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Targeting mitochondrial respiration as a therapeutic strategy for cervical cancer

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ABSTRACT

Targeting mitochondrial respiration has been documented as an effective therapeutic strategy in cancer. However, the impact of mitochondrial respiration inhibition on cervical cancer cells are not well elucidated. Using a panel of cervical cancer cell lines, we show that an existing drug atovaquone is active against the cervical cancer cells with high profiling of mitochondrial biogenesis. Atovaquone inhibited proliferation and induced apoptosis with varying efficacy among cervical cancer cell lines regardless of HPV infection, cellular origin and their sensitivity to paclitaxel. We further demonstrated that atovaquone acts on cervical cancer cells via inhibiting mitochondrial respiration. In particular, atovaquone specifically inhibited mitochondrial complex III but not I, II or IV activity, leading to respiration inhibition and energy crisis. Importantly, we found that the different sensitivity of cervical cancer cell lines to atovaquone were due to their differential level of mitochondrial biogenesis and dependency to mitochondrial respiration. In addition, we demonstrated that the in vitro observations were translatable to *in vivo* cervical cancer xenograft mouse model. Our findings suggest that the mitochondrial biogenesis varies among patients with cervical cancer. Our work also suggests that atovaquone is a useful addition to cervical cancer treatment, particularly to those with high dependency on mitochondrial respiration.

1. Introduction

Cervical cancer is the second-leading cause of cancer-related deaths in women worldwide and its incidence is still increasing in the past decades [1]. Although early cervical cancer can be treated with surgery or radiation, advanced cervical cancer is incurable and new therapeutic approaches are needed. In addition to human papilloma virus (HPV) infection, many other epigenetic and genetic factors are critically involved in cervical cancer transformation and progression, including activation Wnt/β-catenin and deactivation of p53 and RB [2–5]. Transcript profiling has shown intra- and inter-tumor heterogeneity in cervical cancer [6,7]. Hence, targeting common cancer drivers may represent an alternative therapeutic strategy.

Targeting cancer mitochondrial biogenesis by pharmacological or genetic approaches has displayed selective cytotoxicity to cancer cells with minimal effects on normal counterparts due to the unique dependency of cancer cells on mitochondrial respiration [8–11]. Compared to normal cells, cancer cells rely more on mitochondrial functions to generate ATP for growth and survival [12,13]. In addition, tumor stem cells have been found to be more dependent on mitochondrial metabolism than differentiated cells [14]. Bioenergetic analysis of ovarian cancer cell lines also demonstrate that ovarian cancer are highly metabolic [15].

The aim of the present study was to investigate if mitochondrial respiration is essential to cervical cancer. Atovaquone is a FDA-approved anti-parastic drug with its mechanism on targeting cytochrome bc1 complex [16,17]. We investigated the effect of atovaquone on a panel of cervical cancer cell lines and confirmed its action on mitochondrial respiration. Given the differential sensitivity of cervical cancer cell lines to atovaquone, we also investigated if these cell lines differ in their mitochondrial bioenergetics profile. Using live cell measurement of oxygen consumption, we demonstrate the inhibition of mitochondrial respiration as an effective therapeutic strategy for cervical cancer treatment.

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2. Materials and methods

2.1. Cells and drugs

Eight human cervical cancer cell lines SiHa, C33A, ME-180, CaSki, DoTc2, ViBo, HeLa (ATCC, USA) and CaLo (Beijing Institute of Cancer Research) were cultured in RMPI (Sigma, USA) supplemented with 10% fetal bovine serum (Hyclone, UK), 2 mM L-glutamine (Invitrogen, USA) and 1 mM sodium pyruvate (Sigma) in a 5% CO_2 environment at 37 °C. All cell-based assays were performed using cells in the exponential growth phase. Atovaquone (Sigma) and paclitaxel (Sigma) were dissolved in DMSO.

2.2. Cell proliferation and apoptosis assays

Cells (7500/well in a 96-well plate for proliferation assay and 100, 000/well in a 6-well plate for apoptosis assay) were treated with atovaquone for 72 h. The cell proliferation activity was evaluated by using the CellTiter 96 AQueous One Solution Cell Proliferation assay kit (Promega, USA) according to manufacturer's instructions. Apoptotic cells were stained by Annexin V-FITC and 7-AAD (BD Pharmingen, USA) followed by flow cytometry on a Beckman Coulter FC500. The percentage of Annexin V-positive cells was determined by CXP software analysis.

