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The novel piperazine-containing compound LQFM018: Necroptosis cell death mechanisms, dopamine D_4 receptor binding and toxicological assessment

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ABSTRACT

Piperazine is a promising scaffold for drug development due to its broad spectrum of biological activities. Based on this, the new piperazine-containing compound LQFM018 (**2**) [ethyl 4-((1-(4-chlorophenyl)-1H-pyrazol-4-yl) methyl)piperazine-1-carboxylate] was synthetized and some biological activities investigated. In this work, we described its ability to bind aminergic receptors, antiproliferative effects as well as the LQFM018 (**2**)-triggered cell death mechanisms, in K562 leukemic cells, by flow cytometric analyses. Furthermore, acute oral systemic toxicity and potential myelotoxicity assessments of LQFM018 (**2**) were carried out. LQFM018 (**2**) was originally obtained by molecular simplification from LASSBio579 (**1**), an analogue compound of clozapine, with 33% of global yield. Binding profile assay to aminergic receptors showed that LQFM018 (**2**) has affinity for the dopamine D_4 receptor (K_i = 0.26 μM). Moreover, it showed cytotoxicity in K562 cells, in a concentration and time-dependent manner; IC_{50} values obtained were 399, 242 and 119 μM for trypan blue assay and 427, 259 and 50 μM for MTT method at 24, 48 or 72 h, respectively. This compound (427 μM) also promoted increase in LDH release and cell cycle arrest in G2/M phase. Furthermore, it triggered necrotic morphologies in K562 cells associated with intense cell membrane rupture as confirmed by Annexin V/propidium iodide double-staining. LQFM018 (**2**) also triggered mitochondrial disturb through loss of ΔΨm associated with increase of ROS production. No significant accumulation of cytosolic cytochrome c was verified in treated cells. Furthermore, it was verified an increase of expression of TNF-R1 and mRNA levels of CYLD with no involvement in caspase-3 and -8 activation and NF-κB in K562 cells. LQFM018 (**2**) showed *in vitro* myelotoxicity potential, but it was orally well tolerated and classified as UN GHS category 5 (LD_{50} > 2000–5000 mg/Kg). Thus, LQFM018 (**2**) seems to have a non-selective action considering hematopoietic cells. In conclusion, it is suggested LQFM018 (**2**) promotes cell death in K562 cells *via* necroptotic signaling, probably with involvement of dopamine D_4 receptor. These findings open new perspectives in cancer therapy by use of necroptosis inducing agents as a strategy of reverse cancer cell chemoresistance.

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1. Introduction

Piperazine is a promising scaffold for drug development [1] due to its broad spectrum of biological activities [2]. It is present in a large variety of commercially available anticancer drugs such as imatinib mesylate, a tyrosine kinase inhibitor (TKI) that acts by inhibiting specific tyrosine kinases, such as BCR-ABL fusion oncoprotein in chronic myeloid leukemia [3–5].

Chronic myeloid leukemia is a myeloproliferative disorder characterized by the neoplastic transformation of hematopoietic stem cells in the bone marrow and their accumulation in the bloodstream. Its molecular hallmark is the Philadelphia chromosome (Ph), an aberrant fusion gene originated by translocation between chromosomes 9 and 22 which results in a chimeric gene product BCR-ABL [6–8]. The mutations of Ph and overexpression of BCR-ABL oncoprotein can promote resistance to apoptosis induced by conventional chemotherapy, and make chronic myeloid leukemia stem cells capable of escaping from imatinib and other TKI agents [9–12]. Therefore, compounds which also promote other or additional cellular death mechanisms, such as regulated necrosis, could be a new therapeutic option.

In accordance with the Nomenclature Committee on Cell Death (NCCD), regulated cell death occurs as part of physiological programs or can be activated once adaptive responses to perturbations of the extracellular or intracellular microenvironment fail [13,14]. Regulated necrosis, in turn, plays a major role in both physiological scenarios (*e.g.* embryonic development) and pathological settings (*e.g.* ischemic disorders); various types have been characterized, including necroptosis, mitochondrial permeability transition (MPT)-dependent regulated necrosis and parthanatos ([15–18]).

Currently, studies show that necrosis is a regulated process involving a set of transduction pathways and degradative mechanisms [19–21]. In view of that, the term “necroptosis” can be defined as a receptor interacting protein kinase 3 (RIPK3)-dependent molecular cascade promoting regulated necrosis [13]. Necrosis can be characterized by cell volume gain, organelle swelling, plasma membrane rupture and loss of intracellular content, which can lead to inflammation [22,23]. The cellular signaling that triggers necrosis is complex and requires different molecules working in concert. This process can be initiated by death receptors such as the tumor necrosis factor (TNF) receptor family, including TNF, Fas and TNF-related apoptosis-inducing ligand (TRAIL) [24].

Considering this background, this study describes the synthesis of the new piperazine-containing compound LQFM018 (**2**) [ethyl 4-((1-(4-chlorophenyl)-1H-pyrazol-4-yl)methyl)piperazine-1-carboxylate], a closely related analogue of LASSBio579 (**1**), a compound obtained by molecular simplification of atypical antipsychotic clozapine [25]. Moreover, the LQFM018 (**2**)-triggered cell death mechanisms in K562 cells as well as acute oral systemic toxicity and potential myelotoxicity assessments of LQFM018 (**2**) were carried out.

