Chemico-Biological Interactions 293 (2018) 20–27

Contents lists available at ScienceDirect

Chemico-Biological Interactions

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

Toxico-pharmacological evaluations of the small-molecule LQFM166: Inducer of apoptosis and MDM2 antagonist

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ARTICLE INFO

Keywords:
Leukemia
MDM2 inhibitor
p53-independent
Apoptosis
LQFM166

ABSTRACT

Inhibition of p53-MDM2 complex has been emerging as a strategy for antitumoral drug development considering the pro-apoptotic role of functional p53 in tumor cells. In our study, the prototype LQFM166 (2), designed through molecular simplification strategy inspired in the Nutlins compounds, was synthetized, characterized and the mechanisms of cell death were investigated. In addition, we estimated the starting doses for acute oral systemic toxicity tests according to the OECD Guidance Document No.129 - 3T3 NRU. The cytotoxic profile of LQFM166 (2) was determined in K-562 cells, a p53-null cell line, since previous studies also showed activity of LQFM166 (2) on this cells. After 24, 48 or 72 h of compound treatment, using MTT reduction assay, the IC50 values found were 100.1 μM, 56.76 μM and 45.11 μM, respectively. LQFM166 (2) was cytotoxic for leukemia cells in a concentration-time-dependent manner. Cell death mechanisms studies of LQFM166 on K-562 cells, revealed that the compound induced cell cycle arrest, increased the expression of caspase 3/7, 8 and 9, cytochrome c, Bax, p21 and p27. Additionally, a decrease in the expression of the Bcl-2 and cyclin-B1 was observed. The apoptotic inducer profile of the compound was confirmed by phosphatidylserine externalization. Investigation of complexation of p53/MDM2 was carried out by ELISA assay using 3T3 cell, showing a decrease in the p53-MDM2 complex induced by the compound. Furthermore, the cytotoxicity in basal fibroblasts 3T3 was determined to estimate LD50. LQFM166 (2) reduced 3T3 cells viability with the IC50 of 185.3 μM and estimated LD50 of 706.7 mg/kg (category 4 of GHS). The rationally designed of the prototype LQFM166 (2) induced cell death by apoptotic mechanisms in leukemic cells and showed MDM2 complexation antagonism in 3T3 cells.

1. Introduction

The discovery of new anticancer drugs have been based on the concept of targeting specific molecular pathways of tumor cells with consequent cell death and, in the best scenario, preserving normal cells/tissues [1]. In this perspective, the pro-apoptotic transcription factor p53 has being considered a valuable target to development of more specific antitumor compounds. p53 modulates the activation of several genes related to cell differentiation, growth and death, playing a crucial role in tumor progression or inhibition [2]. The natural negative regulator of p53 is the MDM2, an endogenous p53 inhibitor. MDM2 has an E3-ubiquitine-ligase domain that allows targeting p53 by proteasome degradation, decreasing transcriptional activity, intracellular levels and subsequently the effects of this protein [3,4]. The restoring of p53 functions through antagonism of MDM2 by antitumor compounds, emerged as a promising therapeutic target for the development of more effective agents.

Although p53 inhibition is the most known function of MDM2, this regulator has also p53-independent functions on tumor development and progression [5–7]. MDM2 also interacts with several other proteins, such as p21, E2F1 and MDMX influencing different cell signaling pathways [8]. Additionally, TP53 gene is mutated in about 50% of neoplastic disorders. Considering this, the development of new MDM2 inhibitors able to acts directly and/or independently of p53 statuses is a

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https://doi.org/10.1016/j.cbi.2018.07.004
Received 14 February 2018; Received in revised form 19 June 2018; Accepted 2 July 2018
Available online 03 July 2018
0009-2797/

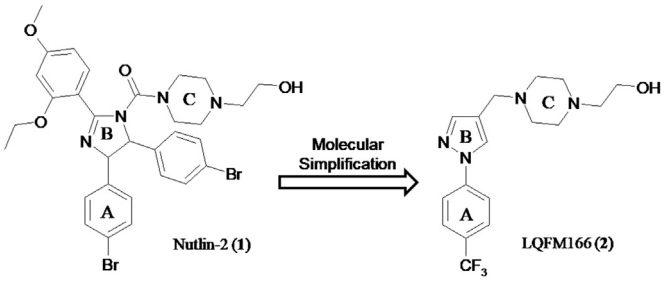

Fig. 1. Structural design of LQFM166 (2) from Nutlin-2 (1).

good strategy to anticancer drug development aiming to extend the spectrum of action of MDM2 inhibitors [9,10].