2.3. Mito stress and glycolytic stress test assays

Cells (20, 000/well in XF24 cell culture plates) were treated with atovaquone for 24 h. Culturing media were replaced by XF assay medium (Seahorse Bioscience, USA) and incubated at 37 °C in a CO_2-free environment for pH stabilization and then transferred to the Seahorse XF24 extracellular flux analyzer for the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) assays as per the XF24 analyzer standard protocol (Seahorse Bioscience). Oligomycin, Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), antimycin A and rotenone, glucose and 2-deoxy-$_D$-glucose are from Seahorse Bioscience. The OCR and ECAR measurements were normalized to protein mass.

2.4. Cellular ATP level

Cells (20, 000/well in 24-well plate) were treated with atovaquone for 24 h. ATP levels were measured by CellTiter-Glo Luminiescent Cell Viability Assay (Promega, WI, US) according to the manufacturer's instructions.

2.5. Mitochondrial mass measurement

Cells were stained with 50 nM Mitotracker Green FM (Invitrogen, CA) for 30 min and then analysed on a Beckman Coulter FC500. The median fluorescence intensity in the FL1 channel was divided by the Forward scatter (FSC) measurement as an estimate of mitochondrial mass.

2.6. Mitochondrial complex activity analysis

Cells (20000/well in 12-well plate) were treated with atovaquone for 24 h. In vitro mitochondrial respiratory complex I, II, III and IV activities were assessed using total cell lysates and were measured using Mitochondrial Complex I, II, III and IV Activity Assay Kits (Novagen, US) according to manufacturers' instructions. The complex activities were determined calorimetrically using Tecan Infinite200 Microplate Reader.

2.7. Generation of mitochondrial DNA-deficient ρ0 cell line

Mitochondria DNA-deficient ρ0 was established according to the method previously described [18]. Briefly, parental cells were growing in RPMI medium containing 10% FBS and selected using 500 ng/ml ethidium bromide (EtBr, Sigma), supplemented with 4 mM L-glutamine, 50 mg/ml uridine (Sigma) for 50 days. The lack of mitochondrial DNA in ρ0 cells was confirmed by determining the transcript level of MT-ND6 and MT-CO2 [19]. ρ0 were maintained in above media without EtBr.

2.8. Cervical cancer xenograft in SCID mouse

SCID mice (6–8 weeks old) were purchased from Shanghai Experimental Animal Center, Chinese Academy of Sciences. All animal experiments were carried out in adherence with the National Institutes of Health Guidelines on the Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of Wuhan University of Science and Technology. Ten million SiHa or ViBo cells were harvested in PBS and implanted into mice flank. Following the development of palpable tumors, the mice were treated with vehicle control (80%/20% saline/DMSO) or intraperitoneal atovaquone at 0.5 mg/kg. Tumor length and width were measured every two days and the volumes were calculated using the formula: length x width^2 x 0.5236.

2.9. Statistical analyses

All experiments in this study were conducted at least three times. The data are expressed as mean and standard deviation (SD). Statistical analyses were performed by unpaired Student's *t*-test, with p-value < 0.05 considered statistically significant.

3. Results

3.1. Cervical cancer cells response differently to atovaquone and paclitaxel

To investigate the biological effects of atovaquone on cervical cancer cells, we performed proliferation and apoptosis assays after drug treatment. Cervical cancers are heterogeneous, both in their pathology and in their molecular profiles [6]. Based on the virus infection profiles, three cell lines which are positive for infection with HPV type 18 (HeLa, CaLo and ME-180), two positive for HPV type 16 (SiHa, CasKi), and three HPV negative (ViBo, DoTc2 and C33A) were used in our study [20].

We found that atovaquone up to 10 μM significantly inhibited proliferation and induced apoptosis of all tested cervical cancer cell lines in a dose-dependent manner (Fig. 1A and B). In addition, the sensitivity to atovaquone varies significantly in cervical cancer cell lines regardless of HPV infection. For example, we found that SiHa (HPV positive) and C33A (HPV negative) were the most sensitive with ~100% growth inhibition and ~80% apoptosis induction whereas ViBo (HPV negative) and HeLa (HPV positive) were the least sensitive to atovaquone with ~60% growth inhibition and ~40% apoptosis induction (Fig. 1A and B).

We next explore if this is a similar response of cervical cancer cell lines to chemotherapeutic agent paclitaxel. Interestingly, we found that the response of cervical cancer cell lines to atovaquone are not correlated with the response to paclitaxel (Fig. 1C and D). We found that ME-180 and ViBo were the most sensitive whereas CaLo and DoTc2 were the least sensitive to paclitaxel (Fig. 1C and D). Taken together, our results suggest that cervical cancer cells response differently to atovaquone regardless of their sensitivity to paclitaxel.

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