Shikonin enhances Adriamycin antitumor effects by inhibiting efflux pumps in A549 cells

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Abstract. Shikonin (SHK) is a natural naphthoquinone pigment isolated from *Lithospermum erythrorhizon,* that has been reported to suppress the growth of a number of cancer cell types. Adriamycin (AD) is typically used as an effective anticancer agent; however, it has the propensity to induce drug resistance. The aim of the present study was to investigate the effects of SHK alone and in combination with AD on lung adenocarcinoma cells and the underlying molecular mechanisms of their effects. Colony formation, MTT and propidium iodide staining assays demonstrated that the co-treatment of A549 cells with SHK and AD significantly decreased cell viability and potently induced apoptosis. The mitochondrial membrane potential was assessed using 5,5', 6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolylcarbocyanine iodide staining and fluorescence microscopy. Cells co-treated with SHK and AD exhibited marked mitochondrial membrane damage. In addition, co-treatment with SHK and AD significantly reduced ATP levels in A549 cells compared with the control. Western blot analysis revealed that SHK enhanced the antitumor effects of AD by inhibiting the expression of ATP-binding cassette transporters. These results suggest that the inhibition of glycolysis could be an effective approach for lung cancer treatment. Therefore, SHK has the potential to be used as an anticancer agent in the treatment of lung adenocarcinoma, and thus warrants further investigation and development.

Introduction

Lung cancer is a major challenge to global public health due to its high epidemiologic incidence (1,2). It is estimated that ~1.8 million patients are diagnosed with lung cancer annually, 80-85% of whom are diagnosed with non-small cell lung cancer (NSCLC), including squamous cell carcinoma, adenocarcinoma and large cell carcinoma (3-5).

Although there have been advances in treatment strategies, the survival rate for lung cancer remains low (6) Surgery is the most effective option for patients with lung cancer; however, in the majority of patients, lung cancer is diagnosed at the advanced stage, and only 30% of patients are eligible for curative resection (7) Therefore, chemotherapy and radiotherapy are considered as alternative options for these patients. However, modern chemotherapy using various antitumor drugs either incompletely kills the malignant cells or causes fatal dysfunction and serious systemic toxicity (8). In addition, tumor drug resistance is a major problem associated with chemotherapy and involves numerous factors, including anticancer drug potency, tumor cell-drug reaction, the tumor microenvironment and tumor cell heterogeneity (9). Numerous apoptosis-inducing agents are also substrates and inducers of drug transporters, including P-glycoprotein (P-gp), multidrug resistance-associated protein 1 (MRP1) and breast cancer resistance protein (BCRP) (10,11). These drug transporters recognize numerous functionally and structurally independent anticancer drugs and, therefore, can expel intracellular drugs efficiently. The overexpression of these proteins can confer cancer cells with multidrug resistance (12,13). The development of strategies to overcome the side effects and drug resistance of antitumor drugs is an ongoing challenge for medical workers. As a broad-spectrum anticancer drug, Adriamycin (AD) is used in chemotherapy to treat a variety of tumor types. However, in addition to bone marrow suppression, myocardial injury and other side effects, drug resistance is an important factor that limits its use (14). Furthermore, the efficacy of AD is associated with drug concentration, which is also associated with drug resistance. The inhibition of tumor cell efflux can effectively increase the sensitivity of tumor cells to chemotherapeutic drugs (15,13). Therefore, agents with high efficacy and few side effects are urgently required in clinical practice.

A number of previous studies have aimed to extract and screen the active components of traditional Chinese herbs in order to develop effective and relatively safe drugs for cancer treatment (16,17). Shikonin (SHK) is the major active ingredient

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isolated from the dried roots of *Lithospermum erythrorhizon*, and possesses a molecular weight of 288 kDa.

The anticancer effects of SHK have been investigated in numerous prior studies (18-24). SHK is not toxic to normal cells (25), but exerts cytotoxic effects against various neoplastic cells, and has not been demonstrated to promote anticancer drug resistance (26). However, its effects against lung cancer remain unclear. The aim of the present study was to investigate the effects of SHK on lung adenocarcinoma cells and the underlying molecular mechanisms of these effects, which may provide an understanding of its novel antitumor functions.

