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Critical role of p21 on olaquindox-induced mitochondrial apoptosis and S-phase arrest involves activation of PI3K/AKT and inhibition of Nrf2/HO-1pathway

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ABSTRACT

Olaquindox, a quinoxaline 1,4-di-N-oxide, is known as an antibacterial agent and feed additive to treat bacterial infections and promote animal growth. However, the potential mechanism of toxicity is still unknown. The present study aims to explore the molecular mechanism of p21 on olaquindox-induced mitochondrial apoptosis and S-phase arrest in human hepatoma G2 cells (HepG2). As a result, olaquindox promoted production of ROS, suppressed the protein expression p21 in p53-independent way and phosphorylated p21. Meanwhile, olaquindox activated AKT and Nrf2/HO-1 pathway, up-regulated Bax/Bcl-2 ratio, disrupted mitochondrial membrane potential (MMP) and subsequently caused cytochrome *c* release and a cascade activation of caspase, eventually induced apoptosis. Olaquindox could induce S-phase arrest in HepG2 cells involved with the increase of Cyclin A, Cyclin E and CDK 2. Furthermore, knockdown of p21 decreased cell viability, enhanced oxidative stress, aggravated olaquindox-induced mitochondrial apoptosis and S-phase arrest involvement of activating PI3K/AKT and inhibiting Nrf2/HO-1 pathway. PI3K/AKT inhibitor (LY294002) and HO-1inhibitor (ZnPP-IX) both increased olaquindox-induced apoptosis and S-phase arrest. In conclusion, knockdown of p21 increased olaquindox-induced mitochondrial apoptosis and S-phase arrest through further activating PI3K/AKT and inhibiting Nrf2/HO-1pathway. Our study provided new insights into the molecular mechanism of olaquindox and shed light on the role of p21.

1. Introduction

Olaquindox, a quinoxaline 1,4-di-N-oxide derivative, is recognized as effective antibacterial drug and feed additive to treat bacterial infectious diseases and improve feed conversion efficiency. However, due to its severe side effects in mutagenicity (Zhang et al., 2015), genotoxicity (Ihsan et al., 2013), hepatoxicity (Zou et al., 2011) and nephrotoxicity (Yang et al., 2015), commission of the European Community has forbidden the application of olaquindox in 1999 (Song et al., 2011), and according to Chinese veterinary pharmacopoeia, olaquindox has been banned used in animal feeding except young swine whose weight is not more than 35 kg (Zhang et al., 2013). The abuse of olaquindox as animal feed additive has polluted the animal-derived food products and water sources. It has been reported that high concentration of olaquindox applied to livestock and poultry could result in residual problem in animal body, which eventually posed a great threat for human health (Pei et al., 2016). According to Agricultural Minister of China regulations, the maximum residue limit (MRL) of olaquindox in porcine muscle is set at 4 μg/kg and in porcine liver at 50 μg/kg. However, the potential toxic residues of olaquindox in edible animal-origin products should arouse much more concern.

In vivo experiments with olaquindox in mice showed that both in liver and kidney were observed toxic side effects (Liu et al., 2011). However, the toxic molecular mechanism of olaquindox has not yet been fully defined. Data discovered that even at a relatively low concentration of olaquindox (6.6 μg/mL) could cause a 12-fold upregulation in mutation frequency (Hao et al., 2006). Previous study proposed that oxidative damage inflicted by ROS was regarded as a plausible mechanism for quinoxaline 1,4-di-N-oxide

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derivative induced toxicity (Wang et al., 2016). Recent studies have shown that the toxic mechanism of olaquindox was the oxidative DNA damage caused by ROS (Zou et al., 2009). There were increasing evidences that olaquindox treatment lead to intracellular ROS generation, which eventually participated in regulating apoptosis in HepG2 cells (Li et al., 2016, 2017; Zhao et al., 2013).

Cell cycle regulatory protein p21, an inhibitor of cyclin-dependent kinase (CDK), is regulated by both p53-dependent and p53-independent pathways. P21 plays a crucial role in drug-induced tumor suppression, and researches show that according to the cell type and cellular environment, p21 could execute pro-apoptotic or anti-apoptotic function in response to anti-tumor agents (Liu et al., 2003). P21 regulated G1 phase cell cycle associated with cyclin E and cyclin A/CDK in p53 dependent way, which caused by issues such as taxol and oncogenic Ras, (Roninson, 2002; Yousefi et al., 2014). Treatment of the wild-type human colon cancer cell with a high concentration of camptothecin resulted in apoptosis, indicating that p21 mediated apoptosis occurred by a p53-independent mechanism (Han et al., 2002). Selenoprotein W plays a major role in the regulation of phosphorylated p21 in cell cycle regulation, which changes the phosphorylation status of Ser-33 and promotes the G1/S phase by p21 down-regulation (Hawkes and Alkan, 2011). There is an inconclusive debate about whether p21 plays a pro-apoptotic or anti-apoptotic role. The function of p21 plays its resistance to apoptosis with the evidence that over-expression of p21 in breast cancer cells reduced cell sensitivity in infrared (IR)-induced apoptosis (Soria and Gottifredi, 2010). Treated with manumycin alone or combined with paclitaxel in thyroid cancer cells was also observed a pro-apoptotic effect of p21 (Yang et al., 2003). Recent study reported that p21 was a two-faced genome guardian depending on cell type, cellular localization, p53 status, and the type and level of genotoxic stress. P21 maintains its genuine signature function as a cell cycle inhibitor and anti-proliferative effector and acquire either oncosuppressive or onco-promoting properties depending on whether it is in a p53-proficient or p53-deficient environment, respectively (Georgakilas et al., 2017).

