Biochimica et Biophysica Acta 1830 (2013) 2779–2789

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

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

Effects of celastrol on human cervical cancer cells as revealed by ion-trap gas chromatography–mass spectrometry based metabolic profiling

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ARTICLE INFO

Article history:
Received 19 April 2012
Received in revised form 4 October 2012
Accepted 29 October 2012
Available online 8 November 2012

Keywords:
Celastrol
Human cervical cancer cell
Apoptosis
Gas chromatography–mass spectrometry
Metabolic profiling
Metabolomics

ABSTRACT

Background: Celastrol, a quinine methide triterpene extracted from a Chinese medicine (*Trypterygium wilfordii* Hook F.), has the potential to become an anticancer drug with promising prospects. Cell culture metabolomics has been a powerful method to study metabolic profiles in cell line after drug treatment, which can be used for discovery of drug targets and investigation of drug effects.

Methods: We analyzed the metabolic modifications induced by celastrol treatment in human cervical cancer cells, using an ion-trap gas chromatography–mass spectrometry based metabolomics combined with multivariate statistical analysis, which allows simultaneous screening of multiple characteristic metabolic pathways related to celastrol treatment. Three representative apoptosis-inducing cytotoxic agents, namely cisplatin, doxorubicin hydrochloride and paclitaxel, were selected as positive control drugs to validate reasonableness and accuracy of our metabolomic investigation on celastrol.

Results: Anti-proliferation and apoptotic effects of celastrol were demonstrated by CCK-8 assay, Annexin-V/PI staining method, mitochondrial membrane potential ($\Delta\Psi m$) assay and caspase-3 assay. Several significant metabolites involved in energy, amino acid and nucleic acid metabolism in HeLa cells induced by celastrol and positive drugs were reported. Our method is proved to be effective and robust to provide new evidence of pharmacological mechanism of celastrol.

Conclusions: The metabolic alterations induced by drug treatment showed the impaired physiological activity of HeLa cells, which also indicated anti-proliferative and apoptotic effects of celastrol and these positive drugs.

General significance: GC/MS-based metabolomic approach applied to cell culture could give valuable information on the systemic effects of celastrol *in vitro* and help us to further study its anticancer mechanism.

1. Introduction

Traditional medicine represents a cornucopia of plant-derived remedies to discover novel lead molecules for the development of new drugs. Celastrol (structure see Fig. 1A), a quinine methide triterpene extracted from a typical Chinese medicine (*Trypterygium wilfordii* Hook F.), has been extensively investigated as a promising drug for the treatment of autoimmune diseases, asthma, chronic inflammation, and neurodegenerative disease [1,2]. In 2006, celastrol is reported for the first time to be a natural proteasome inhibitor and has exhibited a great potential for cancer prevention and treatment [3]. From then on, investigation on therapeutic efficacy of celastrol against various cancer cells has become a hot spot [4–9]. Celastrol can inhibit the proliferation of wide variety of human tumor cells, and prevent their malignant tissue invasion and block angiogenesis [3,4,10,11]. When used in combination therapy, it can also sensitize resistant melanoma cell to temozolomide treatment, and potentiate radiotherapy in prostate cancer cells [12,13]. These studies show that celastrol has the potential to become an anticancer drug with promising prospects.

Several molecular targets of celastrol have been characterized, including heat shock protein (HSP), reactive oxygen species (ROS), vascular endothelial growth receptor (VEGFR), nuclear factor-κB (NF-κB) and so on [14,15]. Interestingly, many of them are centered on the function of IκB kinase enzyme (IKK) complex and NF-κB system [1,11], which is the key regulator in cancer disease [16,17]. However, the NF-κB system is highly integrated with other signaling pathways *via* a variety of protein kinases [18,19], which makes it difficult to explain the mechanism of celastrol's therapeutic effects. Hence, although

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http://dx.doi.org/10.1016/j.bbagen.2012.10.024