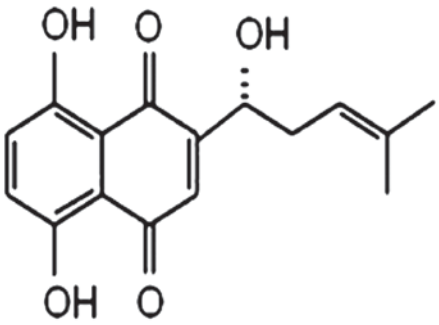

Figure 1. Chemical structure of shikonin.

Materials and methods

Reagents. SHK was purchased from Shanghai Shifeng Biological Technology Co., Ltd. (Shanghai, China). AD was purchased from Zhejiang Hisun Chemical Co., Ltd. (Taizhou, China). MTT was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). The JC-1 and propidium iodide (PI) assay kits were purchased from Beyotime Institute of Biotechnology (Haimen, China), and the ATP assay kit was obtained from Sigma-Aldrich (Merck KGaA). Monoclonal antibodies against MRP-1 (cat. no. sc-13960) and β-actin were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The anti-BCRP antibody (cat. no. MAB4155F) was obtained from EMD Millipore (Billerica, MA, USA) and the anti-P-gp antibody (cat. no. ab98322) was from Abcam (Cambridge, UK).

Cell lines and culture. The human A549 lung adenocarcinoma cancer cell line was obtained from the Central Laboratory of the Central South University (Changsha, China), and cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) without mycoplasma (Sigma-Aldrich; Merck KGaA), penicillin (100 μg/ml) and streptomycin (100 μg/ml; North China Pharmacy Co., Ltd., Shijiazhuang, China) at 37˚C in a humidified incubator with 5% CO_2. The culture solution was replaced every 2 days, and cell morphology and vitality were monitored using an inverted microscope (Olympus Corporation, Tokyo, Japan).

Cell viability assay. A549 cells were counted using a cell counting plate prior to being seeded into 96-well tissue culture plates at a density of $1x10^4$ cells/well, and cultured at 37˚C with 5% CO_2 overnight. Subsequently, the cells were exposed to various concentrations of SHK (0.8, 1.6, 3.2, 6.4, 12.8 or 25.6 μmol/l) or AD (0.375, 0.75, 1.5, 3, 6 or 12 mg/l) and then cultured for 24 and 48 h, respectively control cells were incubated with medium only. The cells were further incubated with PBS containing 5 mg/ml MTT for 4 h at 37˚C, then the MTT solution was removed and replaced with 150 μl dimethyl sulfoxide/well. Following this, the absorbance of the reaction solution was measured using a plate reader at 490 nm.

Colony formation assays. A549 cells were seeded in 6-well plates at a density of $4x10^3$ cells/well and treated with 1.6 μmol/l SHK, 0.75 mg/l AD or both (1.6 μmol/l SHK and 0.75 mg/l AD) for 24 h. Following drug treatment, the cells were inoculated onto the 6-hole culture plate. The number of cells was adjusted to 100 cells/hole. The culture plate was placed in saturated humidity at 37˚C and 5% CO_2 environment for 7 days. Following this, medium was removed and the plates were rinsed with PBS 2 times. Methanol was used to fix the plates for 15 min and air dried once the methanol was discarded. A total of 200 ml/pore 0.5% crystal violet stain was added, and after 20 min at room temperature, the plates were rinsed under water to remove the dye solution. The colonies were visualized under an inverted microscope (Olympus Corporation, Tokyo, Japan), violet and the cell number was counted in 10 fields of view.

PI staining. A549 cells were cultured in 6-well plates ($2x10^6$ cells/well) for 24 h and allowed to attain exponential growth. Then the cells were treated with 1.6 μmol/l SHK, 0.75 mg/l AD, or both for 24 h, stained with 600 μl PI/well for 2 h and then evaluated using flow cytometry (Accuri C6: US; BD Biosciences, Franklin Lakes, NJ, USA). SPSS 19.0 (IBM Corp., Arkmonk, NY, USA) was used to analyse the results of the results.

Intracellular ATP measurement. A549 cells were seeded at $2x10^5$ cells/well in a culture plate for 24 h. Cellular ATP levels were determined using the CellTiter-Glo® Luminescent Cell Viability Assay kit (Promega Corporation, Madison, WI, USA), according to the manufacturer's protocol. The luminescence levels were measured using the luminometer mode on a microplate reader.