2. Materials and methods

2.1. Chemicals

An *In Vitro* Lactate Dehydrogenase Activity Assay kit, a Caspase-3 Colorimetric Assay kit, Dulbecco’s modified Eagle’s medium (DMEM), Roswell Park Memorial Institute (RPMI)-1640 medium, gentamycin, amphotericin B, propidium iodide (PI), RNAse, acetate 2,7-dichlorofluorescein (DCFH-DA), EDTA, bovine serum albumin (BSA), phosphatidylcholine, sodium taurodeoxycholate hydrate, rhodamine 123, granulocyte-macrophage colony-stimulating factor (GM-CSF), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) and ethidium bromide were purchased from Sigma-Aldrich (St. Louis, MO, USA). A CaspaTagTM Caspases-8 *In Situ* Assay kit was obtained from Millipore^{TM} (Temecula, CA, USA). Fetal bovine serum (FBS) and acetic acid were acquired from Gibco (Grand Island, NY, USA) and Cromoline (Diadema, SP, Brazil), respectively. Trypan blue dye, dimethyl sulfoxide (DMSO), ethanol, methanol and Triton X-100 were obtained from Vetec (Rio de Janeiro, RJ, Brazil), while May-Grünwald-Giemsa dye was purchased from Merck (Darmstadt, HE, Germany). Hoechst 33342 dye was acquired from Invitrogen (GrandIsland, NY, USA). Xylasine and ketamine hydrochloride were obtained from Syntec (Cotia, SP, Brazil) and König (Embu-Guaçu, SP. Brazil), respectively. Agar and an FITC Annexin V Apoptosis Detection kit were purchased from BD Bioscience (Franklin Lakes, NJ, USA). The mouse monoclonal anti-human cytochrome c (6H2, sc-13561 PE) and anti-human tumor necrosis factor receptor 1 (TNF-R1) (6A658, sc-73195 FITC) antibodies were acquired from Santa Cruz Biotechnology (Dallas, TX, USA). RNeasy mini, QuantiTect Reverse Transcription, Rotor-Gene SYBR Green PCR and QuantiTect Reverse Transcription kits were purchased from Qiagen (Hilden, Germany). The sunflower oil was obtained from Bunge Alimentos (Gaspar, SC, Brazil).

2.2. Cell cultures

Balb/c 3T3-A31 fibroblasts were kindly donated by Dr. Mari Cleide Sogayar (Chemistry Institute, University of São Paulo, SP, Brazil); while K562 chronic myelogenous leukemia cells were obtained from the Rio de Janeiro Cell Bank (Rio de Janeiro, RJ, Brazil). The 3T3 and K562 cells were cultured in DMEM or RPMI-1640 medium, respectively, supplemented with 10% (v/v) heat-inactivated FBS, 2 mM L-glutamine, 4.5 mM HEPES, 0.17 M sodium bicarbonate, 100 IU/mL penicillin and 100 µg/mL streptomycin in a humidified atmosphere of 5% CO_2 in air at 37 °C.

2.3. Animals

Female Swiss mice, weighing between 30 and 35 g, obtained from the Bioterium at the Federal University of Goiás (Goiânia, GO, Brazil) were used in this study. All efforts were realized to ensure the welfare of mice. Parameters as loss of body weight, food/water consumption and changes in activity and behavior of the animals were daily checked as clinical conditions of animal suffering to determine when the animals must be humanely sacrificed [26]. In addition, mice were acclimatized for a week before beginning the experiments.

The animals were kept under constant environmental conditions with a light-dark (12:12 h) cycle, controlled temperature (23 ± 2 °C), water and food provided *ad libitum*. All procedures and protocols were reviewed and approved by the Research Ethics Committee of the Federal University of Goiás (UFG no. 137/2009). At the end of each experiment, the mice were previously anesthetized with xylazine (10 mg/kg) and ketamine hydrochloride (100 mg/kg) administered intraperitoneally and then euthanized by cervical dislocation [26].

2.4. General

NMR experiments were acquired at room temperature on a BrukerAvance III 500 (11.75 T) spectrometer, using a 5 mm inverse-detection probehead with z-gradient. To acquire ^{1}H and ^{13}C experiments, samples containing 20 mg of LQFM018 (**2**) in $CDCl_3$ and tetramethylsilane (TMS) as internal standard were used. 1D and 2D pulse sequences from the Bruker user library were used for all experiments. Infrared (IR) spectra were obtained on a Nicolet-55a Magna spectrometer using KBr plates. Mass spectra (MS) were obtained with a microTOF III (Brucker Daltonics Bremen, Germany). The sample preparation for MS analysis consisted of diluting 1 µg of sample in 1 mL of methanol. To perform the analysis in positive mode, 1 µl of formic acid was added to the sample. The solution obtained was directly infused at a flow rate of 3 µL/min into the ESI source. ESI(+) source conditions were as follows: nebulizer nitrogen gas temperature and pressure of 2.0 bar and 200 °C, capillary voltage of −4,5 kV, transfer capillary temperature of 200 °C; drying gas of 4 L min−1; end plate offset of