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In vitro genotoxicity and *in vivo* subchronic evaluation of the anti-inflammatory pyrazole compound LQFM021

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ABSTRACT

Scientific evidences have highlighted 5-(1-(3-fluorophenyl)-1H-pyrazol-4-yl)-2H-tetrazole (LQFM021) as a promising anti-inflammatory, analgesic and antinociceptive agent due to its effects on peripheral opioid receptors associated with activation of the nitric oxide/cGMP/K_{ATP} pathway. Despite these important pharmacological findings, toxicity data of LQFM021 are scarce. Thus, this study investigated the *in vitro* genotoxicity of LQFM021 through cytokinesis-block micronucleus assay (OECD Nº 487/2014). Moreover, zebrafish model was used to assess the embryotoxicity potential of LQFM021 using fish embryo toxicity test (OECD Nº 236/2013) with extended exposure to evaluate subchronic larval development. *In vivo* subchronic toxicity of LQFM021 in rats (OECD Nº 407/2008) was also conducted. This compound at the lower concentrations tested (3.1 and 31 μg/mL) did not promote changes in micronuclei frequency in HepG2 cells. However, in the higher concentrations of LQFM021 (310 and 620 μg/mL) triggered a significant increase of micronucleated HepG2 cells, showing an alert signal of potential genotoxicity. Regarding the oral treatment of rats with LQFM021 (62.5, 125 or 250 mg/kg) for 28 days, the main findings showed that LQFM021 promoted renal and liver changes in a dose-dependent manner, being irreversible damage for kidneys while liver tissue showed a recovery after 14 days post treatment. Regarding embryotoxicity, although the lower concentrations used did not show toxicity, the concentration of LQFM021 (39.8 and 100 mg/L) promoted malformations in zebrafish embryo-larvae stage, in especial cardiac tissue changes. In conclusion, anti-inflammatory compound LQFM021 seems to have some limiting factors as a new therapeutic option to be used orally and in high repeated doses, related to those found in the non-steroidal anti-inflammatory drugs (NSAIDs).

1. Introduction

Pyrazole derivatives, which containing an aromatic five membered heterocyclic ring with two nitrogen atoms [19], have gained attention due to their wide applications in several research fields, in especial in drug discovery and development [18].

The broad range of biological activities of pyrazole analogs has allowed the development of several pharmacological agents, including molecules with antibacterial [6], anticancer [21,29], analgesic [1,27], anti-inflammatory [1,6,7] and cardiovascular [10,16] potential. In fact, several anti-inflammatory drugs carrying pyrazole scaffold are already commercially available, such as celecoxib, deracoxib and dipyrone [19]. However, some pyrazole derivatives have been withdrawal from clinical practice or used with

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caution due to the possibility of the occurrence of severe toxic events (e.g. agranulocytosis) [4,22].

5-(1-(3-fluorophenyl)-1H-pyrazol-4-yl)-2H-tetrazole (LQFM021) has demonstrated to be a versatile pyrazole compound. It was originally designed and synthesized through molecular hybridization of phosphodiesterase-3 (PDE-3) inhibitors, milrinone and cilostazol, resulting in a compound with fluorophenyl and tetrazole moieties appended to a pyrazole ring (Fig. 1) [23]. Preliminary studies have demonstrated its potential *in silico* PDE-3 inhibitory and *ex vivo* vasorelaxant activities [23]. Moreover, scientific evidences have highlighted LQFM021 as a promising anti-inflammatory, analgesic and antinociceptive agent.

Regarding the mechanisms involved in its pharmacological activities, it has been reported that *in vivo* antinociceptive effects of LQFM021 are promoted by its effects on peripheral opioid receptors associated with activation of the nitric oxide/cGMP/K_{ATP} pathway [11,12]; whereas *in vivo* analgesic [11] and anti-inflammatory effects [11,13] are due to LQFM021-induced nitric oxide increase [13].

Despite these important pharmacological findings, toxicity data regarding the LQFM021 are scarce. In this sense, this study investigated the *in vitro* genotoxicity of LQFM021 through cytokinesis-block micronucleus assay (MNvit test-cytoB) (OECD Nº 487/2014) in HepG2 cells. Moreover, zebrafish model was used to assess the embryotoxic potential of LQFM021 using fish embryo toxicity test (OECD Nº 236/2013) with extended exposure time for 7 days post-fertilization in order to examine the subchronic embryonic effects. We also investigated the *in vivo* subchronic toxicity of LQFM021 in rats by OECD Nº 407/2008.