Mitochondrial membrane potential. A549 cells were cultured in 12-well plates at a density of $2x10^5$ cells/well for 24 h and allowed to attain exponential growth prior to treatment. Changes in the mitochondrial membrane potential were evaluated using the JC-1 Mitochondrial Membrane Potential Fluorescence Probe kit (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) according to the manufacturer's protocol. The cells stained with JC-1 solution were visualized using an inverted fluorescence microscope (IX-71: Olympus Corporation).

Live cell imaging. *A549 cells were cultured* in 6-well plates ($2x10^5$ cells/well) for 24 h to allow attainment of exponential growth. Then, the cells were treated with 1.6 μmol/l SHK followed by 0.75 mg/l AD for 1 h each. The drug-induced fluorescence in the cells was visualized using a live cell imaging system (Olympus Corporation Live Cell Imaging Workstation IX83).

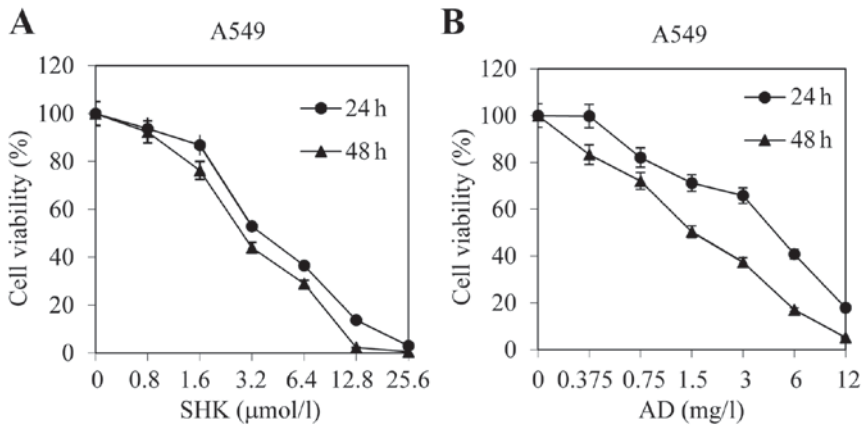

Figure 2. SHK and AD exhibit cytotoxic effects in A549 cells. A549 cells were treated with (A) SHK (0.8, 1.6, 3.2, 6.4,12.8 or 25.6 μmol/l) or (B) AD (0.375, 0.75, 1.5, 3, 6 or 12 mg/l) for 24 and 48 h, respectively. Cell viability was analyzed using an MTT assay. Data are presented as the mean ± standard error of the mean of 4 independent experiments. SHK, shikonin; AD, Adriamycin.

