European Journal of Pharmaceutical Sciences 106 (2017) 231–243

Contents lists available at ScienceDirect

European Journal of Pharmaceutical Sciences

journal homepage: www.elsevier.com/locate/ejps

Pharmacological evaluation and molecular docking of new di-*tert*-butylphenol compound, LQFM-091, a new dual 5-LOX/COX inhibitor

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ARTICLE INFO

Keywords:
Analgesic
Anti-inflammatory
Anti-oxidants
Cytokines
Cyclo-oxygenase
Lipoxygenase

ABSTRACT

Dual 5-LOX/COX inhibitors are potential new dual drugs to treat inflammatory conditions. This research aimed to design, synthesis and to evaluate the anti-inflammatory and antinociceptive effects of the new compound, which is derived from nimesulide and darbufelone lead compounds. The new dual inhibitor 5-LOX/COX has the possible advantage of gastrointestinal safety. A voltammetric experiment was conducted to observe the drug's antioxidative effect. A formalin test, a hot plate test and carrageenan-induced mechanical hyperalgesia were employed to evaluate the analgesic nature of LQFM-091. To evaluate anti-inflammatory activity, we measured edema, leukocyte count, myeloperoxidase activity and cytokines levels in carrageenan-induced inflammation tests. We elucidated the underlying mechanisms by assessing the interaction the with COXs and LOX enzymes by colorimetric screening assay and molecular docking. The lethal dose (LD_{50}) was estimated using 3T3 Neutral Red Uptake assay. Our results indicate that the LQFM-091 prototype is a powerful antioxidant, as well as able to inhibit COX-1, COX-2 and LOX activities. LQFM091 was classified in GHS category 4 ($300 < LD_{50} < 2000$ mg/Kg). This prototype showed analgesic activity in the formalin test and decreased carrageenan-induced mechanical hyperalgesia. Furthermore, LQFM-091 reduced the paw edema induced by carrageenan and reduced the leukocyte count, myeloperoxidase activity, TNF-α and IL-1β levels in the pleural exudate. Another interesting finding was the absence of gastrointestinal lesions. These data indicate that LQFM-091 produced antinociceptive and anti-inflammatory effects while maintaining gastrointestinal safety. Furthermore, this compound presented a safe toxicological profile. Blocked COXs and LOX enzymes are important targets for manipulating the mechanism of this compound.

1. Introduction

Inflammation is a physiological response that begins immediately after tissue injury, and its main goal is to remove the pathogenic agent and to restore the tissue (Sousa et al., 2013). However, a prolonged inflammation may contribute to the pathogenesis of many chronic diseases of inflammatory origin (Gilroy et al., 2004).

In the inflammatory process, activation of cascade of arachidonic acid occurs that leads the production of several inflammatory mediators takes place through the action of cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450s (CYP) enzymes (Dennis and Norris, 2015).

Cyclooxygenase is the key enzyme in the biosynthesis of prostaglandins (PGs) (González-Périz and Clària, 2007). There are also LOX,

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http://dx.doi.org/10.1016/j.ejps.2017.06.006
Received 28 December 2016; Received in revised form 8 May 2017; Accepted 5 June 2017

designated 5-LOX, 12-LOX and 15-LOX. 5-LOX converts arachidonic acid into hydroxyeicosatetraenoic acid (HETE), specifically in 5(*S*)-HETE and leukotrienes (LTs), whereas 12-LOX and 15-LOX generate the corresponding 12 and 15-HETEs, respectively (González-Périz and Clària, 2007; Korotkova and Lundberg, 2014). 15-HETEs are in turn converted to lipoxins—important anti-inflammatory mediators (Bertolini et al., 2002)—by neutrophils and 5-HPETE, or 5-hydroperoxyeicosatetraenoic acid.

Currently, nonsteroidal anti-inflammatory (NSAIDs) constitute the main class of drugs used for the treatment of inflammation and pain. NSAIDs inhibit prostaglandin (PG) synthesis by rendering cyclooxygenase inactive (Mitchell et al., 1993) which are divided into first and second generation. First-generation NSAID inhibitors, both cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2), and second-generation, also known as coxibs, are selective for COX-2 (Vane and Botting, 1998). Both NSAIDs of the first or second generation exert their anti-inflammatory activity, reducing the production of PGs in the inflammatory process (Dennis and Norris, 2015).

Nowadays, there is a consensus that multifactorial diseases can originate from several sources, so looking for drugs with dual profiles that can be expressed through more than one biochemical window would be useful for treating many kinds of diseases (Gilroy et al., 2004; Barreiro and Fraga, 2008). In this sense, dual 5-LOX/COX inhibitors are new, potential dual drugs to treat inflammation and act by blocking the formation of both PGs and LTs but do not affect lipoxin formation (Martel-Pelletier et al., 2003; Lai et al., 2010). Such combined inhibition avoids some disadvantages of selective COX-2 inhibitors which reduces damage caused to gastrointestinal mucosa (Martel-Pelletier et al., 2003).

As reported in the literature, the experimental data for leukotriene modulators, licofelone, darbufelone (**1**) and BF-389, all 5-LOX/COX inhibitors, are considered to have a complementary role of LTs and PGs in the maintenance and regulation of episodes of pain and inflammation (Kulkarni and Singh, 2008). Moreover, nimesulide, a NSAID, has preferential selectivity for COX-2 over COX-1 *in vivo* at full therapeutic doses and induces less gastrointestinal damage than that seen with other NSAIDs (Shah et al., 2001; Warner et al., 1999). Thus, in the scope of a research program aimed at drug development for treatment of inflammatory disease, we describe in the present study the design, synthesis and pharmacological evaluation of LQFM-091 (**3**), a new compound derived from nimesulide (**2**) and darbufelone (**1**), creating a new dual inhibitor of 5-LOX/COX with the possible advantage of -gastrointestinal safety (Fig. 1a).

