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Original Research Article

β-Elemene enhances cisplatin-induced apoptosis of nasopharyngeal carcinoma cells involving an endoplasmic reticulum stress pathway

Hanlin Lv, Yan Wang, Rui Yang, Zezhang Tao*
Department of Otolaryngology-Head and Neck Surgery, Renmin Hospital of Wuhan University, Wuhan, Hubei Province 430060, China

For correspondence:* ***Email:* *ZezhangTaocvg@163.com;* ***Tel:*** *+86-027-88041911-88287*

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Abstract

Purpose: *To investigate whether β-elemene can enhance the anticancer activity of cisplatin in nasopharyngeal carcer (NPC) 5-8F cells and the possible molecular mechanism involved.*
Methods: *The cytotoxicity of β-elemene and its combination with cisplatin in 5-8F cells was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cell cycle arrest was assessed by flow cytometry. Immunoblotting was performed to determine the expression levels of proteins related to the cell cycle (cyclin D1, p21, p27) and to cell apoptosis (Bax, cleaved caspase 9, Bcl-2, and cleaved caspase 3), as well as the endoplasmic reticulum (ER) stress pathway associated proteins.*
Results: *In 5-8F cells, β-elemene (40 μg/mL) and cisplatin (10 mM) exhibited synergistic effects on cell apoptosis and cell cycle arrest. The endoplasmic reticulum stress pathway-related proteins were significantly upregulated after the combination treatment of β-elemene and cisplatin ($p < 0.05$).*
Conclusion: *β-Elemene enhances the antitumor activity of cisplatin in 5-8F cells via a mechanism involving the endoplasmic reticulum stress pathway. Thus, β-elemene is a potential tumor-suppressive agent in the clinical management of nasopharyngeal carcinoma.*

Keywords: *β-Elemene, Cisplatin, Nasopharyngeal carcinoma (NPC), Apoptosis, Endoplasmic reticulum (ER) stress signaling pathway*

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INTRODUCTION

Nasopharyngeal cancer (NPC) is an uncommon oral and maxillofacial malignant tumor, which shows strict geographic distribution [1,2]. Nasopharyngeal carcinoma is a malignancy with a low incidence, but people in southern China are prone to NPC, with a much higher incidence [1,2]. Nasopharyngeal carcinoma is endemic, suggesting that genetic, ethnic, and environmental factors could promote the occurrence of this malignancy [1]. Radiotherapy is a standard treatment approach for nasopharyngeal carcinoma due to its radio-sensitive property [1-3]. However, radiotherapy can give rise to unexpected complications [1,2]. It

also results in high recurrence rates [2,3]. Radiotherapy combined with chemotherapy provides excellent local control, but it does not improve long-term and overall survival [1,3,4]. Moreover, adjuvant chemotherapy is limited by its cumulative toxic effects [4].

β-Elemene is extracted from a herbal medicine, *Rhizoma zedoariae* [5-7]. Emerging *in vitro* studies have shown that β-elemene has antitumor effects, suggesting its potential use for anticancer chemotherapy [5-10]. β-Elemene induce cell apoptosis, suppress tumor metastasis, and reverse multidrug resistance [5-10]. The combination of β-elemene with chemotherapy for cancer treatment appears to be synergistic and less toxic [8]. β-Elemene increases the susceptibility to cisplatin in cancer cells via mitochondrial apoptotic pathways [11], inactivation of c-Jun NH_2-terminal kinase (JNK) [12], and suppression of the signal transducer and activator of transcription 3 (STAT3) [13]. However, the mechanism of cell apoptosis induced by β-elemene still needs to be further elucidated.

Endoplasmic reticulum (ER) stress mediates cell apoptosis, indicating that ER stress pathways are potential targets for treating several diseases [14-17]. The unfolded protein response (UPR) is a well conserved pathway, also named the ER stress response, and this response eliminates unfolded proteins and restores normal ER homeostasis [14-18]. UPR is mediated by three ER stress sensors, namely, activating transcription factor 6 (ATF6), protein kinase RNA-like ER kinase (PERK), and inositol-requiring enzyme 1α (IRE1α) [14-18]. The expression level of CCAAT-enhancer-binding protein homologous protein (CHOP) is low under physiological conditions but its expression is remarkably induced by UPR via the three ER stress sensor proteins [18].

