Cytotoxic and Antitumor Effects of Curzerene from *Curcuma longa*

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**Introduction**
Lung cancer is one of the most threatening malignant tumors to human, which has the highest morbidity and fastest growth rate [1]. It has recently risen to the top in the high incidence rate ranking of malignant tumors all over the world [2]. According to the World Health Organization, lung cancer is responsible for more than a quarter of the total number of cancer deaths. China is facing a more severe situation as it has a high lung cancer incidence rate and mortality. According to the “2012 China Cancer Annals Report” [3], the incidence of lung cancer is 57.63/10 million and the mortality of it is 48.87/10 million.

Chemotherapy is still the main method to treat lung cancer, but it also unavoidably produces critical toxicity and side effects when destroying tumor cells. In recent years, domestic and foreign scholars have been committed to the research of the active constituents of traditional Chinese herbal medicines that have anti-lung cancer efficacy [4] and little toxicity and side effects. The dried rhizomes of *Curcuma longa* (Zingiberaceae) are herbal remedies commonly used in oriental medicines such as Ayurveda and traditional Chinese medicine [5]. Curcumin, one of the key active components from Curcuma rhizomes, attracted great interest for its therapeutical potential in ophthalmology [6] in the past 20 years. Meanwhile, some monomer compounds from the Curcuma rhizomes have been used in clinical cancer treatment, such as β-elemene, which has been listed as the national second class of noncytotoxic anticancer drugs [7] and curcumol, which has been proven to be effective for anti-liver cancer [8]. Curzerene, a sesqui-
_quiterpene, is another active component from Curcuma rhizomes, with a formula of C_{15}H_{20}O (Fig. 1) and a relative molecular weight of 216.32. Curzerene has been reported to have an anti-inflammatory effect and certain anticancer effects [9, 10]. However, the anticancer effects of Curzerene and its mechanisms have been underexplored.

The glutathione S-transferases (GSTs) are a multigene family of drug detoxification enzymes that play an important role in phase II metabolism by catalyzing the conjugation of glutathione to a variety of electrophilic substances [11, 12]. GST isoenzymes are known to modulate cell-signaling pathways, controlling cell proliferation and apoptotic cell death [13]. GSTs can metabolize anticancer drugs, and the abnormal expression of GSTs isoenzyme is related to the drug resistance of cancer cells. Using Phage display cDNA library technology, our previous work indicated that glutathione S-transferase A1 (GSTA1) was one of the tumor antigens of lung cancer [14, 15]. The expression of GSTA1 in the lung adenocarcinoma cells line was much higher than that in control group MRC-5 cells line, meanwhile, the overexpression of GSTA1 could promote the aggressive and metastasis of cancer [15].

This study examined the antineoplastic efficacy and mechanism of curzerene in a lung cancer cell line and tumor-bearing mice model. To our knowledge, there is no literature report on the antineoplastic efficacy of curzerene in a lung cancer model as well as no literature report on the relationship between GSTA1 and curzerene. Findings from this study will be helpful to evaluate possible applications of curzerene as a chemotherapeutic agent.

Results

The potential cytotoxicity of curzerene on human lung cancer SPC-A1 cells was detected by using the MTT assay. The cell viability was determined after SPC-A1 was treated with various concentrations (0, 6.25, 12.5, 25, 50, 100 μM) of curzerene for 24, 48, and 72 h. As shown in Fig. 2, cell inhibition increased in a dose- and time-dependent manner. The half-maximal inhibitory concentration (IC_{50}) for curzerene to SPC-A1 cells at 24, 48, and 72 h was 403.8 μM, 154.8 μM, and 47.01 μM, respectively. The inhibition rate of curzerene (25 μM) was similar to the positive control (100 μM of β-elemene). Compared with the blank control group, the difference is statistically significant (p < 0.05).

A fluorescent inverted microscope was used to detect whether curzerene would affect the morphology of the SPC-A1 cells. As shown in Fig. 3, the nuclei of SPC-A1 cells in the control group (0 μM curzerene) emitted homogenous blue fluorescence (Fig. 3a), which could be explained by an even distribution of the chromatin in the nuclei, while the cells treated with curzerene emitted bright fluorescence due to nuclear condensation (Fig. 3c–g). It was shown that the density of cells treated with higher levels of curzerene, i.e., 25, 50, or 100 μM, significantly decreased. The cell morphology of the positive control (100 μM of β-elemene) (Fig. 3b) was similar to that of curzerene (25 μM; Fig. 3e).

