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ORIGINAL ARTICLE

Anticancer effects of curzerenone against drug-resistant human lung carcinoma cells are mediated via programmed cell death, loss of mitochondrial membrane potential, ROS, and blocking the ERK/MAPK and NF-κB signaling pathway

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Summary

Purpose: *The main objective of the current study was to examine the anticancer effects of Curzerenone - a naturally occurring sesquiterpene against gemcitabine-resistant lung carcinoma cells. The effects of Curzerenone on mitochondrial-mediated apoptosis, ROS, and ERK/MAPK and NF-kB signalling pathways were also investigated in the present study.*

Methods: *Cell proliferation was evaluated by MTT assay. Apoptosis was detected by acridine orange (AO)/ethidium bromide (EB) and DAPI staining as well as flow cytometry using annexin V apoptosis assay. The effects on reactive oxygen species (ROS) as well as mitochondrial membrane potential (MMP) were examined by flow cytometry. Protein expression was examined by western blotting.*

Results: *It was found that Curzerenone induced potent antiproliferative effects against the gemcitabine-resistant lung cancer cells and exhibited an IC_{50} of 24 μM. The anticancer effects of curzerenone were due to the induction of apoptosis which was also associated with alteration of apoptosis-related proteins (Bax,Bcl-2). Curzerenone also caused ROS-mediated alterations in the MMP. Curzerenone induced cell death in gemcitabine-resistant lung cancer cells by activating p38 MAPK/ERK signalling pathway while NF-kB pathway was inhibited in a dose-dependent manner.*

Conclusions: *In conclusion, the current results strongly indicate that Curzerenone may prove a potential anticancer drug candidate against drug-resistant lung cancer.*

Key words: *curzerenone, apoptosis, ROS, lung cancer, Bax, Bcl-2*

Introduction

Plants have provided a number of pharmaceutical agents for the alleviation of human diseases [1]. Anticancer drugs such as etoposide, vincritine and others have been employed for the treatment of cancer [2]. Plants have a sophisticated mechanism to synthesize chemical entities, such as terpenes, to suppress the growth of other plants or pathogenic microbes [3]. These metabolites have also been screened by researchers to develop drugs for the treatment of diseases [4]. Terpenes are naturally produced by plants and other organisms and have been reported to exhibit a wide spectrum of

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bioactivities such as anticancer and antifungal to name a few [5]. Curzerenone is an important sesquiterpene and has been isolated from a number of plants species [6]. However, Curzerenone has not been screened for anticancer activity. Herein, we evaluated for the first time the anticancer effects of Curzerenone against human drug-resistant lung cancer cells. Lung cancer causes significant morbidity and mortality across the globe. It has been reported that more than one and a half million people died of lung cancer across the world in 2012 [7]. The incidence of lung cancer is increasing at an alarming rate and it has been reported that the deaths in lung cancer patients were 3 million in 2003 [8]. The lack of potent chemotherapy and therapeutic targets and late diagnosis are the major obstacles in the treatment of lung cancer [9].

The purpose of this study was to investigate the anticancer effects of curzerenone against gemcitabine-resistant lung carcinoma H69AR cell line and also its effects on apoptosis, mitochondrial membrane potential (MMP), reactive oxygen species (ROS) generation, ERK/MAPK and NG-kB signalling pathway.

Methods

Cell viability assay

The cell lines H69AR and MRC5, which were procured from Chinese Academy of Sciences, Shanghai, China (Dept of Biochemistry and Biology) were kept in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS) at 37°C in a 5% CO_2 incubator. Briefly, at around 70% confluence the H69AR (a drug-resistant variant of lung cancer cell line NCI-H69) and non-cancer MRC5 cells were seeded in 96-well plates and treated with 0-100 µM of Curzerenone. After a 24-h incubation, the cells were incubated with MTT for another 4 h. After this, DMEM was removed and the colored formazan product was solubilized by 200 µl of dimethyl sulfoxide. The viability of the H69AR and the MRC5 cells was then determined by taking absorbance at 570 nm.

Determination of ROS and MMP

The ROS and MMP levels were estimated by culturing of the H69AR cells for 24 h at 37°C and subsequently treating them with varied doses of Curzerenone (0, 4.5, 9 and 18 µM) for 24 h. Next, DMEM was removed and the cells were treated with 5 µM DCH-Da (2', 7'-dichlorodihydrofluorescein diacetate) for estimation of ROS or rhodamine 123 (Rh123) for estimation of MMP by flow cytometry.

