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Furazolidone induces apoptosis through activating reactive oxygen species-dependent mitochondrial signaling pathway and suppressing PI3K/Akt signaling pathway in HepG2 cells

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ABSTRACT

Furazolidone (FZD), a synthetic nitrofuran with a broad spectrum of antimicrobial activities, has been shown to be genotoxic and potentially carcinogenic in several types of cells. However, the proper molecular mechanisms of FZD toxicity remain unclear. This study was aimed to explore the effect of FZD on apoptosis in HepG2 cells and uncover signaling pathway underlying the cytotoxicity of FZD. The results showed that FZD induced apoptosis in HepG2 cells in a dose-dependent manner characterized by nuclei morphology changes, cell membrane phosphatidylserine translocation, poly (ADP-ribose) polymerase (PARP) cleavage and a cascade activation of caspase-9 and -3. FZD could enhance reactive oxygen species (ROS) generation, up-regulate Bax/Bcl-2 ratio, disrupt mitochondrial membrane potential (MMP) and subsequently cause cytochrome c release. Both ROS scavenger (N-acetyl cysteine, NAC) and caspase inhibitors suppressed FZD-induced apoptosis. Furthermore, NAC attenuated FZD-induced ROS generation and mitochondrial dysfunction. Meanwhile, FZD treatment inhibited both the activation and expression of Akt, and PI3K/Akt inhibitor LY294002 promoted FZD-induced apoptosis. On the contrary, PI3K/Akt activator insulin-like growth factor-1 (IGF-1) attenuated lethality of FZD in HepG2 cells. In conclusion, it is first demonstrated that FZD-induced apoptosis in HepG2 cells might be mediated through ROS-dependent mitochondrial signaling pathway and involves PI3K/Akt signaling.

1. Introduction

Furazolidone (FZD) is a nitrofuran derivate with a broad spectrum of antimicrobial actions and has been applied to treat certain bacterial and protozoal infections in human and animals for more than 40 years (Ali, 1999). Due to its potential genotoxicity and carcinogenicity, FZD has been banned as feed additive in food animals in many countries since 1990s (Khong et al., 2004). However, FZD is still used as a feed additive for livestock and aquatic products in some Middle and Far East countries (Chumanee et al., 2009; McCracken et al., 2005). It is also applied to gastrointestinal infectious diseases in human, especially for *Helicobacter pylori* disease (Egan et al., 2008).

The genotoxic effects of FZD are mainly caused by its oxido-reductive metabolism and formation of incomplete reduction products (Raipulis et al., 2005). The reductive metabolites of FZD could bind to proteins as well as glutathione and react with DNA, consequently resulting in cytotoxicity (De Angelis et al., 1999). As an inhibitor of some monoamine oxidases, FZD may cause cardiotoxicity by antagonizing catecholamine removal (Deisher et al., 1994). Hence, FZD is commonly employed to establish animal models of dilated cardiomyopathy (DCM) (Hajjar et al., 1993). However, FZD induces cardiomyocyte apoptosis and mitochondrial impairments in DCM animals (Zhang et al., 2013b). Recent findings (Jiang et al., 2013) show that FZD displays anti-leukemic activity through inducing apoptosis and myeloid cell differentiation in acute myeloid

Abbreviations: AML, acute myeloid leukemia; BSA, bovine serum albumin; DCFH-DA, 2′,7′-dichlorofluorescensin diacetate; DCM, dilated cardiomyopathy; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; FZD, furazolidone; HepG2, hepatoblastoma cell line; IGF-1, insulin-like growth factor-1; MEM, minimum essential medium; MMP, mitochondrial membrane potential; MTT, 3-(4,5-dimetylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; NAC, N-acetyl cysteine; NC, nitrocellulose; PARP, poly (ADP-ribose) polymerase; PBS, phosphate buffered solution; PMSF, phenylmethyl sulfonylfluoride; QRT-PCR, quantitative real-time-PCR; ROS, reactive oxygen species; SDS, sodium dodecylsulfonate.

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leukemia (AML) cells, indicating that FZD is a novel therapeutic strategy in AML patients. Therefore, further study concerning the pharmacological and toxicological properties of FZD is still necessary.

Liver cancer is one of the most prevalent cancers in human and represents the third leading cause of cancer death worldwide (Jemal et al., 2011). Nowadays, the induction of apoptosis has been assessed as a promising approach for destroying cancer cells (Fabregat et al., 2007). Apoptosis is programmed cell death which is mainly mediated through two major pathways, the death receptor-mediated extrinsic pathway and the mitochondrial-mediated intrinsic pathway. In our previous study, FZD could obviously inhibit cell growth in human hepatoblastoma cell line (HepG2) through inducing S phase arrest and DNA damage (Jin et al., 2011). However, whether FZD induces apoptosis in HepG2 cells and its putative mechanisms remain unclear.

