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Combing metabolomics with bioanalysis methods to study the antitumor mechanism of the new acridone derivative 8q on CCRF-CEM cells: 8q induced mitochondrial-mediated apoptosis and targeted the PI3K/AKT/FOXO1 pathway

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ABSTRACT

A novel acridone derivative, N-(2-(dimethylamino)ethyl)-1-((3-methoxybenzyl)amino)-5- nitro-9-oxo-9,10-dihydro-acridine-4-carboxamide (**8q**), which was synthesized in our lab, showed potent anti-leukaemia activity against CCRF-CEM cells. Moreover, *in silico* predictions showed that **8q** conformed to the rule of five and displayed low toxicity. However, the mechanism of anti-leukaemia action remains unclear. The aim of this research was to reveal the probable anti-leukaemia mechanism of **8q** on CCRF-CEM cells. Flow cytometry assay demonstrated that **8q** induced apoptosis. The expression of caspase family proteins results showed that **8q** could only promote cleaved caspase-3, 7 and 9 expressions without affecting cleaved caspase-8 protein, hinting that **8q** induced mitochondrial-mediated apoptosis. Further, we detected 3 indicators of mitochondrial lesions, including increased of Cyt-C release, with a decrease in MMP and ATP levels. Next, metabolomics were introduced to assist in the research of the anti-leukaemia mechanism of **8q**. The metabolomics results showed that 100 nM **8q** could increase the level of GSH, and decrease its oxidation products. These indicated **8q** could influence the ROS, which derived by mitochondria. Then we examined the effect of 8q on intracellular ROS levels. What is particularly interesting is that **8q** inhibited cell ROS stress at low concentration and stimulated ROS stress at high concentration. The pro-apoptosis mechanisms of **8q** were then explored. **8q** significantly decreased anti-apoptotic proteins Bcl-2 and Bcl-xL expression, whereas it up-regulated the pro-apoptotic proteins Bax, Bak, Bad, Bik and Puma expression. In addition, **8q** dramatically inhibited the expression of FASN, which is related to fatty acid metabolism. Furthermore, PI3K, AKT and FOXO1 were inactivated, and the expression of total AKT was also inhibited by **8q** treatment, which promoted intrinsic apoptosis. In conclusion, these findings demonstrate that **8q** can induce mitochondrial lesions and promote mitochondrial-mediated pathway apoptosis by regulating the expression of Bcl-2 family proteins and inhibiting the activity of the PI3K/AKT/FOXO1 signaling pathway.

1. Introduction

Acridine/acridone derivatives with a π-conjugated tricyclic planar structure have been widely used in numerous applications. They were originally used as pigments and industrial dyestuffs due to the conjugated system in their structures. Later, in the past few decades, the applications of acridine/acridone compounds in the field of chemotherapy were more extensive and important.

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For instance, they have been used as antibacterial, anti-virus, anti-psoriatic, anti-malaria and especially anticancer drugs [1–3]. So far, many acridine/acridone drugs have entered clinical/preclinical studies for anticancer therapies, such as Amsacrine (*m*-AMSA), *N*-[2-(dimethylamino)-ethyl]-acridine-4- carboxamide (DACA), and Pyrazoloacridine (PZA) [4]. Clinically, *m*-AMSA is often used in combination with other anti-tumour drugs (cytarabine, mitoxantrone, fludarabin) to treat different types of leukaemia [5]. Despite the increasing number of antitumor application, leukaemia is still an important type of anti-tumour application of acridine/acridone drugs [6].

Recently, with the development of chemotherapy drugs, the treatment of leukaemia has greatly improved, but the side effects of the drugs are still large, especially for children [7]. Therefore, it is still necessary to develop novel anti-leukaemia drugs. Acridine/acridone derivatives are one type of potential precursor compound for leukaemia. Many acridine/acridone derivatives in the research stage have a good inhibitory effect on leukaemia cells [8,9]. For example, the acridine complexes developed by Asanda c. Matsheku were selective towards HL60 cells over normal cells [9]. In addition, the acridine derivative 8 m, which was designed and synthesized by Z Cui, showed good anti-proliferative activity against K562 cells by inhibiting Src and MEK kinases [10].

Acridine/acridone derivatives with potential antitumour mechanisms mainly target DNA and DNA-related enzymes, such as Top I/II [11]. Recently, however, researchers have found some new anti-tumour mechanisms of acridine/acridone compounds. It has been found that acridone compounds can induce apoptosis by inhibiting PARP or AKT- and ERK-induced stabilization of MCL1 [12,13]. Wang et al discovered that the acridone derivative 8a could induce oxidative stress-mediated apoptosis in CCRF-CEM leukaemia cells [14]. In previous work, our group synthesized a series of acridine/acridone derivatives with a nanomolar or micromolar level of anticancer activity against leukaemia cells [15–18]. Among these compounds, it is interesting that compound **8q** (N-(2-(dimethylamino)ethyl)-1-((3-methoxybenzyl)amino)-5-nitro-9-oxo-9,10- dihydro-acridine-4- carboxamide) with a benzyl acridone scaffold showed potent antitumour activity against CCRF-CEM cells at nanomolar concentration levels. However, the anticancer mechanism of **8q** was not yet clear. As a promising anticancer small-molecule compound, it is necessary to further study its anti-leukaemia mechanism. Fully understanding the drugs' mechanism is beneficial for structural optimization and screening of lead compounds. In this study, we dissected the anti-leukaemia mechanism of **8q** in CCRF-CEM leukaemia cells, which is helpful for its further improvement and application.