DAPI (4',6-diamidino-2-phenylindole) and AO/EB staining

The H69AR cells were grown in 6-well plates (0.6×10^6 cells/well) and incubated for 12 h. The H69AR cells were subjected to Curzerenone treatment (0, 12, 24 and 48 µM) for 24 h at 37°C. As the cells cast off, 25 µl cell cultures were put onto a glass slide and stained with DAPI or a solution of AO/EB. The slides were then coverslipped and examined with a fluorescent microscope. The annexin V/propidium iodide (PI) staining was performed as described previously [12].

Western blotting

The H69AR cells were harvested and subjected to washing with ice-cold phosphate buffered saline (PBS). The pellet was then suspended in a lysis buffer at 4°C and then shifted to 95°C. Thereafter, the protein content of each cell extract was checked by Bradford assay. About, 40 µg of protein was loaded from each sample and separated by SDS-PAGE before being shifted to polyvinylidene fluoride membrane. The membranes were then subjected to treatment with tris buffered saline (TBS) and exposed to primary antibodies (anti-PNF-KB, phospho-ERK, ERK) at 4°C. Then, the cells were treated with appropriate secondary antibodies and the proteins of interest were visualised by enhanced chemiluminescence reagent.

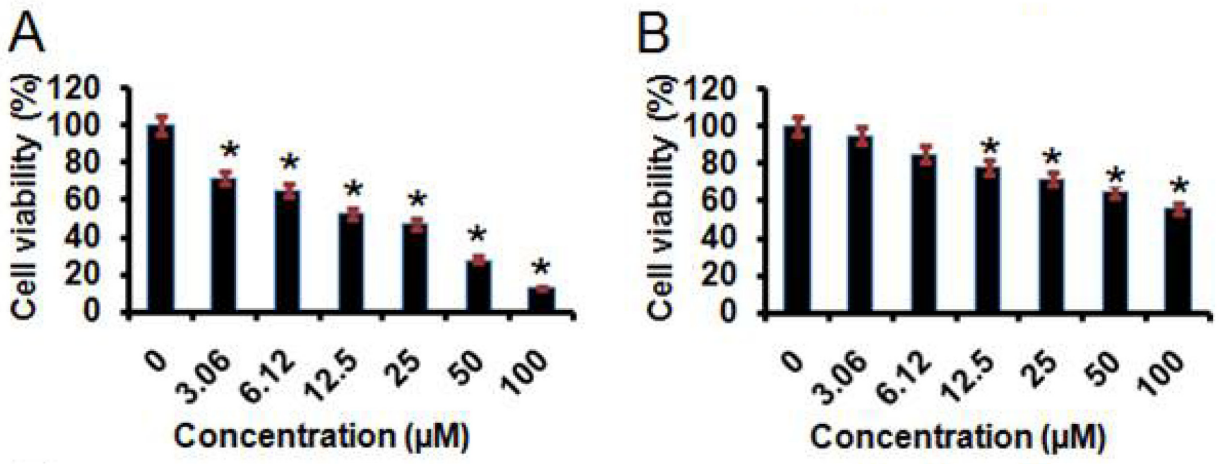

Figure 1. MTT assay showing the effect of Curzerenone on the viability of **(A)** lung cancer H69AR and **(B)** normal MRC5 cells. The experiments were performed in triplicate and shown as mean ± SD (*p<0.05).

Statistics

The experiments were performed in triplicate and the data are shown as mean ± SD. Statistical analysis was done using Students t-test with GraphPad prism 7 software. Values of $p<0.05$ were taken as indicative of significant difference.

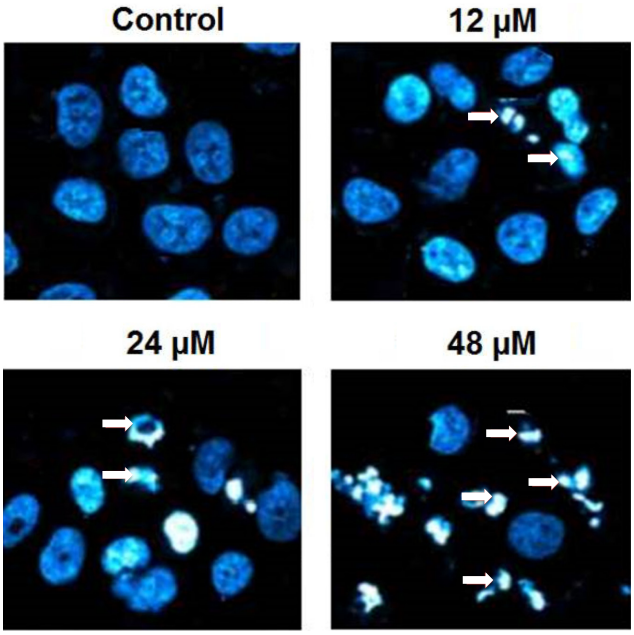

Figure 2. Induction of apoptosis in the H69AR cells by Curzerenone treatment as evidenced from the DAPI staining. Arrows show the apoptotic cells, which increased with increasing doses of the compound. The experiments were performed in triplicate.