In this study, the mechanism of FZD-induced apoptosis in HepG2 cells was investigated. FZD caused the generation of excessive reactive oxygen species (ROS) and the suppression of Akt activation, which resulted in mitochondrial dysfunction and activated caspase cascades. Our findings suggested that FZD induced apoptosis through a ROS- and caspase-dependent mitochondrial and PI3K/Akt signaling pathway in HepG2 cells.

2. Materials and methods

2.1. Materials

Minimum essential medium (MEM) and fetal bovine serum (FBS) were purchased from Life Technologies Corporation (Grand Island, NY, USA). Sodium dodecylsulfonate (SDS), 3-(4,5-dimetylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were all purchased from AMRESCO Inc. (Solon, OH, USA). Furazolidone (FZD), sodium pyruvate and phenylmethyl sulfonylfluoride (PMSF) were all purchased from Sigma–Aldrich (St. Louis, MO, USA). Bovine serum albumin (BSA), N-acetylcysteine (NAC), LY294002, 2′,7′-Dichlorofluorescensin diacetate (DCFH-DA), cell permeable pan caspase inhibitor Z-VAD-FMK, caspase-3 inhibitor Ac-DEVD-CHO, Rhodamine 123, poly (ADP-ribose) polymerase (PARP), mouse monoclonal antibodies against β-actin and GAPDH were all purchased from Beyotime Institute of Biotechnology Co., Ltd. (Haimen, Jiangsu, China). Caspase-9 inhibitor Ac-LEHD-FMK was obtained from Beijing B&M Biotech Co, Ltd (Beijing, China). Rabbit polyclonal antibodies against Akt, caspase-9, Bax and coxIV were purchased from ProteinTech Group, Inc. (Chicago, IL, USA). Rabbit polyclonal antibodies against p-Akt, Bcl-2, caspase-3, mouse monoclonal antibody against cytochrome c and horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Annexin V-FITC apoptosis detection kit was purchased from Nanjing KeyGEN Biotech Co., Ltd (Nanjing, Jiangsu, China). Insulin-like growth factor-1 (IGF-1) was purchased from Novoprotein Scientific Inc. (Shanghai, China). All other reagents were of analytical grade.

2.2. Cell culture

HepG2 cell line was purchased from Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China) and cultured in MEM supplemented with 10% (v/v) FBS, 110 mg/L sodium pyruvate, 100 units/mL penicillin and 100 μg/mL streptomycin. Cells were maintained in a humidified incubator with 5% CO_2 at 37 °C.

2.3. Cell treatment and cytotoxicity assay

FZD was dissolved in DMSO to make a stock solution of 50 mg/mL and further diluted to corresponding concentrations with the cell culture medium. The final DMSO concentration was less than 0.1% (v/v) for each treatment. The cell viability was determined by MTT assay as previously described (Zhang et al., 2013a). Briefly, HepG2 cells were cultured in the medium as mentioned above in 96-well plates at a density of 1×10^4 cells per well. After culture for 24 h, the cells were exposed to different concentrations of FZD (0, 12.5, 25 and 50 μg/mL). Then the medium containing FZD was removed, and cells were incubated in the 100 μL fresh medium supplemented with 10 μL MTT solution (5 mg/mL in phosphate buffered solution, PBS) for 4 h at 37 °C. After gentle removal of the medium, 100 μL DMSO was added into each well to dissolve the formazan crystals. Absorbance at 570 nm was measured with a microplate reader (Molecular Devices, Sunnyvale, CA, USA). The cell viability and cytotoxicity were estimated as the percentage of the control.

2.4. Hoechst 33342/PI staining assay

The HepG2 cells were plated in 6-well plates with a density of 5.0×10^5 cells per well and incubated for 24 h. Then cells were exposed to FZD (0, 12.5, 25 and 50 μg/mL) for 24 h. For the apoptotic morphological examination, cells were stained with fluorescent staining apoptosis detection kit (1 μg/mL Hoechst 33342 and 1 μg/mL PI) (Vigorous Biotechnology, Beijing, China) according to the product manual and observed by the fluorescence microscopy (Leica Microsystems, Wetzlar, Germany). Chromatin condensation indicated cell apoptosis.