2. Material and methods

2.1. Chemicals

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), neutral red (NR), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), sodium bicarbonate, penicillin, streptomycin, RNAse, propidium iodide, ethylenediaminetetraacetic acid (EDTA), trypsin and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hematoxylin and eosin stainings were acquired from Merck (Darmstadt, HE, Germany). Standard granulated chow for mice, xylazine and ketamine hydrochloride were purchased from Presence Nutrição Animal (São Paulo, SP, Brazil), Syntec (Cotia, SP, Brazil) and König (Santana de Parnaíba, SP, Brazil), respectively. EDTA anticoagulant and Instant Prov dyes were acquired from Newprov (Pinhais, PR, Brazil) and Doles (Goiânia, GO, Brazil), respectively. Ethanol, methanol, acetic acid and xylene were acquired from Vetec (Duque de Caxias, RJ, Brazil).

2.2. Obtaining of LQFM021

This compound was synthesized in the *Laboratório de Química Farmacêutica Medicinal* (LQFM), Faculty of Pharmacy, Federal University of Goiás, according to the synthetic route previously described [23]. Briefly, 1-(3-fluorophenyl)-1H-pyrazole-4-carbaldehyde (1 mmol) was added to a mixture of $NH_2OH \cdot HCl$ (1.3 mmol) and NaI (4 mmol) in N,N-dimethylformamide (DMF) (4 mL), at room temperature, followed by heating at reflux temperature for 6 h. After that, precipitate was vacuum filtered, dried and the crude product was purified by chromatography using $CHCl_3$ (mobile phase) to provide 1-(3-fluorophenyl)-1H-pyrazole-4-carbonitrile in 99% of yield. This compound (2.0 g, 12.4 mmol), sodium azide (4.1 g, 62 mmol) and ammonium chloride (3.35 g, 62 mmol) in 35 mL of DMF was heated at reflux temperature for 72 h. The reaction mixture was then poured into water and acidified to pH 5. The product was vacuum filtered and dried. LQFM021 (molecular weight: 230.07) was then obtained as a beige solid in 99% of yield and characterized by High Performance Liquid Chromatography (HPLC) and 1H, and 13C nuclear magnetic resonance (NMR) evaluations as showed by Ref. [23].

2.3. Cell culture

HepG2 liver cells were obtained from the Rio de Janeiro Cell Bank (Rio de Janeiro, RJ, Brazil). Cells were cultured in DMEM supplemented with 10% (v/v) heat-inactivated FBS, HEPES (4.5 mM), sodium bicarbonate (170 mM), penicillin (100 IU/mL) and streptomycin (100 mg/mL), in a humidified atmosphere of 5% CO_2 in air at 37 °C. Cell culture with confluence between 70 and 80% was removed from the 75 cm^2 culture flasks using trypsin/EDTA (0.025%:0.02%, w/w) solution diluted in PBS (1:1, v/v). To conduct the assays, cell viability was previously analyzed using TC20™

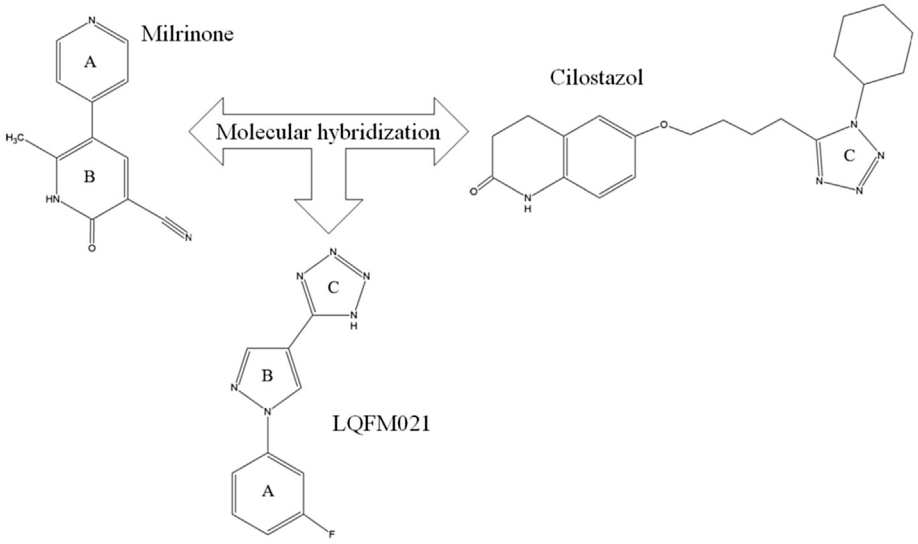

Fig. 1. Chemical structure of 5-(1-(3-fluorophenyl)-1H-pyrazol-4-yl)-2H-tetrazole (LQFM021). It was originally synthesized through molecular hybridization of phosphodiesterase-3 (PDE-3) inhibitors, milrinone and cilostazol, resulting in LQFM021, a compound with fluorophenyl (A) and tetrazole (C) moieties appended to a pyrazole ring (B).