In 2006, Vassilev and coworkers have described the compounds Nutlins as MDM2 antagonists, able to disrupt p53-MDM2 interaction and induce apoptosis in cancer cells restoring the p53 functions [11]. Nutlins has a good antitumor profile in p53 wild-type cells but are not cytotoxic in mutant or null p53 phenotype cells [11,12]. In view of the potential antitumor property of Nutlins, analogues of this compound have been studied. Our research group synthetized and investigated the antitumor properties of LQFM030, a Nutlin analog created using a molecular simplification strategy. In that study, we demonstrated that LQFM030 had antiproliferative and cytotoxic activities on tumor cells, induced the accumulation of p53 protein, promoted cell-cycle arrest and apoptosis, activated caspases and decreased MDM2 protein [13]. Recently, we demonstrated that LQFM030 reduced tumor cell proliferation and VEGF levels along with enhanced survival *in vivo* [14].

Considering the promising effects of LQFM030, in the present study we designed, synthetized, characterized, and investigated the mechanisms of cytotoxicity of the compound LQFM166 (2), a Nutlin derivative, illustrated in Fig. 1. The scaffold A and B present in Nutlin-s (1) were replaced by 1-(4- (trifluoromethyl)phenyl)-1H- pyrazole scaffold and the (2-hydroxyethyl) piperazine (C) scaffold was maintained.

As the first step, this compound showed marked cytotoxic effects against p53-null leukemia K-562 cell line, motivating us to study the mechanisms of cell death induced by LQFM166 (2). Therefore, we hypothesized that this compound could be a MDM2 inhibitor independently of the cell p53 statuses. Moreover, we investigated the cytotoxicity of the LQFM166 (2) using 3T3 fibroblast cell line to estimate starting doses for acute oral systemic toxicity tests according to the OECD Guidance document no 129- 3T3 NRU [15].

2. Material and methods

2.1. Chemicals

Roswell Park Memorial Institute (RPMI)-1640 medium, Dulbecco's modified Eagle's medium (DMEM), heat-inactivated fetal bovine serum (FBS), streptomycin/penicillin solution, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), propidium iodide (PI), RNase, bovine serum albumin (BSA), RIPA buffer, bicinchoninic acid protein assay kit and protease inhibitor cocktail were purchased from Sigma Aldrich (St. Louis, MO, USA). Annexin V Apoptosis Detection kit FITC, ELISA p53/MDM2 Immunoset kit and CaspaTag™ Caspase −3, −7, −8 and −9 In Situ Assay kit were acquired from eBioscience (San Diego, CA, USA), Enzo Life Sciences (Farmingdale, NY, USA) and Millipore™ (Temecula, CA, USA), respectively. The antibodies against Bax, cytochrome-c, p21, p27 and cyclin-B1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibody against Bcl-2 and BD Cytofix/Cytoperm™ solution were acquired from BD Biosciences (San Jose, CA, USA). Dimeltilsulfoxide (DMSO) was acquired from Merck (Darmstad, Germany), while Tween-20 was obtained from Vetec (Rio de Janeiro, RJ, Brazil).