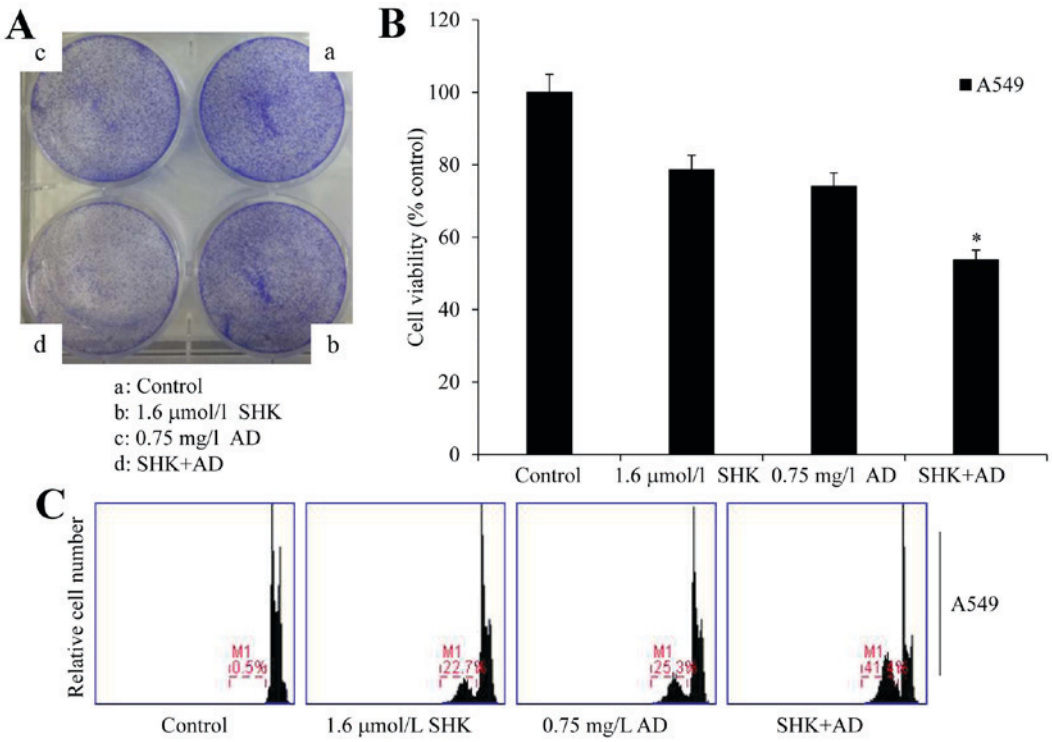

Figure 3. SHK sensitizes A549 cells to AD-induced cell growth inhibition and apoptosis. (A) A549 cells were treated with the control, SHK (1.6 μmol/l), AD (0.75 mg/l) or SHK (1.6 μmol/l) + AD (0.75 mg/l) for 7 days. Cell viability was determined using crystal violet staining. (B) A549 cells were treated with SHK (1.6 μmol/l), AD (0.75 mg/l) or SHK (1.6 μmol/l) + AD (0.75 mg/l) for 24 h, and cell viability was assessed using an MTT assay. Data are presented as the mean ± standard error of the mean of 4 independent experiments. *P<0.01 vs. the control. (C) A549 cells were treated with SHK (1.6 μmol/l), AD (0.75 mg/l) or SHK (1.6 μmol/l) + AD (0.75 mg/l) for 24 h, prior to being analyzed using flow cytometry. SHK, shikonin; AD, Adriamycin.

Western blot analysis. A549 cells were rinsed with ice-cold PBS and lysed with radioimmunoassay precipitation buffer (Shanghai Fankang Biotechnology Co., Ltd., Shanghai, China) for 30 min on ice. The cell lysates were centrifuged at 12,000 x g for 30 min at 4˚C. The supernatant proteins were separated using 6% SDS-PAGE and subsequently transferred to PVDF membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membranes were blocked with 5% skimmed milk + TBST for 1 h at room temperature, and then washed with TBST 3 times. Membranes were then incubated with primary antibodies overnight at 4˚C, followed by the horseradish peroxidase-labeled rabbit anti-mouse IgG (cat. no. PA128568; Invitrogen; Thermo Fisher Scientific, Inc.), while β-actin was used as the loading control. TBST was used to rinse the poly-vinylidene fluoride (PVDF) membranes 3 times for 10 min each time. The PVDF membranes were dipped in methanol for l0 min, and the membranes were allowed to develop in the dark. Following this, the membranes were analyzed using an BIO-RAD gel imaging system (Bio-Rad Technologies, Inc., Hercules, CA, USA). A gel band image analysis system was used to analyze the electrophoresis band density with Quantity One version 4.6.6 software (Bio-Rad Technologies, Inc.), and