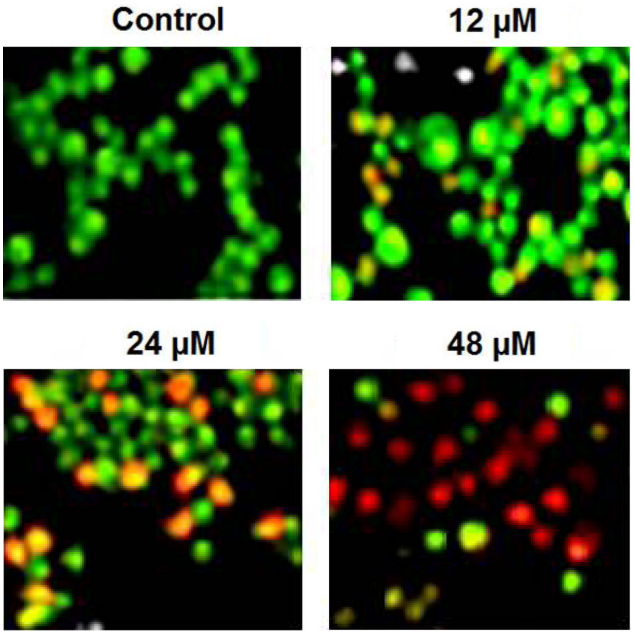

Figure 3. Induction of apoptosis in the H69ARcells by Curzerenone treatment as evidenced from the AO/EB staining. Yellow and red cells are dead apoptotic cells, which increased in a dose-dependent manner. The experiments were performed in triplicate.

Results

Curzerenone inhibits the proliferation of H69AR lung cancer cells

The growth inhibitory effects of Curzerenone were examined on the H69AR lung cancer cells and the normal MRC5 cells by MTT assay at concentration ranging from 0 to 320 μM. Curzerenone was found to halt the growth of the H69AR cells concentration-dependently (Figure 1A). The IC_{50} of Curzerenone against the H69AR cells was 9 μM. On the other hand, the effects of Curzerenone on the proliferation of the MRC5 cells were negligible. The IC_{50} of Curzerenone against the normal MRC5 cells was 80 (Figure 1B).

Curzerenone induces apoptotic cell death of H69AR cells

The apoptosis in the Curzerenone-treated H69AR cells was determined by DAPI and AO/EB staining. The DAPI staining revealed that Curzerenone triggered apoptosis as evidenced from nuclear fragmentation of the Curzerenone-treated H69AR cells (Figure 2). Moreover, the results of the AO/EB staining showed that orange colored cells increased with increase in the concentration of Curzerenone, indicative of apoptotic cell death (Figure 3). Annexin V/PI staining showed that

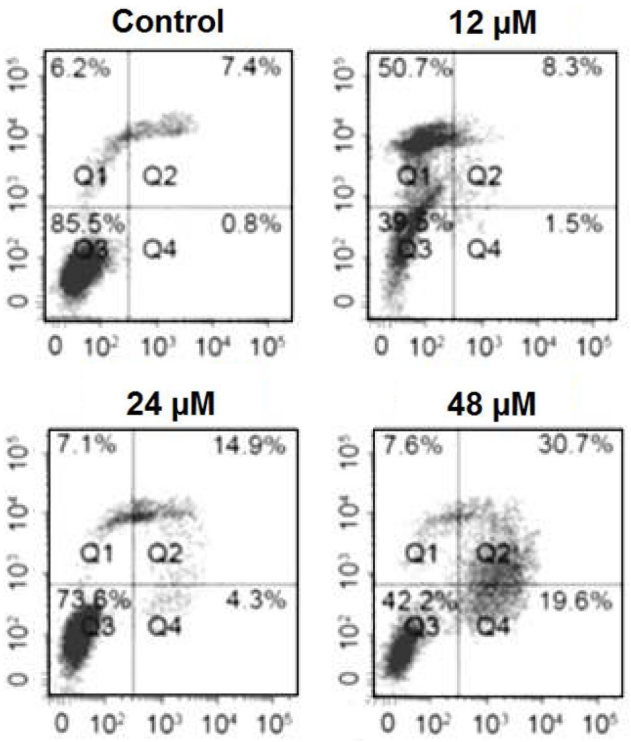

Figure 4. Annexin V/PI staining showing the percentage of apoptotic H69AR cells at each dose of Curzerenone. The Figure indicates that the degree of apoptosis was dose-dependent. The experiments were performed in triplicate.

the apoptotic H69AR cell percentage increased to about 20% at 48 μM concentration of Curzerenone (Figure 4). Furthermore, the expression of Bax was significantly enhanced while that of Bcl-2 was decreased upon Curzerenone treatment (Figure 5).