In order to further investigate whether the loss of cell viability induced by curzerene was associated with apoptosis, annexin V-FITC/PI double staining was performed with the cells followed by flow cytometry analysis. As shown in Fig. 4, cells treated with various concentrations (0, 6.25, 12.5, 25, 50, 100 μM) of curzerene for 48 h exhibited a higher percentage of apoptotic and necrotic cells than that of the control group (Fig. 4A). As shown in Fig. 4B, curzerene induced apoptosis of the cells in a dose-dependent manner. The results were consistent with experimental results determined by MTT. It means that a large dose of cur-
Curzerene can obviously kill SPC-A1 cells, while a small dose of curzerene can induce SPC-A1 cells to apoptosis (▶Fig. 4).

In order to evaluate if the antiproliferative activity of curzerene was related to cell cycle arrest, SPC-A1 cells treated with different concentrations of curzerene for 48 h were analyzed using flow cytometry. As shown in ▶Fig. 5A, curzerene induced cell cycle to arrest at the G2/M phase in SPC-A1 cells in a dose-dependent manner. The percentage of cells arrested in the G2/M phase increased from 9.26% in the control group cells to 17.57% in the cells treated with the highest dose (100 µM) of curzerene. These results suggested that curzerene induced cell apoptosis and G2/M cell cycle arrest of SPC-A1 cells, which may be an expression of its cytotoxicity.

To examine whether curzerene affects the expression of GSTA1, real-time PCR and Western blotting were used to quantitatively analyze the GSTA1 mRNA and protein levels in each group. It was shown that both GSTA1 mRNA and protein expression levels of the cells treated with various concentrations (100, 25, 6.25 µM) of curzerene for 48 h were significantly lower than those of the blank control group (p < 0.05; ▶Fig. 6).

BALB/c nude mice were used as an in vivo model to assess the antineoplastic efficacy of curzerene (▶Fig. 7). During the period of delivery, no death occurred in the experimental group of animals. All tumor-bearing mice suffered from weight gain (▶Fig. 7a). The liver organ coefficients of curzerene in 45 mg/kg−1 and 15 mg/kg−1 were higher than the model control group, but other organ coefficients were not significantly different when compared with the model control group (▶Fig. 7b). The tumor grow curve results showed that tumors in the model control group grew faster than those in the treatment group of curzerene or β-elemene (▶Fig. 7c). After 12 days of continuous intraperitoneal administration, tumor weights and volume were measured and the inhibition rates were calculated (▶Fig. 7d). The inhibition rates for tumor growth in the high-, medium-, and low-dose groups of curzerene were 58.94%, 32.58%, and 19.71%, respectively. The results strongly suggest that curzerene-mediated inhibition of tumor growth in SPC-A1 cell-bearing mice is closely correlated with the enhanced apoptosis in tumor cells.
Discussion

Curzerene is a sesquiterpene originally isolated from the traditional Chinese herbal medicine *C. longa*. It was reported for the first time that curzerene significantly inhibited the secretion of TNF-α inflammation factors from the THP-1 cells, which indicated that curzerene may have potential application values in the treatment of inflammatory diseases [16]. It was also reported that curzerene, within the concentration range of 5–30 µM/L, inhibited the release of nitric oxide by macrophages after being activated by lipopolysaccharide [17]. Although there are some reports on the pharmacological effects of curzerene, there are few studies of curzerene on anti-non-small cell lung cancer (NSCLC). Therefore, it is of great interest to identify the anti-non-small cell lung cancer effects and mechanism of curzerene.

GSTA1 plays an important role in the detoxification of genotoxic substances as well as in the biotransformation of xenobiotics; it arises from normal constituents of living organisms [18]. GSTA1 can metabolize anticancer drugs. Under the catalysis of GSTA1, GSH combines with chemical drugs, thus reducing the cytotoxic effect of chemical drugs [19]. In this study, we detected the GSTA1 protein levels and mRNA levels of the curzerene groups, which showed that both levels in the curzerene groups are much lower than those of the control group (p < 0.05), suggesting that the expression of GSTA1 is inhibited by curzerene.

