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Vosaroxin induces mitochondrial dysfunction and apoptosis in cervical cancer HeLa cells: Involvement of AMPK/Sirt3/HIF-1 pathway

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ABSTRACT

Vosaroxin is a quinolone-derivative anticancer agent with inhibitory activity on type II DNA topoisomerases (TOP2). The aim of the present study was to investigate its cytotoxic effect and potential molecular mechanisms in human cervical cancer HeLa cells. Vosaroxin decreased cell viability and increased lactate dehydrogenase (LDH) release in a dose- and time-dependent manner in HeLa cells, but not in normal cervical epithelial cells. Vosaroxin also induced apoptosis and increased caspase-3 activity in HeLa cells. These effects were accompanied by increased mitochondrial reactive oxygen species (ROS) generation, lipid peroxidation, mitochondrial swelling and reduced ATP production. Western blot analysis showed that vosaroxin significantly reduced hypoxia-inducible factor 1α (HIF-1α) protein levels. However, it had no effect on HIF-1α protein degradation and HIF-1α mRNA levels. The results showed that vosaroxin inhibited the synthesis of HIF-1α protein and interfered with the dimerization of HIF-1α and aryl hydrocarbon receptor nuclear translocator (ARNT). In addition, vosaroxin stimulated mitochondrial enzyme activities and superoxide dismutase 2 (SOD2) deacetylation via activating (Sir2 like protein 3) Sirt3. More importantly, vosaroxin-induced inhibition on HIF-1α and its cytotoxic effects, as measured by cell viability, LDH release and apoptosis, were partially prevented by Sirt3 knockdown or the AMP-activated protein kinase (AMPK) inhibitor compound C. Overall, vosaroxin is demonstrated to be a chemotherapeutic agent targeting the Sirt3/HIF-1 pathway and could be beneficial for inducing cytotoxicity in human cervical cancer cells.

1. Introduction

Cervical cancer remains the second most common gynecologic cancer worldwide. Each year, more than 500,000 individuals suffer from new diagnosed cervical cancers, and approximately 250,000 women die of cervical cancer globally [1]. The risk factors for cervical cancer include human papillomavirus (HPV) infections, multiple sexual partners, long-term use of oral contraceptives and cigarette smoke exposure [2]. To date, hysterectomy followed by platinum and taxol-based chemotherapies are still standard therapeutic paradigms, but the effects are not satisfactory, especially for the patients with chemoresistance [3].

Type II DNA topoisomerases (TOP2) are ubiquitous enzymes that play important roles in maintaining normal DNA topology and chromosome condensation [4]. They are essential to the survival of proliferating cells, and generating enzyme-mediated DNA damage by targeting TOP2 is an effective strategy for cancer chemotherapy [5,6]. Vosaroxin (SNS-595, voreloxin) is a quinolone-derivative chemotherapeutic agent with a naphthyridine core similar to quinolone antibiotics [7]. It induces replication dependent DNA damage by intercalating DNA and inhibiting TOP2, causing cancer cell apoptosis [8]. Vosaroxin can induce apoptosis in both p53-independent and P-glycoprotein drug pumps-independent manner, thereby avoiding two common dug resistance mechanisms [9]. The efficacy and safety of vosaroxin in combination with cytarabine have been demonstrated in patients with relapsed/refractory acute myeloid leukemia (AML) in a phase III, randomized, multicenter, double-blind, placebo-controlled study (VALOR) [10]. In addition, vosaroxin has been shown to exert anticancer effects in experimental models of Lewis lung carcinoma, M5076 ovarian sarcoma, breast, bladder, pancreas, colon, and gastric cancer [11–13]. However, its effect on cervical cancer cells has not been determined.

Hypoxia is frequently observed in solid tumors and also one of the major obstacles for effective cancer therapies. Hypoxia-inducible factors (HIFs) are the key factors that control hypoxia-inducible pathways by regulating the expression of a vast array of genes. Over the past few decades, several HIF-1 inhibitors with anticancer effects have been described in the literature, most of which are nonselective and target

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redundant signaling pathways implicated in HIF-1 activation [14]. Sirtuins (or Sir2-like proteins) are a family of NAD^+-dependent deacetylases which regulate signaling pathways involved in cellular proliferation and differentiation, metabolism, response to stress, and cancer [15]. Among seven members of Sirtuins, Sirt3 is localized in mitochondria and may act on numerous substrates to activate fat oxidation, amino-acid metabolism and electron transport. Previous studies demonstrated that SIRT3 shows a dual role in cancer, as it can act as a tumor suppressor or a tumor promoter, depending on the cellular context [16]. Due to the important role of Sirt3 in mitochondrial function [17,18], investigating Sirt3 related signaling under hypoxia rises as a possible target to develop new therapeutic strategies against cancer.

In the present study, the cytotoxic effect of vosaroxin was investigated in human cervical cancer HeLa cells. We also investigated the potential underlying molecular mechanisms with focus on the AMP-activated protein kinase (AMPK)/Sirt3/HIF-1 pathway.