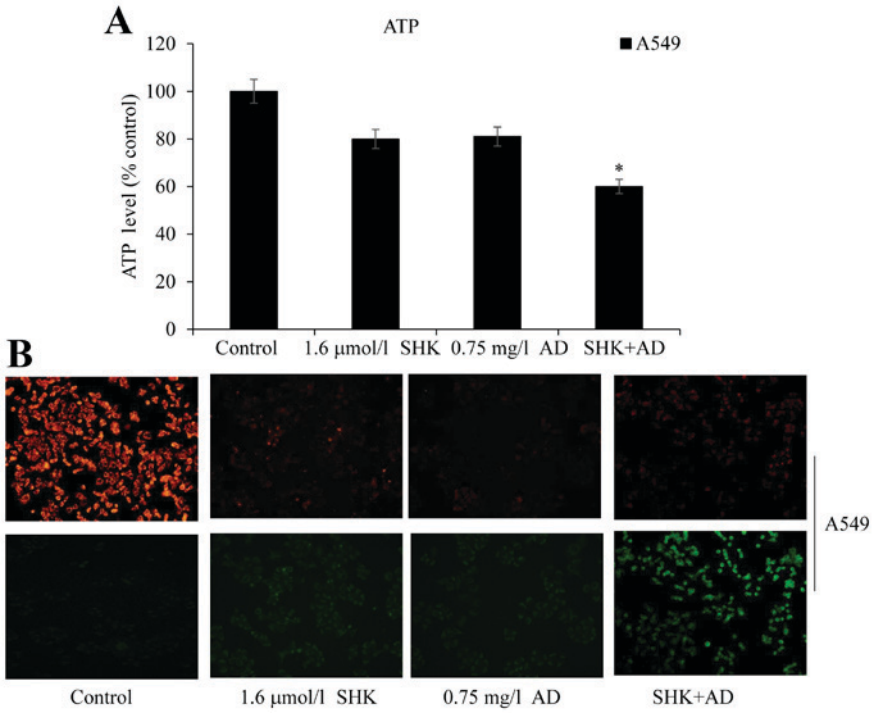

Figure 4. SHK disrupts the mitochondrial membrane potential and inhibits ATP generation in A549 cells. (A) Intracellular ATP levels were evaluated in A549 cells treated with SHK (1.6 μmol/l), AD (0.75 mg/l) or SHK (1.6 μmol/l) + AD (0.75 mg/l) for 5 h. Data are presented as the mean ± standard error of the mean of 3 independent experiments. *P<0.05 vs. the control. (B) A549 cells were treated with indicated concentrations of SHK (1.6 μmol/l), AD (0.75 mg/l) or SHK (1.6 μmol/l) + AD (0.75 mg/l) for 24 h, and the mitochondrial membrane potential was assessed using JC-1 staining and fluorescence microscopy (magnification, x200). When mitochondrial membrane potential is intact, cells fluoresce red. In contrast, when it is destroyed, cells fluoresce green. SHK, shikonin; AD, Adriamycin.

the ratio of target protein band density and internal reference band optical density was used as the relative expression for each group of proteins.

Statistical analysis. All statistical analyses were performed using an unpaired Student's t-test or an analysis of variance followed by the Student-Newman-Keuls test in SPSS 19.0 (IBM Corp., Armonk, NY, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

SHK and AD induce cytotoxicity in A549 cells. The chemical structure of SHK is illustrated in Fig. 1. To investigate the cytotoxicity of SHK and AD, A549 cells were treated with various concentrations of the agents for 24 and 48 h, respectively. The MTT assay results revealed that SHK and AD each significantly decreased the viability of A549 cells in a dose-dependent manner compared with the control group (Fig. 2A and B).

SHK sensitizes A549 cells to AD-induced cell growth inhibition and apoptosis. To determine whether SHK sensitized *A549* cells to chemotherapeutic agents, cells were co-treated with AD and SHK. Colony formation, MTT and PI staining assays demonstrated that SHK administered in combination with AD significantly decreased cell viability when compared with the control group and with the cells treated with SHK or AD alone (P<0.01), and also potently induced the apoptosis of A549 cells (Fig. 3A-C). The survival rate of the cells treated with SHK and AD was 53.77%, which was statistically different from that of the single treatment groups, at 78.68% for SHK and 74.07% for AD (P<0.01). These results suggest that SHK sensitizes A549 cells to AD-induced cell growth inhibition and apoptosis.

SHK damages the mitochondrial membrane potential and inhibits ATP generation in A549 cells. Mitochondrial membrane potential loss and the consequent production of reactive oxygen species (ROS) are the common landmark events of early apoptosis (27). ROS interact with mitochondrial antioxidants and induce apoptosis by releasing cytochrome *c* from the mitochondria. Furthermore, ROS inhibit the production of ATP, which in turn further increases apoptosis and ROS generation (28,27). Treatment of A549 cells with SHK or AD alone did not significantly decrease ATP levels compared with the control group; however, co-treatment with SHK and AD significantly decreased ATP levels in the A549 cells when compared with the control (P<0.05; Fig. 4A). To further assess changes in mitochondrial membrane potential, JC-1 was used as a fluorescent marker. When the mitochondrial membrane potential is intact the cells fluoresce red, whereas cell dysfunction induces a green fluorescence (29). SHK-treated and SHK/AD co-treated A549 cells exhibited clear mitochondrial membrane potential damage with green fluorescence following JC-1 staining; the co-treated cells exhibited a bright green fluorescence and the control cells exhibited a bright red fluorescence (Fig. 4B). Taken together, these results suggest that SHK adversely affects the mitochondrial membrane potential and decreases ATP generation in A549 cells.