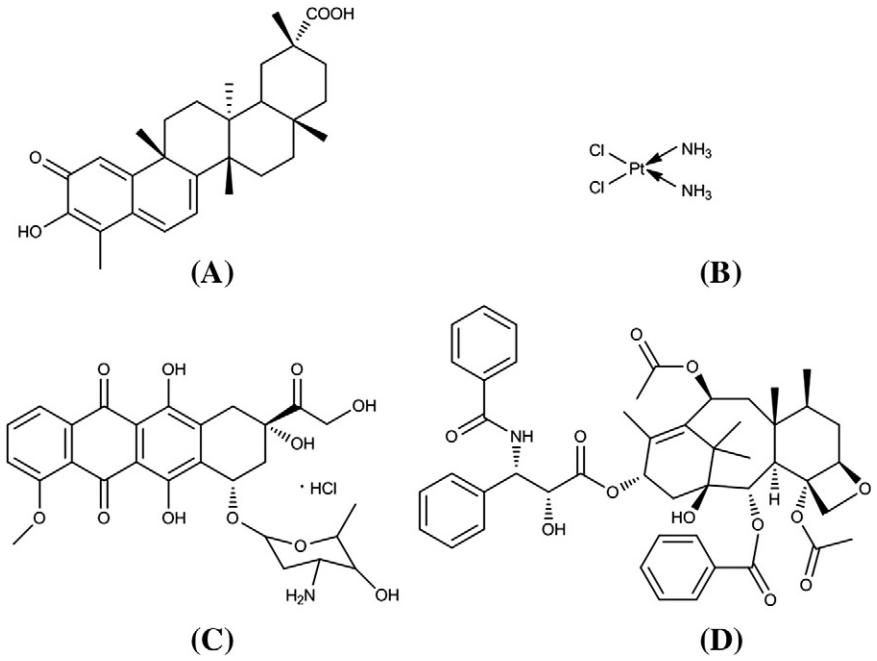

Fig. 1. Chemical structures of celastrol (A), cisplatin (B), doxorubicin hydrochloride (C) and paclitaxel (D).

investigations focusing on celastrol's effects on specific cellular pathways have revealed a number of targets in a diverse array of *in vitro* models [14,15], there is still lack of a thorough insight into its anticancer effects from a global view.

Metabolomics seeks to characterize the metabolic profile of a biological system. Since the panel of metabolites with relatively low molecular weight are downstream products of biomolecular processes, their identity and concentration in living biological system can provide biochemical signatures for globally tracking the physiological effects, and exploring the drug effects [20–22]. Particularly, because cancer cells have several specific metabolic features, such as high enzyme activities, high phosphometabolite levels and high energy metabolism (for an overview see http://www.metabolic-database.com/html/tumor_metabolome_overview.html), cell culture metabolomics has been instrumental in finding further susceptible biomarkers for cancer diagnosis or drug treatment [23–26].

Apoptosis is an important phenomenon in cancer therapy and represents a common mechanism of drug effect [27]. Therefore, investigation on biomarkers indicative of early apoptosis is crucial in theranostics of cancer therapy [28]. Importantly, celastrol has been reported to induce apoptosis in many cancer cells. However, the metabolic intervention of celastrol on cancer cells has not been revealed, whereas this is clearly very meaningful for exploring its mechanism of action in preventing and treating cancer. These considerations prompted us to study the metabolic modifications induced by celastrol treatment in cancer cells.

Comparing to LC-MS and NMR, GC–MS remains a good choice for metabolomic study, since it has been proved of high selectivity and reproducibility with relatively low cost, and a number of structure databases are available [29]. The present study aimed to design a fast, robust and reliable GC–MS analysis system for metabolite measurements in cancer cells, for the purpose of providing a global view of celastrol's effects. We report for the first time several metabolites indicative for early apoptotic processes in HeLa cells culture induced by celastrol using ion-trap gas chromatography–mass spectrometry. Meanwhile, in order to validate reasonableness and accuracy of our metabolomic investigation on celastrol, we selected three representative apoptosis-inducing cytotoxic agents, namely cisplatin, doxorubicin hydrochloride and paclitaxel (structures see Fig. 1B to D) as positive control drugs. In our study, cell fate and apoptosis were determined by CCK-8 assay, Annexin-V/PI staining method, mitochondrial membrane potential assay and caspase-3 assay.

2. Materials and methods

2.1. Reagents

Dulbecco's modified Eagle's medium (DMEM) was purchased from HyClone Thermo scientific (Beijing, China). Fetal bovine serum (FBS) was obtained from GIBCO BRL (Grand Island, NY, US). Celastrol, cisplatin, doxorubicin hydrochloride and paclitaxel were all purchased from Sigma-Aldrich (St. Louis, MO, US). Celastrol and positive control drugs were prepared from stock solutions in dimethyl sulfoxide (DMSO). The stock solutions were kept frozen in aliquot at −20 °C and thawed immediately prior to each experiment. Methoxylamine hydrochloride, N-methyl-N-(trimethylsilyl)-trifluoracetamide (MSTFA), pyridine, trimethyl-chlorosilane (TMCS) and ribitol (used as internal standard) were purchased from Sigma-Aldrich (St Louis, MO, US).

2.2. Cell culture

Human cervical cancer HeLa cells line was purchased from Chinese Academy of Sciences (Shanghai, China). Cells were routinely cultured in DMEM, supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), streptomycin (0.1 mg/mL) and were maintained in a humidified atmosphere of 5% CO_2 at 37 °C. Cells were passaged every 3–4 days. For drug treatment, appropriate amounts of celastrol and positive control drugs were added to culture medium to achieve the appropriate concentrations and then incubated for the indicated time periods.

2.3. CCK-8 assay to determine cell viability

To evaluate the percentage of viable cells after different treatments, the Cell Counting Kit-8 (CCK-8) assay (Beyotime Biotech, China) was performed. Cells were seeded in 96-well plates at a density of 2000 cells/well. On the next day, cells were incubated with different concentrations of celastrol. After appropriate incubation time, 10 μL CCK-8 was added to each well. After another 1 h of incubation at 37 °C, absorbance was measured at 480 nm (A_{480}) with the Synergy™ 4 Mulit-Detection