2. Material and methods

2.1. Animals

Experiments were performed using female *Swiss* albino mice (25–30 g) aging 7 weeks, 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 they lived under a 12 h light/dark cycle, in compliance with the International Guiding Principles for Biomedical Research Involving Animals. The animals were acclimated for 7 days before beginning the experiments. All experimental protocols were developed according to the principles of ethics and animal welfare designated by the Ethics Commission on Use of Animals (CEUA). The experimental protocols were approved by CEUA of the UFG (number: 37/14).

2.2. Drugs, chemicals and kits

The chemicals used in this study were acetic acid (Cromoline Química Fina LTDA, Diadema, SP, Brazil), carrageenan (Sigma Chemical, USA), DMSO (dimethyl sulfoxide – Vetec, Rio de Janeiro, RJ, Brazil), Evan's blue (Merck, São Paulo, SP, Brazil), formaldehyde (Synth, Brazil), morphine sulfate pentahydrate (MW: 758.83-Dimorf®, Cristalia, SP, Brazil), nimesulide (MW: 308.31), (Sigma–Aldrich, USA), sodium heparin (Parinex®, Hipolabor, MG, Brazil). Peroxidase, hydrogen peroxide (0.0005%, p/v), sodium azide and *o*-dianisidine 2HCl were also utilised (Sigma Chemical, USA). Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, trypsin/EDTA, fetal bovine serum (FBS) and neutral red (NR) dye were purchased from Sigma-Aldrich (St. Louis, MO, USA). The TNF-alpha Elisa Ready-Set-GO Mouse Kit (no. 88–7324) of eBioscience was employed. “Colorimetric COX (ovine) inhibitor screening assay” kits (no. 760111, and Cayman Chemical) and Lipoxygenase (soybean) inhibitor screening assay kits (no. 760700, Cayman Chemical Company) were also used. Treatments with the vehicle, LQFM-091(**3**) and nimesulide (**2**) were administered orally by gavage. Morphine treatments were performed by subcutaneous injection.

2.3. Chemistry

2.3.1. General

Reactions were monitored by thin-layer chromatography 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 Nuclear magnetic resonance (NMR) spectra were recorded in the indicated solvent on a Bruker Avance III 500 MHz spectrometer, in $CDCl_3$. Chemical shifts are quoted in parts per million downfield from tetramethylsilane, and the coupling constants are in Hertz. All assignments of the signals of ^{1}H and ^{13}C NMR spectra are consistent with the chemical structures of the products described. Infrared spectra were recorded on a Perkin- Elmer Spectrum Bx-II FT-IR System spectrophotometer instrument as films on KBr discs. Mass spectra (MS) were obtained with a Q-Exactive (Thermo Scienific, Bremen, Alamanha). The sample preparation for mass spectrometry analysis consisted of diluting 0.5 mg of each sample in 1 mL of methanol. To perform the analysis in negative mode, 1 μL of ammonium hydroxide was added to the samples. The solution obtained was directly infused at a flow rate of 5 $\mu L \cdot min^{-1}$ into the electrospray Ionisation (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 275 °C. Melting points were performed using a Marte melting point apparatus, and the results were uncorrected. The organic solutions were dried over anhydrous sodium sulfate, and organic solvents were removed under reduced pressure in a rotary evaporator. Methanol of analytical grade was supplied by J.T. Baker (Phillipsburg, NJ). Potassium phosphate and acetate were obtained from Vetec (Rio de Janeiro, Brazil). All electrolyte solutions were prepared using analytical grade reagents and double distilled water.

2.3.2. Synthesis step. Synthesis of 2,6-di-tert-butyl-4-((4-nitrophenylamino)methyl)phenol (3) (Luo et al., 2004)

A stirred heterogeneous mixture of 3,5-Di-*tert*-butyl-4-hydroxybenzaldehyde (**4**) (1.0 mmol), 4-nitroaniline (**5**) (1.0 mmol), and $NaBH_3CN$ (0.5 mmol) in 5 mL of MeOH was added $ZnCl_2$ (0.5 mmol) in one portion. The mixture was stirred at a room temperature for 2 h. In turn, MeOH was then evaporated, and the residue was partitioned between water and CH_2Cl_2. The combined organic layers were dried (Na_2SO_4), concentrated *in vacuo*, and the crude product was purified by chromatography using hexane:ethyl acetate as a mobile phase to 2,6-di-*tert*-butyl-4-((4-nitrophenylamino)methyl)phenol (**3**) and as a yellow solid in 75% of the yield, M.P. > 150 °C, *Rf* = 0.96 (hexane:ethyl acetate, 70:30): IR_{max} (KBr) cm^{-1}: 3621 (*ν* O—H), 3384 (*ν* N—H), 1599 and 1362 (*ν* NO_2) and 831 (*ν* Ar 1,4); ^{1}H NMR (500.13 MHz) $CDCl_3$ *δ*: 8.11 (2H, dd, *J* = 6.58 and 2.02 Hz, H-5 and H-7), 7.14 (2H, s, H-2′ and H-6′), 6.59 (2H, dd, *J* = 6.58 and 2.02 Hz, H-4 and H-8), 5.27 (1H, l, OH), 4.66 (1H, ls, NH), 4.27 (2H, d, *J* = 3.6 Hz, H-1), 1.44 (18H, s, H-7′ and H-8′); ^{13}C NMR (125.76 MHz) $CDCl_3$ *δ*: 153.3 (C-4′), 152.9 (C-3), 137.8 (C-6), 136.2 (C-3′ and C-5′), 136.0 (C-1′), 126.4 (C-5 and C-7),