Curzerenone prompted the generation of ROS in H69AR cells

The effects of Curzerenone were also investigated on the ROS levels of the H69AR cells at 0, 12, 24 and 48 μM concentrations. The results showed that Curzerenone caused significant increase in the ROS levels of the H69AR cells. The ROS levels increase from 100% in the control to about 175% at 48 μM concentration of Curzerenone in the H69AR cells (Figure 6).

Curzerenone decreased MMP levels in H69AR cells

The effects of Curzerenone were also evaluated on the MMP levels of the H69AR cells at 0, 12, 24 and 48 μM concentrations. The results revealed that Curzerenone caused significant decrease in the MMP levels of the H69AR cells. The MMP levels decreased from 100% in the control to about 25% at 48 μM concentration of Curzerenone-treated H69AR cells (Figure 7).

ERK/MAPK and NF-κB signaling pathway

The effects of Curzerenone were examined on the ERK/MAPK and NF-kB signalling pathways. The results showed that Curzerenone caused significant inhibition of the expression of p-ERK and p-p38 with no apparent effects on the total ERK and p38 (Figure 8). Similarly, the expression of NF-kB was decreased in H69AR cells upon treatment with Curzerenone and these effects exhibited concentration-dependent trend (Figure 9).

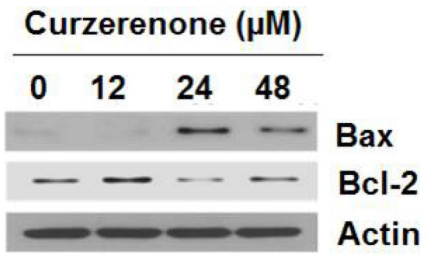

Figure 5. Effect of Curzerenone on the expression of apoptosis-associated proteins as depicted by western blot analysis. The compound led to increase of the expression of Bax and decrease in the expression of Bcl-2. The experiments were performed in triplicate.

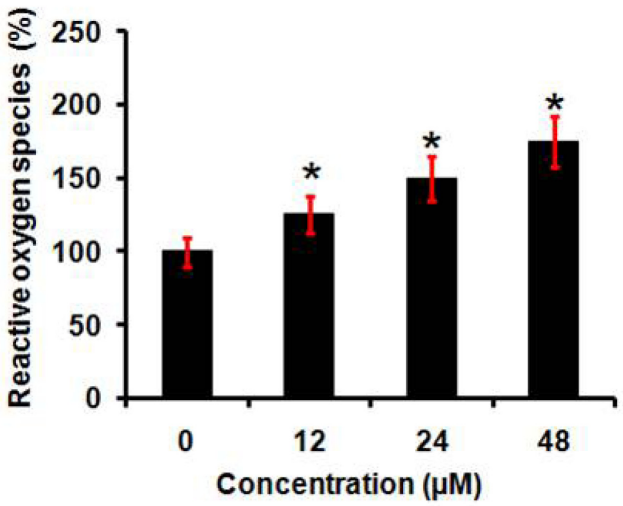

Figure 6. Production of ROS in Curzerenone-treated H69AR cells as depicted by flow cytometry. Curzerenone led to increase of ROS levels in cancer cells in a dose-dependent manner (*p<0.05). The experiments were performed in triplicate.

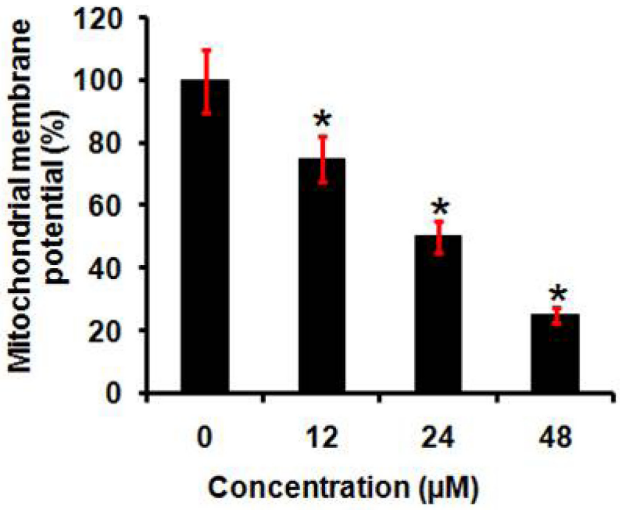

Figure 7. Curzerenone-treated H69AR cells showing decrease in MMP levels in a dose-dependent manner (*p<0.05). The experiments were performed in triplicate.