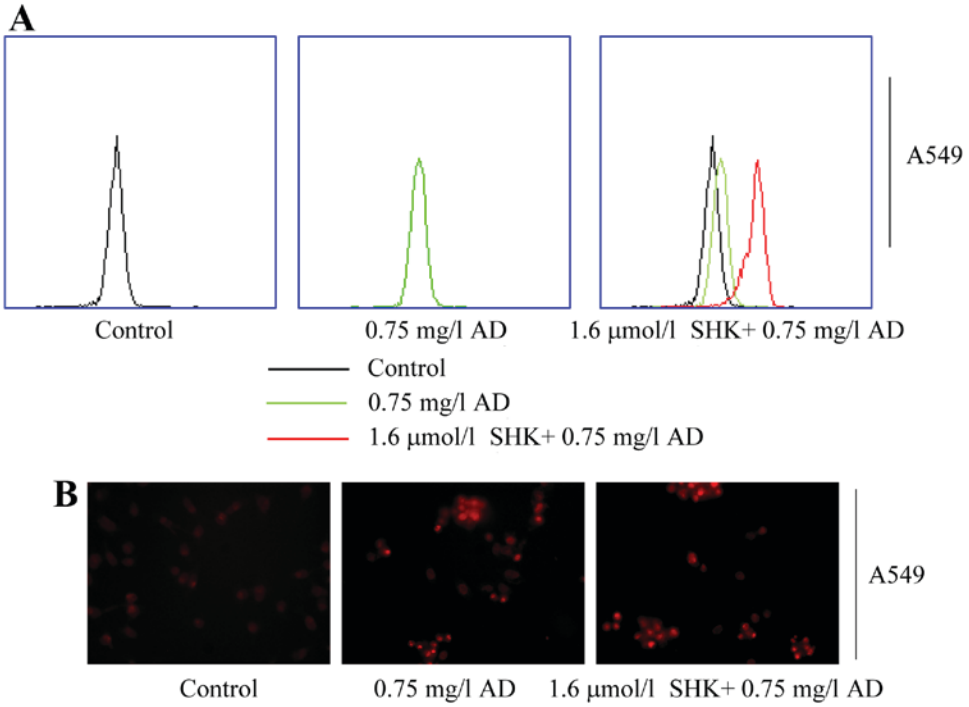

Figure 5. SHK enhances AD accumulation and reduces efflux from A549 cells. (A) A549 cells were cultured in the presence or absence of SHK (1.6 μmol/l); 30 min later, AD (0.75 mg/l) was added and the intracellular fluorescence levels were analyzed by flow cytometry following incubation for 30 min. Then the cells were further incubated in the presence or absence of SHK (1.6 μmol/l) for 1 h, and intracellular fluorescence levels were analyzed by flow cytometry. (B) A549 cells were treated with SHK (1.6 μmol/l), AD (0.75 mg/l) or SHK (1.6 μmol/l) + AD (0.75 mg/l) for 24 h, and cell fluorescence was detected using a live cell imaging system (magnification, x200). SHK, shikonin; AD, Adriamycin.

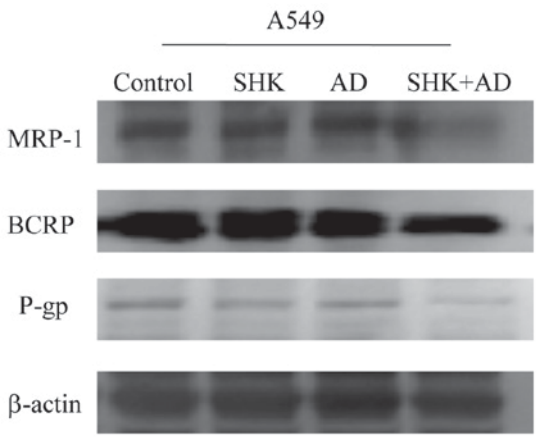

Figure 6. SHK enhances the antitumor effects of AD by inhibiting ABC transporter expression. A549 cells were treated with the indicated concentrations of SHK (1.6 μmol/l), AD (0.75 mg/l) or SHK (1.6 μmol/l) + AD (0.75 mg/l) for 24 h. At the end of each treatment, total cell lysates were subjected to western blot analysis. The expression of MRP-1, BCRP and P-gp was monitored. SHK, shikonin; AD, Adriamycin; MRP-1, multidrug resistance-associated protein 1; BCRP, breast cancer resistance protein; P-gp, P-glycoprotein.