AKT plays an important role in many cellular processes, such as cell growth, cell cycle and apoptosis, which control several downstream signaling pathway including p53, p21and cyclinD1(Franke et al., 2003). In AKT pathway, p21 is directly phosphorylated on Thr-145 by AKT and the interaction between p21 and PCNA is disturbed, leading to the formation of PCNA-DNA polymerase holoenzyme complex, and promotes DNA replication (Kreis et al., 2015; Zhou et al., 2001). Nuclear factor erythroid 2-related factor 2 (Nrf2) is considered as a transcription factor regulating anti-inflammatory and anti-oxidant protein (Dai et al., 2016). Nrf2-dependent proteins heme oxygenase 1 (HO-1) had effects on the protection against oxidative stress, modulation of inflammation and regulation of apoptosis (Loboda et al., 2016). Previous study indicated that p21 exerted antioxidant function was mediated through stabilizing the Nrf2 protein to activate Nrf2 pathway (Chen et al., 2009). In the present study, we aimed to investigate the role of p21 in olaquindox induced apoptosis and cell cycle arrest in HepG2 cells. Our findings shed insight into the molecular mechanisms of olaquindox and provided fundamental data for a subsequent toxicity study in vivo.

2. Materials and methods

2.1. Chemicals and reagents

Olaquindox (purity $\geq$ 98%), propidium iodide (PI), Zinc protoporphyrin IX (ZnPP-IX) (HO-1inhibitor), hydrogen peroxide (H2O2) and 3-(4,5-Dimethyl-2-thiazolyl) −2,5-diphenyl-2H-tetrazolium bromide (MTT) were obtained from Sigma-Aldrich (St. Louis, MO, USA). X-tremeGENE HP DNA Transfection Reagent was purchased from Roche (Basel, Switzerland). Dulbecco's modified Eagle's medium (DMEM) was acquired from Invitrogen (Gibco, Grand Island, NY, USA). Fetal bovine serum (FBS) was acquired from HyClone (Logan, UT, USA). Dimethyl sulfoxide (DMSO) tween-20, sodium dodecyl sulfonate (SDS), trypsin, Tris−HCl, and Tris Base were purchased from AMRESCO Inc. (Solon, OH, USA). Pifithrin-α (p53 inhibitor), DCFH-DA probe, LY294002 (PI3K/AKT inhibitor), RNase A and PMSF were purchased from Beyotime Institute of Biotechnology (Haimen, China). All other chemicals and reagents were of reagent grade.

2.2. Cell culture and treatment

The HepG2 cell line and 293 T cell line were gotten from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in DMEM complemented with 10% fetal bovine serum, 100 U/mL penicillin and streptomycin at 37 °C in a humidified atmosphere containing 95% air and 5% CO_2. Olaquindox was freshly prepared and dissolved in DMEM to make concentrations of 200, 400, 800 μg/mL.

2.3. Western blotting assay

Cells were washed twice with cold PBS and lysed in a lysis buffer for 15 min at 4 °C. Then, the lysates were ultrasonicated by ultrasonic apparatus (Branson, Missouri, USA). The concentrations of protein were identified by spectrophotometer NanoDrop 2000c (Thermo Scientific, MA, USA). Loading buffer was added into each sample and denatured at 100 °C for 8 min. Cellular protein was in charge into sodium dodecyl sulfate-polyacrylamide gel for electrophoresis. After that, proteins were taken to nitrocellulose membranes and blocked with 5% non-fat milk for 2 h. Then, running gel was transported to nitrocellulose membranes. After being blocked with 5% non-fat milk for 2 h, the membranes were washed with tris buffered saline tween and incubated with primary and secondary antibodies. Finally, membranes were measured by ECL luminescent detection kit (ABclonal Biotech Co. Ltd., Cambridge, MA, USA). Western blot density was evaluated by the ImageJ 1.46 software (National institutes of Health, USA). The primary antibodies were performed as followed: rabbit polyclonal antibodies against p21 (1:1000; abcam, Cambridge, MA, USA), phosphorylation-p21 (Thr 145) (1:1000), *p*-Akt (Ser473)(1:1000), p53 (1:1000; Santa Cruz, CA, USA), Bax (1:3000), Bcl-2 (1:1000), HO-1 (1:1000), Nrf2 (1:1000) (ProteinTech Group, Inc., Chicago, IL, USA), caspase 3 (1:500), caspase 9 (1:500; Cell Signaling Technology, Beverly, MA, USA) and poly (ADP-ribose) polymerase (PARP) (1:1000; Beyotime, Haimen, China), mouse polyclonal antibodies against β-actin (1:1000) and Cytochrome C (Cyt *c*) (1:2000; Zhongshan Golden Bridge, Beijing, China). The secondary antibodies were anti-rabbit IgG (1:5000) and anti-mouse IgG (1:5000) (Zhongshan Golden Bridge, Beijing, China).

2.4. Lentiviral packaging

The shRNA sequence of p21 and scramble is 5′-GAT GGA ACT TCG ACT TTG T-3′ and 5′-TGA CAT GAT AAT ACT CTC T-3'. The interference sequences were inserted into the pLKO.1 plasmid. 293T cells (1×10^5 cells/well) were seeded in a 6-well plate. After 24 h, cells were transfected with pLKO.1, delta-8.2, and VSVG plasmids using X-tremeGENE HP DNA transfection reagent (Roche, Basel, Switzerland). After 48 h, lentiviral particles were collected in order to infect the target HepG2 cells. Twenty-four hours after infection, cells were re-suspended, and exposed to1 μg/mL