Journal of Pharmaceutical and Biomedical Analysis 160 (2018) 344–350

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Proteomic analysis of evodiamine-induced cytotoxicity in thyroid cancer cells

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ARTICLE INFO

Article history:
Received 18 March 2018
Received in revised form 27 July 2018
Accepted 4 August 2018
Available online 9 August 2018

Keywords:
Evodiamine
Proteomics
2D-DIGE
MALDI-TOF
Thyroid cancer

ABSTRACT

Evodiamine is a natural product extracted from herbal plants such as *Tetradium* which has shown to have anti-fat uptake and anti-proliferation properties. However, the effects of evodiamine on the behavior of thyroid cancers are largely unknown. To determine if evodiamine might be useful in the treatment of thyroid cancer and its cytotoxic mechanism, we analyzed the impact of evodiamine treatment on differential protein expression in human thyroid cancer cell line ARO using lysine-labeling two-dimensional difference gel electrophoresis (2D-DIGE) combined with mass spectrometry (MS). This study demonstrated 77 protein features that were significantly changed in protein expression and revealed evodiamine-induced cytotoxicity in thyroid cancer cells involves dysregulation of protein folding, cytoskeleton, cytoskeleton regulation and transcription control. Our work shows that this combined proteomic strategy provides a rapid method to study the molecular mechanisms of evodiamine-induced cytotoxicity in thyroid cancer cells. The identified targets may be useful for further evaluation as potential targets in thyroid cancer therapy.

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1. Introduction

Evodiamine is a natural product isolated from herbal plants such as *Tetradium* and shown to be harmless to humans [1]. It has long been used to reduce fatty acid synthesis and exhibit anti-tumor activity [2,3]. Evodiamine has also been reported to show anti-tumor and inhibitory effects on lung cancer [4], liver cancer [5], colon cancer [6], thyroid cancer [7] and ovarian cancer [8] via blockade of the cell cycle, inhibition of DNA synthesis, activation of caspases and induction of apoptosis. It has also been demonstrated that evodiamine acts as an anti-metastatic drug in human breast and colon cancers via inhibition of cell migration, arrest of cell cycle progression and induction of cell apoptosis. Recently, the generation of reactive oxygen species (ROS) has been reported to be an important factor in evodiamine-induced apoptosis of cancer cells [9]. However, the detailed mechanisms of evodiamine-induced cytotoxicity remains poorly understood.

Proteomics is a powerful tool to monitor protein expression changes in response to drug treatment. 2-DE remains an important technique in proteomics for global protein profiling within biological samples and plays a complementary role to LC–MS-based analysis [10]. However, reliable quantitative comparison between gels remains the primary challenge in 2-DE analysis. A significant improvement in gel-based protein detection and quantification was achieved by the introduction of 2D-DIGE, where several samples can be co-detected on the same gel using differential fluorescent labeling. This approach alleviates gel-to-gel variation and allows comparison of the relative amount of resolved proteins across different gels using a fluorescently-labeled internal standard. Moreover, the 2D-DIGE technique has the advantages of a broader dynamic range of detection, higher sensitivity and greater reproducibility than traditional 2-DE [10]. This innovative technology relies on the pre-labeling of protein samples on the

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https://doi.org/10.1016/j.jpba.2018.08.008
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amino group of lysine resudues with fluorescent dyes (Cy2, Cy3 and Cy5) before electrophoresis. Each dye has a distinct fluorescent wavelength, allowing pairs of experimental samples and an internal standard to be simultaneously separated in the same gel. The internal standard, which is a pool of an equal amount of all samples, helps to provide accurate normalization and spot matching and increases statistical confidence in relative quantification across gels [11–14].

The aim of the current study was to use a proteomic approach combining lysine 2D-DIGE and MS to investigate the inhibitory effects of evodiamine on thyroid cancer cells. ARO cells were used as a model system to clarify the molecular effects of evodiamine including the investigation of evodiamine-induced proteome alteration of intracellular proteins.

2. Materials and methods

2.1. Chemicals and reagents

Generic chemicals including evodiamine were purchased from Sigma-Aldrich (St. Louis, USA), while reagents for 2D-DIGE were purchased from GE Healthcare (Uppsala, Sweden). All primary antibodies were purchased from Genetex (Hsinchu, Taiwan) and anti-mouse, and anti-rabbit secondary antibodies were purchased from GE Healthcare (Uppsala, Sweden). All the chemicals and biochemicals used in this study were of analytical grade.

2.2. Cell lines and cell cultures

The thyroid cancer cell line ARO (anaplastic thyroid carcinoma cell line) was obtained from Chiayi Christian Hospital, Chiayi, Taiwan and was maintained in Dulbecco's Modified Eagle's medium (DMEM) supplemented with fetal calf serum (FCS) (10% (v/v)), L-glutamine (2 mM), streptomycin (100 μg/mL), and penicillin (100 IU/mL) (all from Gibco-Invitrogen Corp., Paisley, UK). All cells were incubated at 37 °C and CO_2 (5%).

2.3. MTT cell viability assay

The detailed MTT experimental procedure has been reported in our previous study [15].

2.4. Immunoblotting analysis

Immunoblotting analysis was used to validate the differential abundance of mass spectrometry identified proteins between ARO cells and ARO cells treated with evodiamine. The detailed experimental procedure has been described in our previous study [12].