EXPERIMENTAL

Human cell lines and reagents

The 5-8F cells were purchased from ATCC. Roswell Park Memorial Institute 1640 medium, trypsin-EDTA (0.25 %), fetal bovine serum (FBS), and MTT were supplied by Thermo Fisher Scientific (Gibco® Life Technologies, Gaithersburg, MD, USA). β-Elemene and cisplatin were supplied by Sigma-Aldrich. Antibodies against cyclin D1, p21, p27, Bax, Bcl-2, PERK, IRE1α and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were provided by Santa Cruz Biotechnology. Antibodies against cleaved caspase 3 and cleaved caspase 9 were provided by Cell Signaling Technology. The primary antibodies against ATF6, ATF4, and CHOP were provided by Thermo Fisher Scientific (Invitrogen™, Carlsbad, CA, USA).

MTT assay

Cells (8×10^4 cells/mL) were added to a 96-well plate (100 µL/well) and supplemented withβ-elemene and cisplatin. The plate was maintained in 5 % CO_2 at 37 °C for 20 h, 40 h or 60 h, respectively. Subsequently, added 50 µL/well of freshly prepared MTT solution (5 mg/mL). Incubated the plate at 37 °C for 4 h. Added 10 % SDS 100 µLper well. The optical density was measured at $A_{570\ nm}$.

Flow cytometry

Cells (8×10^4 cells/mL) were added to a 6-well plate (3 mL/well). After the treatment for 48 h. The culture medium from each well was collected into tubes, separately. The attached cells were detached using 0.25 % trypsin. After rinse with phosphate-buffered saline (PBS), then were collected and resuspended in 300 µL binding buffer and mixed with 5 µL Annexin V-PE/7-AAD and 5 µL propidium iodide. Incubated at room temperature for 10 min (in dark). Percent apoptotic cells was evaluated within 1 h using a flow cytometer.

Western blotting

Cells were detached by scratching, and cell pellets were harvested and rinsed with PBS. Lysed cells in radioimmunoprecipitation assay (RIPA) buffer with supplementary protease and phosphatase inhibitors. Supernatant was collected after centrifugation at 10,000 × *g* × 10 min. Bicinchoninic acid assay (BCA assay) was used for determination of protein concentrations. Proteins were separated using SDS-PAGE. Transferred the proteins to PVDF membranes. Five percentage (w/v) dry non-fat milk was used for block for 1 h at 25 °C. Incubated with primary antibodies at 4 °C overnight. Washed with 1 × TBST. Incubated with secondary antibodies at 25 °C for 1 h. Washed with 1 × TBST.

Protein bands were detected using enhanced chemiluminescence using glyceraldehyde-3-phosphate dehydrogenase as the internal control for protein quantitation. The information and preparations of the antibodies against the specific proteins are as follows: Mouse monoclonal GAPDH antibody (sc-47724, dilution 1:500 in 1× TBST); mouse monoclonal cyclin D1 antibody (sc-8396, dilution 1:500 in 1× TBST);

mouse monoclonal p21 antibody (sc-6246, dilution 1:500 in 1× TBST); mouse monoclonal p27 antibody (sc-1641, dilution 1:500 in 1× TBST); cleaved caspase-3 antibody (CST, #9664, dilution 1:1,000 in 1× TBST); cleaved caspase-9 antibody (CST, #7237, dilution 1:1,000 in 1 × TBST); Bax antibody (sc-7480, dilution 1:500 in 1 × TBST); Bcl-2 antibody (sc-7382, dilution 1:500 in 1× TBST); mouse monoclonal PERK antibody (sc-377400, dilution 1:500 in 1 × TBST); mouse monoclonal IRE1α antibody (sc-390960, dilution 1:500 in 1× TBST); mouse monoclonal ATF6 antibody (Thermo Fisher Scientific, #MA1-25358, dilution 1:1,000 in 1 × TBST); rabbit polyclonal ATF4 antibody (Thermo Fisher Scientific, #PA5-27576, dilution 1:1,000 in 1 × TBST); mouse monoclonal CHOP antibody (Thermo Fisher Scientific, # MA1-250, dilution 1:1,000 in 1× TBST). The secondary antibodies were mouse IgGκ BP-HRP (Cruz Marker™, sc-516102-CM, dilution 1:5,000 in 1 × TBST), and mouse anti-rabbit IgG-HRP (sc-2357, dilution 1:5,000 in 1× TBST).

Statistical analysis

GraphPad software was applied for statistical analysis. Data are expressed as mean ± standard error of the mean (SEM, n = 3), while $p < 0.05$ was considered statistically significant.