2. Materials and methods

2.1. Cell culture

Human cervical cancer HeLa cells were obtained from the Cell Bank of the Chinese Academy of Sciences, and human normal cervical epithelial cells (#CP-H059) were purchased from Procell (Wuhan, Hubei, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Gaithersburg, MD, USA) containing 10% fetal bovine serum (FBS Gibco, Gaithersburg, MD, USA) and 100 IU/mL penicillin and streptomycin and incubated at 37 °C with 20% O_2/5% CO_2 for normoxia or 1% O_2/5% CO_2 for hypoxia.

2.2. Cell viability assay

Cell viability assay was performed using WST-1 assay (Roche, Basel, CH) according to a previously published method [19]. Briefly, cells were cultured at a concentration of 3×10^5 in microplates in a final volume of 100 μl/well culture medium. After various treatments, 10 μl WST-1 was added into each well and incubated for 4 h at 37 °C. Then, 100 μl/well culture media and 10 μl WST-1 was added into one well in the absence of cells, and its absorbance was used as a blank position for the enzyme-linked immunosorbent assay (ELISA) reader at 450/655 nm. The cells were shaken thoroughly for 1 min on a shaker and the absorbance of the samples was measured using a microplate reader.

2.3. Lactate dehydrogenase (LDH) release assay

For LDH assay, the supernatant from each sample was incubated with the reduced form of nicotinamide-adenine dinucleotide (NADH) and pyruvate (Sigma, St. Louis, MO, USA) for 15 min at 37 °C and the reaction was stopped by adding 0.4 M NaOH. The activity of LDH was calculated from the absorbance at 490 nm, and the background absorbance from the culture medium that was not used for any cell cultures was subtracted from the absorbance measurements. The results were presented as the percentage of the enzymatic activity (U) of control.

2.4. TUNEL staining

Apoptosis in HeLa cells were detected by the *In Situ* TUNEL Kit (Roche, Basel, Switzerland) according to the manufacturer's instructions. The cells were mounted using Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA) and the cells were counted in at least 10 different high-power fields and the apoptotic rate was calculated.

2.5. Measurement of caspase-3 activity

The activity of caspase-3 in HeLa cells was determined by a kit according to the manufacturer's instructions (#CASP3C, Sigma, St. Louis, MO, USA). Briefly, after being harvested and lysed 10^6 cells were mixed with 32 μl of assay buffer and 2 μl of 10 mM Ac-DEVD-pNA substrate. Absorbance at 405 nm was measured after incubation at 37 °C for 4 h. Absorbance of each sample was determined by subtraction of the mean absorbance of the blank and corrected by the protein concentration of the cell lysate.

2.6. Measurement of mitochondrial ROS generation

Mitochondrial superoxide production was measured by using the Mitosox Red Kit (Invitrogen, Carlsbad, CA, USA). Briefly, cells were incubated with Mitosox Red for 10 min in a CO_2 incubator at 37 °C. Mitosox was added to the medium to a final concentration of 5 μM after treatment and cells were rinsed with the perfusion buffer before imaging. Following incubation, Mitosox Red fluorescence intensity was acquired at 510/580 nm on an Olympus FV10i Confocal Microscopy equipped with a digital cooled charged-coupled device camera.

2.7. Measurement of lipid peroxidation

Malonyl dialdehyde (MDA) and 4-hydroxynonenal (4-HNE), two index of lipid peroxidation, were determined by using assay kits (#MAK085 and #870608H) following the manufacturer's instruction (Sigma, St. Louis, MO, USA). The absorbance of the samples was measured by a microplate (ELISA) reader.

2.8. Measurement of ATP synthesis

The ATP synthesis in HeLa cells was determined by a kit according to the manufacturer's instructions (#MAK190, Sigma, St. Louis, MO, USA). HeLa cells were subjected to fission and centrifuged at 12 000 g for 5 min. In 24-well plates, 100 μl of each supernatant was mixed with 100 μl ATP working dilution. The luminescence was measured using a monochromator microplate reader at 340 nm. The ATP release levels were expressed as a fold of the luminescence levels in the control cells.

2.9. Measurement of mitochondrial swelling

Mitochondria were isolated with mitochondria isolation kit (Thermo Scientific) following the manufacturer's instructions. Briefly, cells were trypsinized and washed with PBS, then re-suspended in the isolation buffer. The cells were then centrifuged at 700 g for 10 min at 4 °C. The supernatant was transferred to a new 1.5 ml tube and centrifuged at 12,000 g for 15 min at 4 °C. The supernatant (cytosol fraction) was transferred to a new tube. The isolated mitochondria in the pellet were re-suspended for experimental use. Mitochondrial swelling was measured following a previously published protocol [20]. Briefly, isolated mitochondria were suspended in fresh swelling buffer (0.2 M sucrose, 10 mM Tris-MOPS, pH 7.4, 5 mM succinate, 1 mM phosphate, 2 μM rotenone, and 1 μM EGTA-Tris, pH 7.4) at 0.5 mg/ml. The swelling of mitochondria was monitored by a decrease in absorbance at 540 nm in the presence of $CaCl_2$ (200 μM) up to 20 min.

2.10. Short interfering RNA (siRNA) and transfection

The specific siRNA targeted Sirt3 (Si-Sirt3, sc-61556) and control siRNA (Si-control, sc-37007) were purchased from Santa Cruz. Transient transfection was performed using the Lipofectamine 2000 (Invitrogen, USA) in 6-well plates following the manufacturer's instructions. Forty-eight hours after transfection, the cells were collected for further experiments.