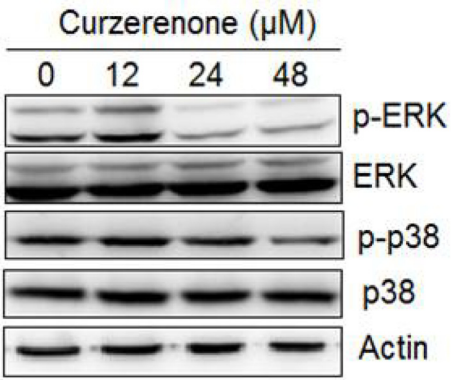

Figure 8. Western blot analysis showing the effect Curzerenone on the MAPK/ERK signalling pathway in H69AR cells. The compound caused significant inhibition of the expression of p-ERK and p-p38 with no apparent effects on total ERK and p38. The experiments were performed in triplicate.

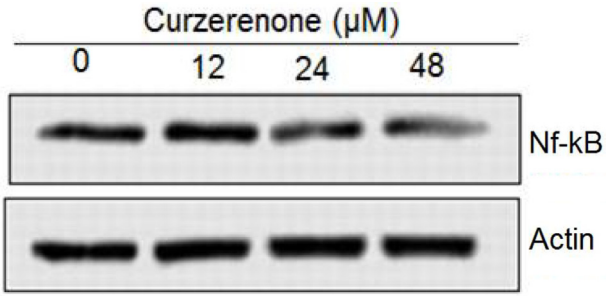

Figure 9. Western blot analysis showing that Curzerenone caused reduction in the expression of NF-kB signalling pathway in H69AR cells. The experiments were performed in triplicate.

Discussion

Lung cancer is one of the devastating cancers that imposes huge disease burden on the world. Accounting for about 25% of all the cancers, lung cancer is the most prevalently detected cancer across the world and is responsible for about 20% of cancer-related mortality [13]. Besides, the adverse effects of the anticancer agents used for the treatment of lung cancer affect the overall health of the patients [13]. Therefore, there is urgent need to explore new drugs or to identify effective therapeutic targets. Plants serve as a diverse repository of chemical entities that may prove to be essential in the development of safer systemic therapy for the treatment of lung cancer [2]. Herein, the anticancer effects of Curzerenone were examined against the H69AR lung cancer and normal lung MRC5 cells. It was found that Curzerenone exerted dose-dependent growth effects on the H69AR cells. However, Curzerenone showed relatively minimal toxic effects on the normal MRC5 cells. These results are in agreement with relevant investigations wherein Sesquiterpenes, such as β-caryophyllene, have been shown to suppress the growth of cancer cells [14]. Apoptosis prevents the development of drug resistance, helps the killing of the abnormal or cancer cells and enables the maintenance of tissue homeostasis [15,16]. We performed AO/EB and DAPI staining of the Curzerenone-treated H69AR cells and it was found that Curzerenone triggered apoptosis in the H69AR cells. The Curzerenone-induced apoptosis was also accompanied by upsurge of Bax and decline in Bcl-2. It has previously been reported that terpenes induce apoptosis in cancer cells [17].

Several of the compounds isolated from different plant species have been reported to interfere with the ROS levels in cancer cells [12]. In the current study, it was found that Curzerenone triggered the formation ROS in the H69AR cells and this was also accompanied by disruption of MMP.

MAPK/ERK and NF-kB pathways have emerged as amazing targets for anticancer drugs and it is believed that drugs targeting these pathways may prove beneficial in the treatment of several types of cancers [10,11]. In this study we found that Curzerenone could block both of these pathways, suggesting the anticancer potential of Curzerenone.

Conclusion

Curzerenone inhibits the growth of the drug-resistant lung cancer cell lines via induction of mitochondrial apoptosis, generation of ROS and disruption of MMP. As such curzerenone may be utilised for the development of systemic therapy for lung cancer. However, evaluation of Curzerenone against more cell lines and under *in vivo* conditions is required.

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Conflict of interests

The authors declare no conflict of interests.

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