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A dynamic study on reversal of multidrug resistance by ginsenoside Rh_2 in adriamycin-resistant human breast cancer MCF-7 cells

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ABSTRACT

The quartz crystal microbalance (QCM) dynamic measurements indicate that ginsenoside Rh_2 (G-Rh_2) could inhibit the proliferation of adriamycin-resistant human breast cancer MCF-7 cells (MCF-7/ADR) in a concentration-dependent way. The combined treatment of G-Rh_2 with adriamycin (ADR) at non-effect dosage resulted in the higher inhibition efficiencies and the increased cell-death velocity, suggesting excellent ability of G-Rh_2 for reversal of multidrug resistance in MCF-7/ADR cells. The cytotoxic effect of the ADR–G-Rh_2 combination was evaluated with the modified Bürgi formula (Jin equation) based on the QCM responses. It presented apparent synergism, indicating the potential ability of G-Rh_2 in tumor therapy. Fluorescent microscopic inspection and methyl thiazolyl tetrazolium (MTT) assay were also carried out and exhibited the comparable results to QCM analysis. The present work may lay an experimental foundation for the application of ginsenosides in cancer therapy, especial in multidrug resistance research.

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1. Introduction

Multidrug resistance (MDR) means that tumor cells growing in the presence of a single anti-cancer drug may become resistant to a wide range of structural dissimilar drugs. This resistance to therapy is correlated to the presence of some molecular "pumps" in tumor-cell membranes that actively expel chemotherapy drugs from the interior [1]. The resistance development of tumor cells to chemotherapeutic drugs is a major obstacle in the treatment of human cancer [2]. It affects patients with a variety of blood cancers and solid tumors, including breast, ovarian, lung and gastrointestinal tract cancers. Design and development of drugs that can reverse multidrug resistance in tumor cells have been one of important targets in cancer researches. Doctors usually chose drug combination in actual therapies in order to intensify therapeutic effect, avoid MDR phenomenon and reduce drug toxicity. Many reports on synergistic effect induced by drug combination have been presented [3–5].

Ginseng has been employed as a costly invigorant for thousands of years in Asian countries including China, Korea and Japan. Ginsenosides are the major effective components of ginseng and contain a similar basic structure, composed of gonane steroid nucleus having 17 carbon atoms arranged in four rings. They can be divided into two main types, the 20(S)-protopanaxatriol and 20(S)-protopanaxadiol family, according to the existence of the hydroxyl group at C-6 or not. Ginsenosides have displayed a wide variety of biological activities including immunomodulatory effects, anti-inflammatory and anti-tumor activity [6–8]. Ginsenoside Rh_2 (G-Rh_2) is isolated from red ginseng [9] and belongs to protopanaxadiol type. It exhibits notably low toxicity and few side-effects over chemotherapeutic agents [10]. Some investigations have reported that G-Rh_2 not only inhibits cell-growth via induction of the cell differentiation, but also induces Gl phasearrest and/or S phase-prolongation in tumor cell cultures [11,12]. It can inhibit growth of human colorectal cancer HCT116 and SW480 cells [13], human breast cancer MCF-7 and MDA-MB-231 cells [14] as well as hepatoma SK-HEP-1 cells [15]. Furthermore, it can also induce apoptosis in such cell lines as rat C6 glioma cells [16], human neuroblastoma SK-N-BE(2)-C cells [17] and human malignant melanoma A375-S2 cells [18]. Researchers are pleasantly surprised to find that G-Rh_2 has remarkable synergy effects to some chemotherapeutic agents, even at a non-effect dosage [19,20]. The reason for this phenomenon is usually ascribed to the potential interaction between G-Rh_2 and efflux transporters such as P-glycoprotein [21] and breast cancer resistance protein [22]. These membrane transporter proteins are one of key factors leading to MDR of tumor cells. So it is expected that G-Rh_2 has influence on multidrug resistance in tumor cells.

Conventional optical methods including microscopy observation, methyl thiazolyl tetrazolium (MTT) colorimetry and flow cytomertry (FCM) in the cell investigation have some drawbacks, e.g., multistep operation and inapplicability for real-time or

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continuous monitoring besides their virtues including good reliability and accuracy. The quartz crystal microbalance (QCM) not only can provide information about mass loading but also can reveal the physico-chemical properties including density and viscosity near the electrode. As a powerful tool to monitor adsorption processes or reactions at solid/liquid interfaces in chemical and biological research, the QCM has been widely used for analyses of proteins, enzymes, antibody/antigen, nucleic acids and bacteria [23,24]. Generally, adherent cells are of tenfold μm-scale size in diameter, being much thicker than the characteristic extinction depth of the QCM shear wave ($\delta \approx 0.188$ μm for 9 MHz crystal in water [25]). The influences of resonant frequency on cell growth can be basically ignored. Due to its satisfactory performance, e.g., non-destruction measurement, high sensitivity, facile operation and dynamic monitoring, QCM has been successfully employed to monitor living cell attachment and incubation [26–29]. In our previous work, the antitumor effects of drugs and the synergistic cytotoxicity of the drug combination on hepatic cancer cells Bel7402 by QCM measurement were investigated, respectively [30]. It has proved that QCM is one of the most powerful tools available for drug toxicology research.