2.5. 2D-DIGE, gel image analysis, protein staining, in-gel digestion and MALDI-TOF MS analysis

For 2D-DIGE analysis, protein samples were labeled with N-hydroxy succinimidyl ester-derivatives of the cyanine dyes Cy2, Cy3 and Cy5. Briefly, 150 μg of protein sample in triplicate was minimally labeled with 375 pmol of either Cy3 or Cy5 for comparison on the same 2-DE gel. To facilitate image matching and cross-gel statistical comparison, a pool of all samples was also prepared and labeled with Cy2 at a molar ratio of 2.5 pmol Cy2 per μg of protein as an internal standard run on all gels. The detailed experimental procedures have been described in our previous study [16–19].

For protein extraction, colloidal coomassie blue G-250 stained gels were used to visualize CyDye-labeled protein features in 2-DE followed by excised post-stained gel pieces. The gel pieces were washed three times in acetonitrile (50%), dried in a Speed-Vac for 20 min., reduced with dithiothreitol (10 mM) in ammonium bicarbonate (5 mM) pH 8.0 for 45 min at 50 °C and then alkylated with iodoacetamide (50 mM) in Ammonium bicarbonate (5 mM) for 1 h at room temperature in the dark. The gel pieces were then washed three times in acetonitrile (50%) and vacuum-dried before reswelling with 50 ng of modified trypsin in Ammonium bicarbonate (5 mM). The pieces were then overlaid with 10 μl of Ammonium bicarbonate (5 mM) and trypsinized for 16 h at 37 °C. Supernatants were collected, peptides were further extracted twice with trifluoroacetic acid (5%) in acetonitrile (50%) and the supernatants were pooled. Peptide extracts were vacuum-dried, resuspended in 5 μl ddH_2O, and stored at −20 °C prior to MS analysis.

For mass spectrometry analysis, extracted proteins were cleaved with a proteolytic enzyme to generate peptides, then a peptide mass fingerprinting (PMF) database search following MALDI TOF mass analysis was employed for protein identification. Briefly, 0.5 μl of tryptic digested protein sample was first mixed with 0.5 μl of a matrix solution containing α-cyano-4-hydroxycinammic acid at a concentration of 1 mg in 1 ml of acetonitrile / trifluoroacetic acid / H_2O (50% : 0.1% : 49.9%, v/v/v), spotted onto an anchorchip target plate and dried. The peptide mass fingerprints were acquired using an Autoflex III mass spectrometer in reflector mode. The algorithm used for spectrum annotation was SNAP (**S**ophisticated **N**umerical **A**nnotation **P**rocedure). This process used the following detailed metrics: Peak detection algorithm: SNAP; Signal to noise threshold: 25; Relative intensity threshold: 0%; Minimum intensity threshold: 0; Maximal number of peaks: 50; Quality factor threshold: 1000; SNAP average composition: Averaging; Baseline subtraction: Median; Flatness: 0.8; MedianLevel: 0.5. The spectrometer was also calibrated with a peptide calibration standard (Bruker Daltonics) and internal calibration was performed using trypsin autolysis peaks at *m/z* 842.51 and *m/z* 2211.10. Peaks in the mass range of *m/z* 800–3000 were used to generate a peptide mass fingerprint that was searched against the Swiss-Prot/TrEMBL database (released on 2016_10) with 552,884 entries using Mascot software v2.5.1 (Matrix Science, London, UK). The following parameters were used for the search: *Homo sapiens*; tryptic digest with a maximum of 1 missed cleavage; carbamidomethylation of cysteine, partial protein N-terminal acetylation, partial methionine oxidation and partial modification of glutamine to pyroglutamate and a mass tolerance of 50 ppm. Identification was accepted based on significant MASCOT Mowse scores ($p < 0.05$), spectrum annotation and observed versus expected molecular weight and p*I* on 2-DE.

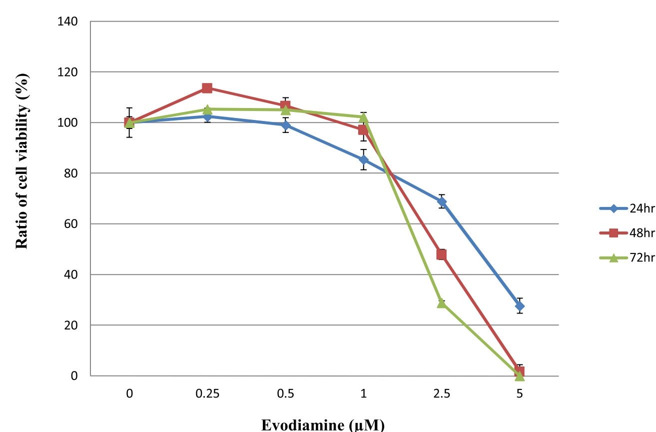

Fig. 1. Evodiamine-induced loss of cell viability. MTT-based viability assays were performed where 10,000 ARO cells were plated into 96-well plates in medium containing 10% FBS. After 24 h, the cells were treated with the indicated concentrations of evodiamine for a further 24 h, 48 h and 72 h. Cells were incubated with MTT and then DMSO added and the plates shaken for 20 min followed by measurement of the absorbance at 540 nm. Values were normalized against untreated samples and are the average of 4 independent measurements +/- the standard deviation.

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