2. Materials and methods

2.1. Reagents and materials

Compound **8q** was synthesized by our group and solubilized in DMSO at 2.5 mM stock. Acetonitrile and methanol were purchased from Fisher (HPLC grade, Fairlawn, USA). Formic acid was purchased from Tedia (HPLC grade, Tedia Co., USA). Distilled water was filtered through a Milli-Q system (Millipore, USA). RPMI-1640 was purchased from Corning (New York, USA). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, USA). Penicillin-Streptomycin was obtained from North China Pharmaceutical, China. CCRF-CEM leukaemia cells (Human T cell lymphoblast-like cell line), K562, QSG-7701, MCF-10 A, MDA-MB-231, HepG2 and MCF-7 cells were purchased from the Chinese Academy of Sciences Cell Bank (Shanghai, China). Specific cells information are shown in Table S1. MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide) was bought from Sigma (Sigma-Aldrich Co., USA). ATP Assay Kit, BCA Protein Assay Kit, rhodamine 123 (Rh123), ROS Assay Kit and Annexin V-FITC/PI apoptosis detection kit were purchased from Beyotime Institute of Biotechnology (Shanghai, China).

2.2. In silico druggability and toxicity prediction

Druggability and toxicity parameters for compound **8q** were predicted *in silico* using Advanced Chemistry Development, Inc. (ACD/Labs, ACD/Percepta Platform, version 14.0.0), and Osiris Property Explorer (http://www.organic-chemistry.org/prog/peo).

2.3. Cell culture and viability assay

All cells were cultured in 2D monolayers without collagen. CCRF-CEM, K562, QSG-7701 and MCF-10 A cells were maintained in RPMI-1640 medium, MDA-MB-231 cells were maintained in L-15 medium, and HepG2 and MCF-7 cells were maintained in DMEM medium. All media were supplemented with 10% FBS, penicillin-streptomycin solution (100 U/mL penicillin and 100 μg/mL streptomycin). CCRF-CEM, K562, QSG-7701, MCF-10 A, HepG2 and MCF-7 cells were incubated at 37 °C in an atmosphere of 5% CO_2, while MDA-MB-231 cells were incubated in an atmosphere of 0% CO_2. When the cell plated overnight (about 12–16 hours), corresponding drug treatment started.

Cell viability was determined with an MTT assay. The cells (6×10^3 - 3×10^5 cells/well) were treated with vehicle or with a different concentration of **8q** for 24 and 48 h. Then, 20 μl of MTT solution (5 mg/mL) was added to each well in 190 μl of medium, and the plates were incubated for 4 h at 37 °C A total of 100 μl of DMSO was added to each well after removal of the media. The absorbance was measured with a Benchmark microplate reader (Molecular Devices Corporation, USA) at a wavelength of 490 nm. Percent viability was calculated as the ratio between the **8q**-treated cells and the untreated control cells, and the half maximal inhibitory concentration (IC_{50}) was measured with Origin 7.5.

2.4. Metabolomics analysis conditions

The extraction of samples and the detection of metabolites were completed as previously described [14]. In brief, CCRF-CEM cells were exposed to 100 nM **8q** and were harvested using 4:1 (v/v) cold methanol/water after 24 h. Then, a UPLC/Q-TOF MS method was employed to separate and analyze the samples. Subsequently, data were processed using the MarkerLynx software, and metabolite markers were identified using the HMDB database (http://www.hmdb.ca/), Lipid Maps database (http://www.lipidmaps.org) and METLIN (http://metlin.scripps.edu/) database with tandem mass spectrometry. Some standards of metabolic interest were also used to confirm their structures. Finally, the changes in metabolic pathways were analyzed by the Biochemical Pathways (http://biochemical-pathways.com/) and MetaboAnalyst 3.0 (http://www.metaboanalyst.ca/) tools.

2.5. Flow cytometric assays

Cell apoptosis was measured with Flow cytometric assays using the Annexin V-FITC/PI apoptosis detection kit after 0–800 nM **8q** treatment for 24 h at 37 °C. The experimental procedure was followed according to the instructions of the manufacturer.

2.6. Western blot analysis

Proteins were separated by electrophoresis on a 12% SDS polyacrylamide gel and transferred to polyvinylidene difluoride membranes (Millipore, USA). The membranes were blocked with