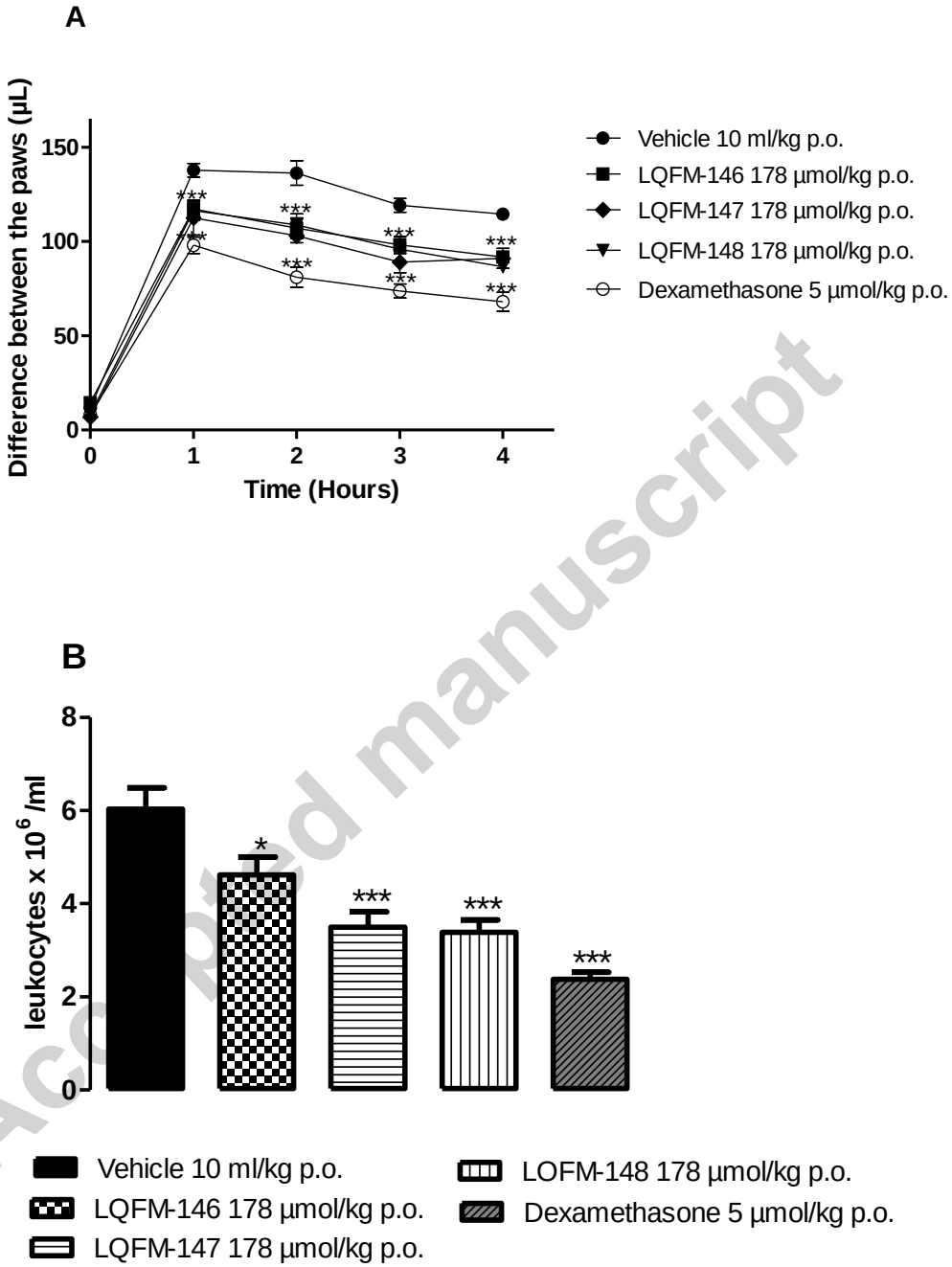
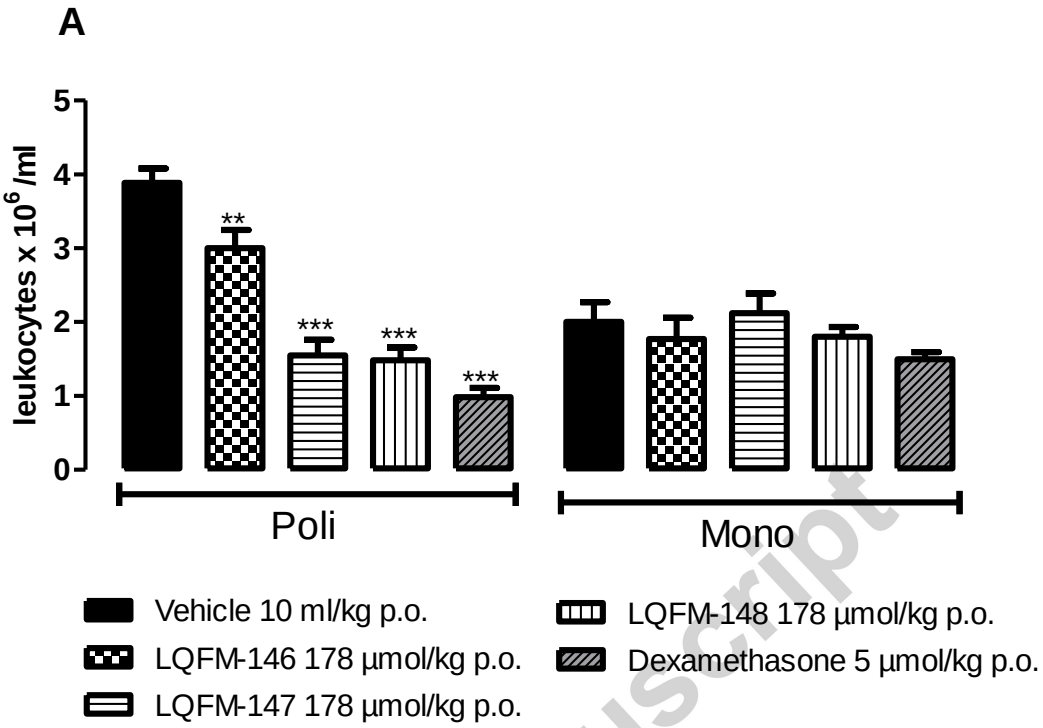