SHK enhances AD accumulation and reduces efflux in A549 cells. The anticancer activity of SHK, as well as inhibition of the cellular efflux of AD, requires depletion of the cellular ATP pool as the ATP-binding cassette (ABC) transporters are ATP-dependent (30). To assess whether SHK enhanced AD accumulation through reduced ATP production, the effects of glycolysis inhibition on AD accumulation and efflux were evaluated in A549 cells. Flow cytometry analysis revealed that SHK markedly increased intracellular AD levels in A549 cells (Fig. 5A); a similar result was obtained using a live imaging system (Fig. 5B). These results collectively suggest that SHK enhances AD accumulation and reduces efflux partly by reducing ATP levels in A549 cells.

SHK enhances the antitumor effects of AD by inhibiting ABC transporter expression. To confirm the effect of ATP reduction in A549 cells, the expression of MRP1, BCRP and P-gp was analyzed in A549 cells. Co-treatment with SHK and AD resulted in a marked decrease in MRP1, BCRP and P-gp expression when compared with the control and either treatment administered alone (Fig. 6).

Discussion

Multi-drug resistance (MDR) constitutes a unique and critical spectrum of drug resistance (21,31) with serious therapeutic consequences. MDR development is typically associated with the development of a series of structurally-associated compounds for cancer treatment, leading to the development of structural and functional cross-resistance (31). Despite the continuous introduction of novel chemotherapeutic agents, overcoming MDR remains a challenge in cancer chemotherapy (32,33). Targeting cancer cell metabolism for cancer prevention and therapy is an emerging topic of research; compared with the majority of normal differentiated cells, cancer cells possess distinct metabolic requirements, including the production of energy primarily via glycolysis even in the presence of oxygen (34,35). This phenomenon is known as the 'Warburg effect' and has received increasing

attention since 2011 (36). Tumor cell metabolism results in the glycolysis-dependent production of cellular ATP due to mitochondrial dysfunction, hypoxia, tumor cell signal transduction or metabolic enzyme expression (37).

ABC transporters mediate cytotoxic drug active efflux and, following repeated chemotherapy cycles, cancer cells can develop MDR (38). Tumor cells proliferate rapidly and require numerous proteins, nucleic acids, lipids and ATP for their survival (39). Cancer cells are more dependent on the glycolytic pathway for ATP generation, compared with normal cells, and they eventually acquire drug resistance, typically due to the aberrant expression of drug-expelling ABC transporters (12). The ATP-dependence of drug transporters for activity (40,41) suggests that glycolysis inhibition may increase the concentration of chemotherapeutic agents in cancer cells (42,43). The most widely studied transporters, including MRP1, BCRP and P-gp, are able to transport a variety of structurally-unassociated chemotherapeutic compounds from cancer cells, thereby inducing MDR (44).

Previous studies have suggested that certain chemicals isolated from Chinese medicinal herbs may exhibit antitumor activity by inducing the apoptosis of cancer cells (16,17,45). Results from the present study demonstrated that SHK exerts antitumor effects by decreasing cell viability, inducing cell apoptosis and inhibiting ATP generation in A549 cells. The half-maximal inhibitory concentration of SHK in A549 cells, as determined using an MTT assay, has previously been determined to be 3.52±0.17 μg/ml (46). To confirm the effect of ATP reduction in A549 cells, the expression of MRP1, P-gp and BCRP was analyzed in the present study following combination treatment with SHK and AD. The results indicated that combination treatment with SHK and AD markedly decreased the expression of MRP1, P-gp and BCRP. Furthermore, SHK efficiently enhanced the cytotoxicity of AD against A549 cells by decreasing the levels of ATP, potentially leading to a decrease in activity of the ATP-dependent efflux pumps. The data from the present study suggest that SHK enhances the antitumor effect of AD through inhibiting ATP generation in A549 cells. These results indicate that the inhibition of glycolysis may be an effective therapeutic approach for the treatment of lung cancer.

In conclusion, the results of the present study collectively suggest that SHK may be a novel and attractive therapeutic candidate for tumor treatment in clinical practice. However, further studies are required in order to identify the precise molecular mechanisms underlying the effects of SHK in A549 cells.

Acknowledgements

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