In the study, the cell cycle of SPC-A1 cell distribution changed after treatment with curzerene, with an increase of the G2/M phase and a decrease of the G0/G1 phase and S phase cells proportion. The results indicate that curzerene blocked the SPC-A1 cells in the G2/M phase, preventing the damaged DNA from copying into the G0/G1 phase and thus leading to the death of hyperplastic cells.
The in vivo study of curzerene on tumor-bearing nude mice showed that curzerene significantly inhibited the growth of transplanted tumors on nude mice in a dose- and time-dependent manner. The body weight data of nude mice treated with curzerene demonstrated that the mice in both curzerene groups and the model group grow normally, which may indicate that curzerene has limited toxicity and side effects in vivo. The positive drug β-elemene is already listed as an antitumor medicine. However, β-elemene showed a lower antitumor function both in vivo and in vitro when compared with curzerene. This may remind us that curzerene is a potential potent anti-lung adenocarcinoma drug and we will research the toxicity of curzerene in future experiments.

As curzerene is a potent anticancer reagent, and also a high concentration component of Curcuma rhizomes, which are commonly used as spices in cooking, the toxicity of curzerene would be limited. Therefore, it is of great interest tests that curzerene is combined with other standard chemotherapeutic agents in the treatment of NSCLC. Curzerene plus carboplatin/cisplatin for the treatment of NSCLC would be a preliminary attempt in our future study.

Materials and Methods

Cell lines and animals
The cell line (human lung cancer cell SPC-A1) used in the study was obtained from Shanghai Institute of Biochemistry and Cell. For each experiment, cells were maintained in RPMI 1640 medium supplemented with 10% FBS. BALB/c-nude mice (age 4 weeks, weight 18–22 g), half male and half female, were purchased from Guangdong Laboratory Animal Center [Experimental Animals Certificate: SCXK (Yue)2013–0002]. The animal handling protocol was reviewed and approved by the Institutional Animal Care and Utilization Committee (IACUL) of the Experimental Animal Center of Guangdong Pharmaceutical University (approval no. SYXX (Yue)2012–0125, approval date June 3, 2014).

Reagents and instruments
Purified curzerene (>98% by HPLC) was obtained from Dshare Pharmaceutical Science & Technology Co., Ltd. Purified β-elemene (>96% by HPLC) was obtained from Shanghai GaoLang Chemical Technology Co., Ltd. RPMI and FBS were purchased from Gibco. MTT was purchased from Genview. Hoechst 33258 kit, cell cycle detection kit, and Annexin V/PI kit were purchased from Kaiji Co. Ltd. RNAiso Plus, PrimeScript™ II 1st Strand cDNA Synthesis Kit, and SYBR® Premix Ex Taq™ II were purchased from Takara Dalian. GSTA1 and GAPDH primers were purchased from Sangon Biotech. Primary antibody anti-GSTA1 (mouse anti-human IgG), anti-β-actin (Mouse anti human IgG), secondary antibodies, and goat anti-mouse IgG H&L (HRP) were from Abcam. The plate reader was from Bio-Rad Laboratories and the flow cytometer was from Beckman Coulter, Inc. The fluorescence microscope was from Leica.

Cell culture and MTT assay
The antiproliferative activity of curzerene on SPC-A1 cells was determined by the MTT assay. Cells were seeded in a 96-well plate. After 24 h of attachment, the cells were treated with various concentrations (0, 6.25, 12.5, 25, 50, 100 µM) of curzerene and the
positive control (100 µM of β-elemene) once for 24, 48, and 72 h, respectively. Curzerene was dissolved in ethyl alcohol absolute (EtOH) and the drug treatment including 0.1% EtOH. After incubation, MTT solution was added to each well. After 4 h of incubation at 37 °C in 5% CO2, 200 µL DMSO were added after abandoning the supernatant. The absorbance at 570 nm was measured with a microplate reader for the cell viability rate. Cell proliferation was calculated, and the experiment was repeated three times.

**Apoptosis assay by Hoechst 33258**

The SPC-A1 cells seeded in a 24-well plate were treated with curzerene (0, 6.25, 12.5, 25, 50, 100 µM) and the positive control (100 µM of β-elemene) for 48 h. After incubation, the supernatant was discarded, and cells were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature, washed three times with PBS, exposed to H33258 at 15 µM for 30 min at room temperature, and washed three times with PBS. Then the plate was dried at room temperature, and visualized using a fluorescence microscope.