It can be imagined that the combination of chemotherapy drug with ginsenoside Rh_2 should exhibit different cytotoxicity to individual component and affect multidrug resistance in multidrug resistant tumor cells. To the best of our knowledge, to date there are no reports on application of QCM for dynamic study on the effect of G-Rh_2 on growth of multidrug resistant tumor cells. In this study, the antitumor effects of adriamycin, G-Rh_2 and their combination on adriamycin-resistant human breast cancer cells MCF-7 were investigated with the QCM measurement, fluorescent microscopic inspection and MTT assay, respectively. The cytotoxicity of the drug combination were also evaluated

2. Materials and methods

2.1. Chemicals and instruments

Adriamycin-resistant human breast cancer MCF-7 cells (MCF-7/ADR) were obtained from XiangYa Central Laboratory of Central South University, China and was routinely cultured using RPMI-1640 growth medium (from Gibco) supplementing with 10% newborn calf serum and 1 μmol L^{-1} ADR. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Amresco. A pH 7.4 phosphate buffer solution consisting of 136.7 mmol L^{-1} NaCl, 2.7 mmol L^{-1} KCl, 9.7 mmol L^{-1} Na_2HPO_4, and 1.5 mmol L^{-1} KH_2PO_4 was used in cell culture. Doxorubicin hydrochloride (adriamycin, ADR) was purchased from Zhejiang Hisun Pharmaceutical Co., Ltd. (China). 20(S)-Ginsenoside Rh_2 was obtained from National Institutes for Food and Drug Control (China), and was of biochemical reagent grade and at least 95% pure as determined by HPLC. It was dissolved in ethanol and further diluted with growth medium. Its stock solution (320 μmol L^{-1}) was stored at −20 °C. Other chemicals were of analytical reagent grade.

The QCM sensor consisted of a thin AT-cut quartz crystal wafer with one gold electrode (6-mm diameter) on each side. The 9-MHz crystal with Au electrodes was mounted between two biocompatible silicon O-rings to allow only one side of the electrode to be exposed to the liquid. The reaction chamber above the crystal was held with a 1 mL chlorinated polyethylene centrifugal tube. The device, being covered, was placed in a humidified CO_2 incubator controlled at 5% CO_2 and 37 °C to prevent evaporation from the culture holder. The quartz crystal electrodes were wire-extended to a research QCM (Maxtek Inc., USA) to achieve simultaneous recording of resonant frequency (f_0) and motional resistance (R_1). The cell-modality observation was performed with an inverted optical microscope (OLYMPUS CKX41, Japan). The pictures of stained cells were observed on an inverted fluorescence microscope (NIKON Eclipse Ti-S, Japan).

2.2. Cell culture and measurement procedures

After being sterilized with 75% ethanol under UV light for 0.5 h, the QCM culture-chamber was washed thrice with PBS. Then 800 μL of the growth medium was added and the entire culture-chamber was put into the incubator. As soon as the QCM readout became steady, 200 μL of the culture medium containing a controlled number of fleshly trypsinized cells was added evenly. In order to diminish changes in liquid density and viscosity caused by difference in temperature, the cell-suspending medium was also adjusted to 37 °C before its introduction. The Δf_0 and ΔR_1 responses were simultaneously monitored up to two days. The drug was introduced at 24 h when the cells were in their growth phases. The crystal regeneration was achieved by dealing with trypsin for 24 h, followed by washing with chromic–sulphuric acid and water in sequence for several times. After cleaning in this way, the QCM electrode could be used repeatedly with good recovery of its initial f_0 and R_1 values.

2.3. MTT assay

The metabolic activity of MCF-7/ADR was determined in triplicate using the MTT cell viability assay. Cells were seeded at a density of 5×10^4 cells $well^{-1}$ and incubated with drugs. After 24 h, cells were washed with PBS and then 0.2 mL growth medium and 50 μL MTT reagents (2 mg mL^{-1}) were added into each well. The cells were allowed to grow for another 4 h until a purple precipitate was visible. The medium was then removed and 150 μL dimethyl sulphoxide was added. The well was vibrated for 10 min to completely liberate the crystals. Finally, the absorbance was measured at 570 nm.

3. Results and discussion

3.1. The modality characterization of MCF-7/ADR cells

Fig. 1 shows the microscopic photos of MCF-7/ADR cells and MCF-7 cells in logarithmic growth phase. MCF-7/ADR cells adhered on the substrate were closely interconnected, presenting vague cellular outline. They owned the increased contact area and reduced height profile than normal MCF-7 cells that were regular polygon- or spindle-shaped. So nucleus and vesicles of MCF-7/ADR cells could be dimly observed. These information mean that the drug resistant cells have changed greatly in order to survive in the presence of ADR.

3.2. QCM dynamic monitoring of the effect of G-Rh_2 and ADR on MCF-7/ADR cells' growth

Fig. 2 shows typical responses of Δf_0 and ΔR_1 to 5×10^4 MCF-7/ADR cells' adhesion, spreading and proliferation on Au electrode and the effect of 1 μmol L^{-1} ADR on cell growth. For comparison, a control experiment was performed by adding 1 mL growth medium without cells. It can be observed that both Δf_0 and ΔR_1 did not change significantly in the following 48 h in the absence of cells. Before the cell addition, the QCM was initially equilibrated with the blank growth medium for 1 h, until stable baselines for Δf_0 and ΔR_1 were achieved. It can be observed that conspicuous decrease in frequency and increase in resistance were presented in 3 h following the cell introduction, corresponding to the adhesion phase of cells. Subsequently the inflexions appeared in QCM-signal curves and continuous frequency decrease and resistance increase were further found, revealing the gradual advent of the latent

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