Fig. 4



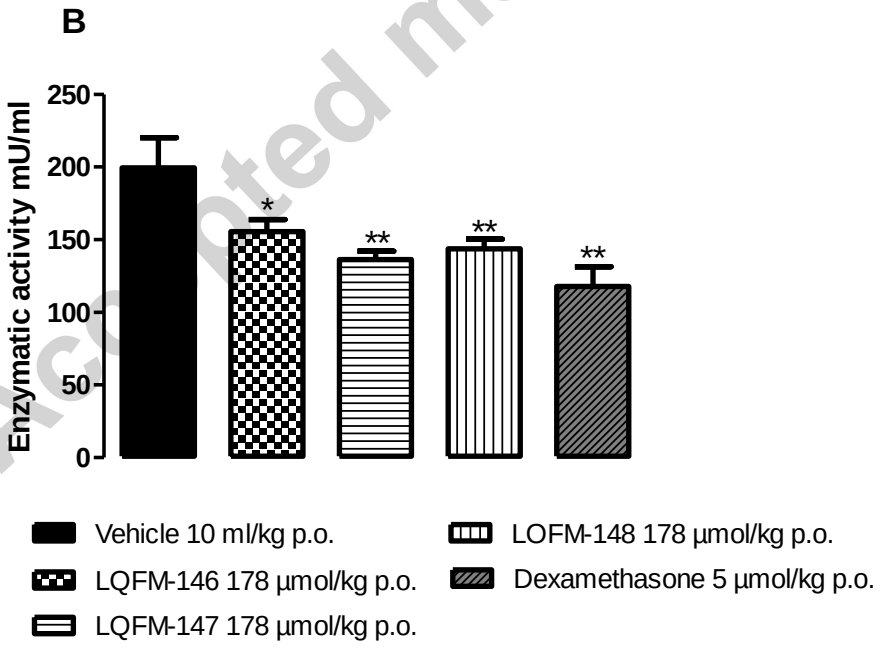


Fig. 5