**Determination of cell apoptosis by Annexin V/PI double staining**

Apoptosis was assessed by using the PI/Annexin V double staining method. Logarithmic growth phase of the SPC-A1 cells were seeded in 6-well plates at a density of 1 × 10^5 cells/mL. After cells were treated with curzerene (0, 6.25, 12.5, 25, 50, 100 µM) and the positive control (100 µM of β-elemene) for 48 h, cells were collected and centrifuged (2000 r/min, 5 min), the supernatant was discarded, 500 µL of binding buffer were added to the suspended cells, and then 5 µL Annexin V-FITC and 5 µL PI were added and mixed at room temperature away from light for 5 min. The apoptosis rate was detected in a flow cytometer.

**Determination of cell cycle by PI staining**

Cell cycle distribution was analyzed using flow cytometry. SPC-A1 cells were seeded in 6-well plates at a density of 1 × 10^5 cells/mL. After cells were treated with various concentrations (0, 6.25, 12.5, 25, 50, 100 µM) of curzerene and the positive control (100 µM of β-elemene) for 48 h, the cells were harvested, washed twice with PBS, and cells were fixed with 70% ethanol at 4°C for 1 h and centrifuged. The pellet was treated with RNase (20 µg/mL) at room temperature for 30 min and then incubated with PI (50 µg/mL) for 30 min. The ModFit 3.1 program was used to determine the percentage of cells stalled at each phase of the cell cycle, namely, the G0/G1 phase, S-phase, and G2/M phase.

**RT-PCR analysis of GSTA1 mRNA levels**

The SPC-A1 cells were seeded in 6-well plates with a density of 1 × 10^5 cells/mL. The six wells were processed in the following six different ways, respectively: the control group (serum free medium), the solvent control group (0.1% EtOH in serum), the positive control group (100 µM of β-elemene), the high-dose group (100 µM of curzerene), the medium-dose group (25 µM of curzerene), and the low-dose group (6.25 µM of curzerene). Cells were collected after treatment for 48 h. Total RNA of the cells was isolated with RNAiso Plus following the manufacturer’s protocol. First-strand cDNA was synthesized with a PrimeScript™ 1st Strand cDNA Synthesis Kit according to the manufacturer’s instructions, using 1 µg of total RNA. Primers for GSTA1 were de-
signed as follows: Forward primer, 5’-GCCTCCATGACTGGTATT-3’; Reverse primer, 5’-CCTGGAAGATGTGGATGGGAT-3’. Real-time PCR was performed following the manufacturer’s protocol of SYBR® Premix Ex Taq™ II. Gene expression levels were normalized to those of GAPDH.

**Protein extraction and Western blot**

The cultivation and the groups of SPC-A1 cells were the same as the RT-PCR experiment. Cells were collected after treatment for 48 h and used to extract protein. Thirty µg of protein were separated by 12% SDS-PAGE and transferred to polyvinylidene-difluoride membranes. Non-specific binding sites were blocked by incubating in TBS Tween-20 buffer containing 5% milk for 2 h at room temperature, and then incubated with GSTA1 primary antibodies overnight at 4 °C. After three washes in TBST, the membranes were incubated with GSTA1 secondary antibody for 1 h at 37 °C. The intensity of the pooled sample bands was determined by densitometric analysis using Image J software.

**In vivo antitumor efficacy study**

All the mice were injected in the right flank subcutaneously with SPC-A1 cells (2 × 10⁶ cells in 100 µL per site) at right forelimb arm-
pit. When the tumors grew to approximately 4–5 mm in diameter, the tumor-bearing mice were randomly allocated to five groups. Curzerene was dissolved in ETOH and diluted in a saline injection to its final concentration containing 1% ETOH, and then it was injected intraperitoneally (i.p.). All drugs were i.p. injected at 0.1 mL/10 g, once every day for 12 consecutive days. Body weights of the mice were measured every day and the tumor volumes were calculated according to the following formula: 0.5 × length × width × width. Mice were sacrificed with adequate anesthesia. Their organs weights were measured, including the heart, liver, spleen, lung, kidney, and tumor.

**Statistical analyses**

All experimental data are presented as the mean ± standard error of the mean (SEM). The value of 50% inhibitory concentration (IC₅₀) was calculated with GraphPad Prism 5.0. Data from two groups were analyzed by two-way ANOVA. P < 0.05 was considered to indicate a statistically significant difference.

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**Conflict of Interest**

The authors declare no conflict of interest.

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**References**