2.5. Flow cytometric analysis of apoptosis

HepG2 cells were plated into 6-well plates and exposed to different concentrations of FZD (0, 12.5, 25 and 50 μg/mL) or FZD (50 μg/mL) with different inhibitors (pan caspase inhibitor, caspase-3 inhibitor, caspase-9 inhibitor, NAC and LY294002) or PI3K/Akt activator IGF-1 for 24 h, respectively. Cells were subsequently harvested with 0.25% trypsin without EDTA, washed twice with cold PBS and re-suspended in 500 μL binding buffer. Then cells were incubated with 5 μL annexin V-FITC (40 μg/mL) and 5 μL propidium iodide (40 μg/mL) in the dark for 10 min at room temperature and detected by BD FACSAria™ flow cytometry (Becton Dickinson, San Jose, CA, USA).

2.6. Measurement of caspase-3/7 and caspase-9 activities

The effects of FZD on caspase-3/7 and -9 activities were determined using the Caspase-Glo® 3/7 assay kit and Caspase-Glo® 9 assay kit (Promega Corp., Madison, USA), respectively. Briefly, HepG2 cells (1.5×10^4 cells per well) cultured in white-walled 96-well plates were exposed to FZD (0, 12.5, 25 and 50 μg/mL) or FZD (50 μg/mL) with NAC, LY294002 and IGF-1 respectively for 24 h. Then Caspase-Glo® 3/7 or 9 reaction solutions (200 μL per well) were added. After incubation for 2 h at room temperature in the dark, luminescence was measured by a fluorescent microplate reader (Molecular Devices, Sunnyvale, CA, USA). The luminous intensity detected as relative light units was proportional to caspase activity.

2.7. Measurement of mitochondrial membrane potential (MMP)

The changes of MMP have been considered to be an indicator of mitochondrial damage (Zhu et al., 2013). After FZD treatment for 24 h, cells were incubated with 10 μg/mL rhodamine 123 in the dark for 30 min at 37 °C prior to harvest, and then washed with PBS. The fluorescence intensity of the cells was determined by flow cytometry.

2.8. Measurement of intracellular ROS generation

The intracellular ROS was measured with cell-permeant probe DCFH-DA. Briefly, after FZD treatment for 24 h, cells were incubated with 10 μmol/L DCFH-DA in the dark for 20 min at 37 °C prior to harvest, and then washed with PBS. The fluorescence intensity of the cells was determined by flow cytometry.

2.9. Western blotting analysis

Western blotting was conducted as previously described with some modifications (Deng et al., 2013). After FZD treatment, the HepG2 cells were harvested using scraper and lysed in 100 mM Tris–HCl, pH 7.4, 2% (m/v) SDS, 10% (v/v) glycerol, 1 mM PMSF. Cell lysates were centrifuged at $14{,}000 \times g$ for 15 min at 4 °C and supernatants were subjected to Western blotting analysis. The protein concentrations were subsequently measured using the BCA protein assay kit (Beyotime Inst. Biotech, Jiangsu, China). The cytosolic and mitochondrial fractions were prepared using cell mitochondria isolation kit (Beyotime Inst. Biotech). Proteins (20 to 100 μg) were separated by 8–15% SDS–PAGE and transferred to nitrocellulose (NC) membrane (Applygen Technologies Inc. Beijing, China) using a Mini Trans-Blot Cell Systems (Bio-Rad, Hercules, CA, USA). The membranes were blocked with 5% (w/v) non-fat milk for 2 h at room temperature, and then incubated with primary antibodies at a dilution of 1:200 to 1:1000 in TBST (0.1% Tween-20) at 4 °C, overnight. After being washed three times with TBST, the membranes were incubated for 1 h with corresponding horseradish peroxidase-conjugated secondary antibodies diluted in TBST ($1{:}10^4$). The blots were detected using western luminescent detection kit (Vigorous Biotechnology, Beijing, China). The load protein was normalized to β-actin, GAPDH (for Akt and p-Akt only) or coxIV (for mitochondrial cytochrome c). The immunoblots were quantified by ImageJ software (National Institute of Mental Health, Bethesda, MD, USA).

2.10. Quantitative real-time (QRT)-PCR analysis of mRNA for Akt

HepG2 cells were treated with FZD (0, 12.5, 25 and 50 μg/mL) for 24 h. Total RNA was extracted using TRIzol ® reagent (Life Technologies, Grand Island, NY, USA) according to the manufacturer's instructions. QRT-PCR was performed using an AB7500 real-time PCR instrument (Applied Biosystems, Foster City, CA, USA). Reactions were