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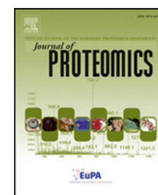

Quantitative analysis of differential protein expression in cervical carcinoma cells after zeylenone treatment by stable isotope labeling with amino acids in cell culture

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ABSTRACT

Cervical carcinoma is a malignant tumor that poses a serious threat to women's health and survival. Approximately 10–25% of cervical cancers are adenocarcinomas (ACs). AC has high rates of recurrence and mortality, while there is no effective treatment for now. Zeylenone (Zey), which is isolated from an ethanol extract of the leaves of *Uvaria grandiflora* Roxb. of the family *Annonaceae*, has shown potent inhibitory activity against various tumor cells, including cervical carcinoma cells. To gain insight into the molecular mechanism underlying the effect of Zey on AC, we quantified protein expression changes in AC cells treated with Zey. We used stable isotope labeling with amino acids in cell culture (SILAC) in combination with high-performance liquid chromatography–electrospray ionization tandem mass spectrometry (HPLC–ESI–MS/MS) and bioinformatics analysis to compare protein expression profiles in HeLa cells before and after Zey treatment. Of 1805 differentially expressed proteins identified, 229 were screened as key protein molecules and classified into nine categories. Profiling of differentially-expressed proteins contributed to our understanding of the molecular mechanism by which Zey induces HeLa cell apoptosis. Using this method, candidate targets can be identified for developing new drugs against cervical carcinoma.

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1. Introduction

Cervical cancer is a tumor of the cervical epithelium and the second most common gynecologic malignancy, accounting for 250,000 to 280,000 deaths each year [1]. Approximately 75–85% of cervical cancers are squamous cell carcinomas (SCC), and the remaining 10–25% are adenocarcinomas (AC) [2]. Improvements in the efficiency of screening programs in the last three decades decreased the incidence of and mortality from SCC in the developed world; however, these programs fail to detect AC efficiently [3]. Current treatments for cervical cancer include surgery, radiation or chemoradiotherapy. However, up to 35% of cervical cancer patients show recurrent or persistent disease after initial treatment [4]. AC, which has a higher incidence of lymphatic and hematogenous metastasis in its early stages, shows a poorer response to conventional therapy than SCC [5]. Cisplatin-based chemoradiation therapy, which remains the gold standard treatment for advanced and metastatic cervical cancer, is limited by its severe side-effects such as bone marrow depression, neutropenia, thrombocytopenia and anemia [6]. Thus, the development of novel therapeutic strategies that replace platinum-based chemotherapy is important, in particular the identification of agents with novel mechanisms of action in AC.

Zeylenone (Zey) is a naturally occurring cyclohexene oxide isolated from *Uvaria grandiflora* Roxb. that has shown potent antitumor effects. Zey displays strong cytotoxic activity against various tumor cell lines, and has low toxicity against normal cell lines. Zey is equally potent against the drug-sensitive mammary carcinoma MCF-7 cell line and the multidrug resistant MCF-7/ADM subline [7–10]. Because Zey is an ester-containing compound and its benzoate ester bonds are hydrolyzed and inactivated rapidly in the blood owing to the presence of esterases, it is regarded as a cytotoxic soft drug. Polymeric micelles loaded with Zey showed an excellent anticancer effect against a model of A549 lung cancer in vivo [11]. Recent studies by our group [9,10] indicated that Zey induces apoptosis of human prostate carcinoma PC3 cells and acute lymphoblastic leukemia Reh and RS4;11 cells in a dose-dependent manner. Zey induces apoptosis via mitochondria- and Fas-mediated caspase-dependent apoptosis pathways, which are associated with the Bcl-2 protein family. However, the anticancer activity

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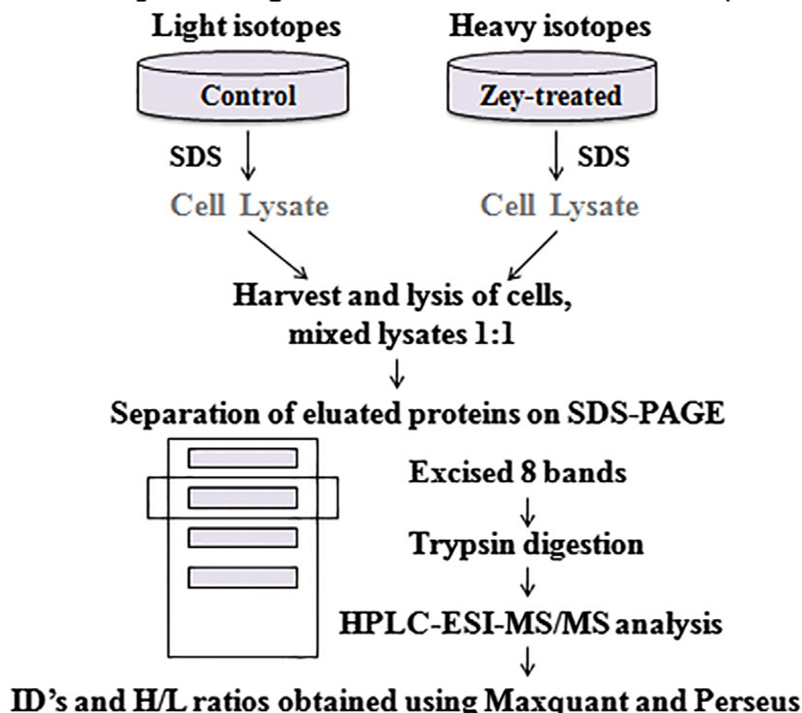

