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Proteomics analysis demonstrating rosmarinic acid suppresses cell growth by blocking the glycolytic pathway in human HepG2 cells

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ABSTRACT

Rosmarinic acid (RA), isolated from herbal balm mint plants, has demonstrated potent anti-tumor properties against liver cancer. However, the precise underlying mechanisms remain unclear. This study aimed to investigate the molecular mechanisms of RA in HepG2 cells. RA anti-tumor activity was assessed using 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide (MTT) and lactate dehydrogenase (LDH) assays, and Hoechst 33258 staining. Apoptosis and the cell cycle distribution were evaluated by flow cytometry. A proteomics approach was used to identify differentially expressed proteins following RA treatment in HepG2 cells, and quantitative reverse transcription–quantitative polymerase chain reaction was used to validate the results. Bioinformatics analysis was also implemented to further understand the identified proteins, and western blotting was used to analyze the associated proteins. Our results suggested that RA treatment significantly inhibits the viability of HepG2 cells. The MTT and LDH assays indicated dose-dependent decreases in cell proliferation following RA treatment. Hoechst 33258 staining and flow cytometry analysis showed that RA exhibits an apoptosis-inducing effect and induces cell cycle arrest in G1. The proteomics analysis successfully identified 16 differentially expressed proteins. Bioinformatics analysis indicated that the identified proteins participated in several biological processes and exhibited various molecular functions, mainly related to inactivation of the glycolytic pathway. Further western blotting analysis showed that RA could downregulate the expression of glucose transporter-1 and hexokinase-2, leading to the suppression of glucose consumption and generation of lactate and ATP. Taken together, our study found that RA exhibits significant cytotoxic effects by inhibiting cell proliferation and inducing apoptosis and cell cycle arrest, possibly by blocking the glycolytic pathway in human HepG2 cells.

1. Introduction

Hepatocellular carcinoma (HCC) is currently one of the most common potentially aggressive human malignant cancers and the third cause of tumor-related deaths worldwide, accounting for more than 800,000 mortalities every year [1]. At early stages, tumors can be curable by surgical therapy, liver transplantation, or ablation, and 5-year survival rates greater than 50% can be attained [2]. At advanced stages, systemic chemotherapy is the primary treatment, although the existing chemotherapy regimens suffer from a number of issues, including poor therapeutic effect, drug resistance, and adverse reactions [3]. Thus, the search for novel efficient targeted agents or new therapeutic strategies are urgently necessary for the treatment of HCC. Thus, increasing attention has been focused on traditional Chinese medicine (TCM) to explore new cancer approaches that are promising and effective for patients with HCC [4].

TCMs from medicinal plants have been shown to be an excellent source of chemotherapeutic agents with various biological activities and great potential therapeutic value [5], and research in the field of TCM is in high demand to help humans overcome many diseases, particularly cancers [6,7]. TCM, which is comprised mainly of natural products, has attracted increasing public attention recently, and many researchers have explored novel therapeutic targets against HCC [8]. Rosmarinic acid (RA; Fig. 1) is a water-soluble polyphenol hydroxyl

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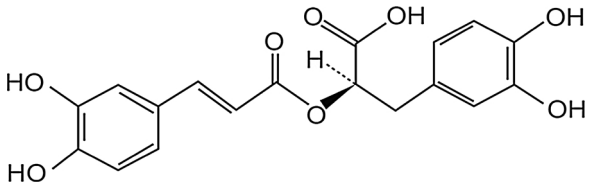

Fig. 1. Chemical structure of RA.

Table 1
Primer sequences for qRT-PCR.

GENE	Forward primer sequence	Reverse primer sequence
PHB	GCGTGGTGAACTCTGCTCTA	TGTACCCAGGGGATGAGGAA
DLST	CTGTCACACGGAAGCTAGCG	ATCCCCAGGATGGCAGACTGA
EEF1A1	CTCCACTTGGTCGTTTTGCTG	GCAGACTTGGTGACTTTGCC
LDHA	GTCAGCAAGAGGGAGAAAGC	TCCAAGCCACGTAGGTCAAG