2.2. Synthesis of LQFM166 (2)

Synthesis of 2-(4-((1-(4-(trifluoromethyl)phenyl)-1H-pyrazol-4-yl) methyl) piperazin-1-yl)ethanol (2) [16]. To a stirred heterogeneous mixture of 1-(4-(trifluoromethyl)phenyl)-1H-pyrazole-4-carbaldehyde (**5**) (251 mg, 1.0 mmol), 1-(2-hydroxyethyl)piperazine (**6**) (130 mg, 1.0 mmol), $ZnCl_2$ (68 mg, 0.5 equiv), in 5 mL of MeOH was added $NaBH_3CN$ (31 mg, 0.5 equiv) in one portion. The mixture was stirred at 70 °C for 2 h. In turn, MeOH was then evaporated and the residue was partitioned between water and CH_2Cl_2. 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 (SiO2, hexane/AcOEt = 6:4) to 2-(4-((1-(4-(trifluoromethyl)phenyl)-1H-pyrazol-4-yl)methyl)piperazin-1-yl)ethanol (**2**) (240 mg, 68%) as a beige solid, m.p. = 108-110 °C, Rf = 0.37 (CH_2Cl_2/MeOH = 95:5). IR_{max} (KBr) cm^{-1}: 3500–300 (υ O-H), 2858 (υ C-H), 1618 (υ C=C), 1330 (υ CF_3), 830 (υ Ar-1,4); ^1H NMR (500.13 MHz) $CDCl_3$/TMS (δ): 8.01 (1H, *s*, H-5′); 7.88 (2H, *m*, H-6); 7.88 (2H, *m*, H-2); 7.78 (2H, *m*, H-5); 7.78 (2H, *m*, H-3); 7.77 (1H, *s*, H-3′); 3.63 (2H, *t*, *j* = 5.21, H-8″); 3.60 (2H, *s*, H-6′); 2.58 (2H, *t*, *j* = 5.21, H-7″); 2.58 (8H, *m*, H-6″); 2.58 (8H, *m*, H-5″); 2.58 (8H, *m*, H-3″); 2.58 (8H, *m*, H-2″). 2D NMR (HSQC/HMBC - 125,76 MHz) $CDCl_3$/TMS (δ): 142.5 (C-3′); 142.4 (C-1); 128.2 (C-4); 126.5 (C-5); 126.5 (C-3); 126.2 (C-5′); 120.3 (C-4′); 120.2 (C-7); 118.4 (C-6); 118.4 (C-2); 59.0 (C-7″); 57.3 (C-8″); 52.5 (C-6″); 52.5 (C-5″); 52.5 (C-3″); 52.5 (C-2″); 52.1 (C-6′). MS: $[M+H]^+ m/z$ of 353.1580.

Reactions were monitored by TLC using commercially available precoated plates (Whatman 60 F254 silica) and the developed plates were examined under UV light (254 and 365 nm). ^1H 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 of TMS and the coupling constants are in Hertz. All assignments of the signals of ^1H 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. Melting points were assessed using a Marte melting point apparatus, and the results were uncorrected. The organic solutions were dried over anhydrous sodium sulphate and organic solvents were removed under reduced pressure in a rotary evaporator. 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 the 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 with nitrogen gas; pressure of 0.4 bar and temperature of 200 °C; capillary voltage of −4 kV, transfer capillary temperature of 200 °C; drying gas of 4 L.min−1; end plate offset of −500 V; skimmer of 35 V and collision voltage of −1.5 V. Each spectrum was acquired using 2 microscans. The resolving power is: m/Δm50% 16,500.00, where Δm50% is the peak full width at half-maximum peak height. Mass spectra were acquired and processed with Data Analysis software (Brucker Daltonics, Bremen, Germany).

2.3. Cells culture

K-562 leukemia cell line was cultured in suspensions using RPMI-1640 medium and Balb/c 3T3-A31 fibroblast cell line was cultured in DMEM, both supplemented with 10% (v/v) of FBS and 100 IU/mL penicillin and 100 mg/mL streptomycin. The cell cultures were maintained in an incubator under 5% CO_2 atmosphere, at 37 °C and controlled humidity. Fibroblasts were disaggregated from the culture flasks using trypsin (0.025%)/EDTA (0.02%) solution. The cell lines were obtained from Cell Bank of Rio de Janeiro (Rio de Janeiro, Brazil). Cell