Fig. 1. Depiction of the experimental design workflow used for the multiplexed comparative analysis of the Zey treated and control cell line samples.

of Zey in cervical cancer is largely unknown and its molecular mechanisms remain elusive.

The present study was designed to investigate the potential anticancer properties of Zey in human AC and the underlying molecular mechanisms. Stable isotope labeling with amino acids in cell culture (SILAC), a quantitative proteomics technique for the discovery of small molecular targets [12], was used to identify Zey-induced protein alterations in cultured human AC HeLa cells. Differentially expressed proteins in response to Zey were apoptosis-associated, chaperones and ribosomal proteins. Further functional studies revealed that Zey promoted HeLa cell apoptosis by inducing Bcl-2 activation and by downregulating the expression of 14-3-3 family, Hsp70 family and ribosomal proteins. Studies on the mechanism make foundations for future clinical treatment of AC.

2. Material and methods

2.1. Reagents

Zey was provided by Professor Yonghong Liao (Medicinal Plant Research Institute of the Chinese Academy of Medical Science, Beijing, China). The purity of Zey was >97.5% by high-performance liquid chromatography (HPLC). A 20 mM stock solution of Zey in DMSO was prepared and sub-packaged at −20 °C. Before the experiments, the stock solution was diluted to the desired concentration (DMSO < 0.1%) using cell culture media.

2.2. Cell lines

All cell lines were purchased from the Cell Culture Center of the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences and Cell Resources Center, Shanghai Institutes for Biological Sciences (Shanghai, China). The cells were passaged and preserved in our laboratory. The cell lines used in our experiments were as follows: human cervical cancer cell lines, HeLa and Caski; human hepatic carcinoma cell line, HepG2; human breast cancer cell lines, MCF-7 and MX-1; human lung cancer cell line, A549; human prostatic cancer cell line, PC-3; human gastric cancer cell line, BGC823; human oral epidermoid carcinoma cell line, KB; human prostatic stromal myofibroblast cell line, WPMY-1; and normal human bladder cell line, SV-HUC-1.

2.3. Main reagents

DMEM, RPMI-1640, trypsin, penicillin, streptomycin, fetal serum (Gibco, Carlsbad, CA, USA), MTT, DMSO, DAPI (Sigma, St. Louis, MO, USA), fluorescent dye JC-1, chemiluminescence detection liquid (Invitrogen, Carlsbad, CA, USA), Annexin V-FITC/PI Apoptosis Detection Kit (Beijing Biosea Biotechnology Co., Ltd., Beijing, China), AO/EB, and crystal violet (Ameresco, Solon, OH, USA) were used in the experiments.

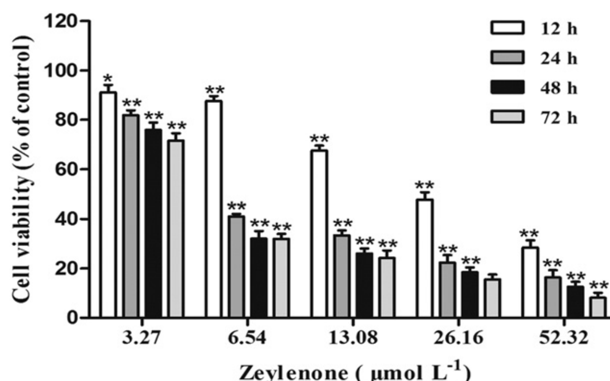

Fig. 2. Anti-proliferative effect of Zey in HeLa cells by MTT assay. (A) Chemical structure of Zey. (B) HeLa (6×10^3/well) cells were seeded into 96-well microplates and treated with various concentrations of Zey (3.27, 6.54, 13.08, 26.16 and 52.32 μmol/L) for 12, 24, 48 and 72 h.

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