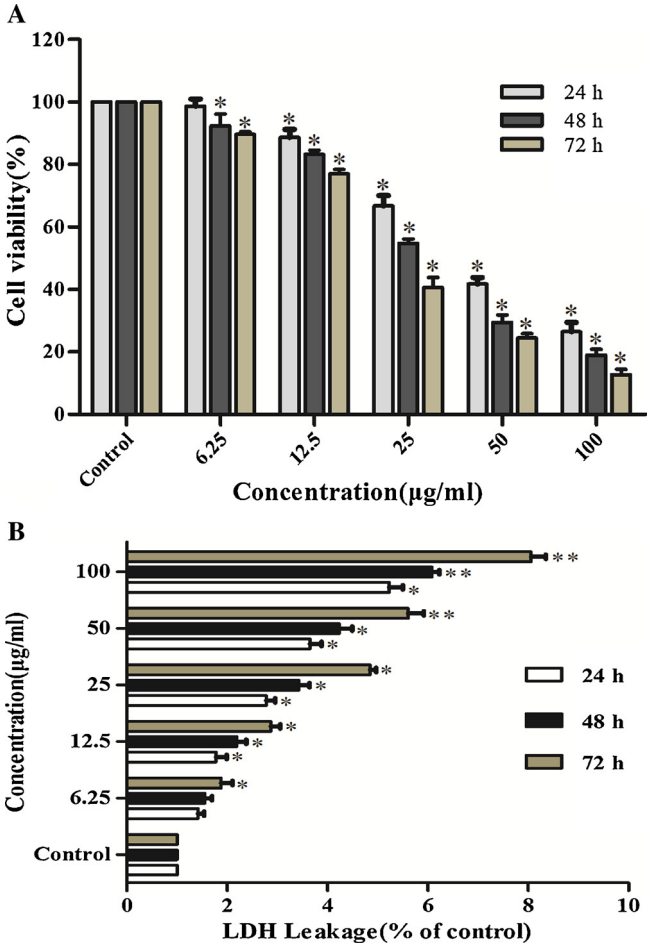

Fig. 2. The cytotoxicity of RA on HepG2 cells. **A.** HepG2 cells were treated with different concentrations of RA for different time points (24, 48, and 72 h), and then cell viability was measured using the MTT assay. The results showed RA could inhibit HepG2 cells proliferation in a time- and dose-dependent manner with respect to control cells (*$P < 0.05$). **B.** Cytotoxicity was measured using the LDH release assay kit. LDH release was significantly increased compared to untreated cells (*$P < 0.05$, **$P < 0.01$). The data were represented by mean ± SD of three experiments, and each experiment was conducted in triplicate.

acid that exists in many Chinese traditional herb drugs, especially in labiatae, boraginaceae, and umbelliferae, with the structure of the ester of caffeic acid and 3-(3,4-dihydroxyphenyl) lactic acid [9]. RA has demonstrated numerous biological activities, including antioxidant, anti-inflammation, anti-virus, anti-mutagenic, and anti-cancer properties [10]. Some studies have shown that RA exhibits anti-tumor effects in vitro and can be applied for the treatment of colon cancer, breast cancer, prostate cancer, ovarian carcinoma, and gastric carcinoma [11,12]. In addition, treatment of liver cancer HepG2 cells with RA caused an increase in apoptosis and a decrease in Bcl-2 mRNA levels [13]. However, the specific mechanisms of action underlying the anti-cancer effects of RA in HepG2 cells remain elusive.

TCM has been widely applied for thousands of years in China and other Asian countries to prevent, diagnose, and cure many diseases. However, TCM is a typical complex material system, comprising raw materials of various origins and leading to various chemical constituents in TCM. In addition, TCM diversity, in turn, leads to expression in multiple effector organs, with multiple roles and multiple targets; thus, studying their mechanisms of action is difficult [14]. The application of systems biology to the study of TCM has provided new ideas and methods for the study of complex systems and has become a focus in the study of the modernization of TCM [15]. Proteomics technology, an important component of systems biology, has gained considerable attention in studying the mechanism of action of TCM, identifying effective drug targets, and developing new drugs [16]. Proteomics approaches mainly consist of two-dimensional electrophoresis (2-DE), mass spectrometry, and bioinformatics [17]. In addition, genomics and proteomics are considered other effective tools to study biological systems [18]. It is also a powerful approach to identify the molecular targets of TCM and investigate the underlying mechanisms of diseases.

In the present study, the cytotoxic effects of RA on HepG2 cells were examined using 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide (MTT) and lactate dehydrogenase (LDH) assays, Hoechst 33258 staining, and flow cytometry. Proteomics, bioinformatics, western blotting, and enzyme-linked immunosorbent assays were carried out to investigate and confirm the mechanism of action in HepG2 cells following RA treatment. The data in this study provide many theoretical bases to understand the molecular mechanisms of the cytotoxic effects of RA in HepG2 cells and will help to develop tools for the diagnosis and prognosis of cancers.

2. Materials and methods

2.1. Reagents and chemicals

RA was purchased from Sigma-Aldrich (St. Louis, MO, USA) and its purity was determined by high-performance liquid chromatography to be ≥ 98%. RA was split into 100 μg per tube and dissolved in 1 ml of culture medium before use, a condition that maintained the stability of RA. The structure of RA is shown in Fig. 1. RPMI-1640 medium, fetal bovine serum (FBS), and antibiotics were obtained from Hyclone (Thermo Fisher Scientific, Waltham, MA, USA). All reagents used in 2-DE were obtained from Bio-Rad Laboratories (Milan, Italy), and the silver staining chemicals were purchased from CWBIO (Beijing, China). The annexin V-FITC/propidium iodide (PI), LDH release assay, and ATP assay kits were obtained from Beyotime Co. (Hangzhou, China). Glucose and lactate assay kits were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Antibodies against β-actin, glucose transporter-1 (Glut-1), and hexokinase-2 (HK-2) were obtained from Cell Signaling Technology (Danvers, MA, USA). Other chemical reagents, unless otherwise indicated, were obtained from Sigma-Aldrich.

2.2. Cell culture and treatment

HepG2 cells were purchased from the cell bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in RPMI-1640 medium supplemented with 10% FBS and 1% antibiotics at 37 °C in 5% CO_2 and 95% air for all cell culture experiments. When the cells