RESULTS

β-Elemene enhances cytotoxic effects of cisplatin in 5-8F cells

Cytotoxic effect of β-elemene was tested by MTT assay. The 5-8F cells were treated with β-elemene for 24, 48, and 72 h. Cell growth was inhibited by β-elemene in a time- and concentration-dependent manner (Figure 1 A). β-Elemene was very cytotoxic at a concentration of 60 µg/mL. Thus, 40 µg/mL was applied in the subsequent experiments. β-elemene was able to promote the cytotoxicity of cisplatin in 5-8F cells (Figure 1 B).

β-Elemene promotes cell cycle arrest induced by cisplatin

Flow cytometric analysis showed that the combination of β-elemene and cisplatin led to enhanced distribution of cells in the G0/G1 phase and a decreased distribution in S-phase, when compared to those cells received single treatment, indicating the cooperative effect of cisplatin combined with β-elemene on the cell cycle arrest of 5-8F cells (Figure 2 A). Immunoblotting analysis revealed that cyclin D1 expression was significantly reduced by the combination with β-elemene and cisplatin, which instead significantly upregulated the protein levels of p21 and p27, when compared to those cells receiving only single treatments (Figure 2 B).

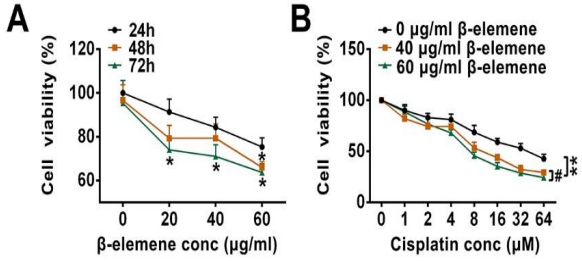

Figure 1: β-Elemene affects the viability of 5-8F cells. (A) Cell viability of 5-8F cells treated with β-elemene. $^{*}p < 0.05$ *vs* 24 h by ANOVA analysis. (B) Cell viability after the treatment of indicated doses of cisplatin combined with β-elemene (0, 40, or 60 µg/mL) for 48 h; $^{*}p < 0.05$ *vs* 0 µg/mL β-elemene by ANOVA analysis. (A, B) MTT assays were performed and cell viability (%) was calculated. Data are mean ± SEM (n = 3)

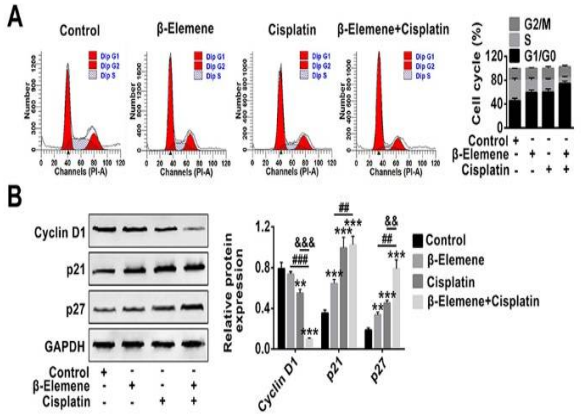

Figure 2: β-Elemene enhanced cell cycle arrest of 5-8F cells. The cells were treated with 40 µg/mL β-elemene or 10 mM cisplatin or their combination for 48 h at 37 °C. (A) The cell cycle was evaluated by flow cytometric analyses. Bars are mean ± SEM of three independent experiments. (B) Immunoblotting analysis was performed to evaluate the expression levels of G0/G1-associated proteins, e.g., cyclin D1, p21, and p27. GAPDH served as a reference protein. Histograms represent the relative densitometric values of indicated proteins relative to GAPDH, Data are mean ± SEM (n = 3). $^{**}P < 0.01$ *vs* control, $^{***}p < 0.001$ *vs* control, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$, by ANOVA. GAPDH = glyceraldehyde 3-phosphate dehydrogenase

β-Elemene enhances induction of cell apoptosis by cisplatin in 5-8F cells

To determine whether the cooperative effect of cisplatin combined with β-elemene or cisplatin

alone on the cell cycle arrest in 5-8F cells was involved cell apoptosis, flow cytometric analysis was used to monitor the apoptosis rate of 5-8F cells. The proportion of apoptotic cells was significantly increased in the combination group, comparing to those of the single treatment group (Figure 3 A). Examination of apoptosis-related proteins using immunoblotting analysis revealed that the pro-apoptotic proteins were significantly upregulated while bcl-2 was significantly reduced in the combined treatment, comparing to those of the single treatment group (Figure 3 B). These results suggest that β-elemene enhanced the induction of 5-8F cell apoptosis by cisplatin.

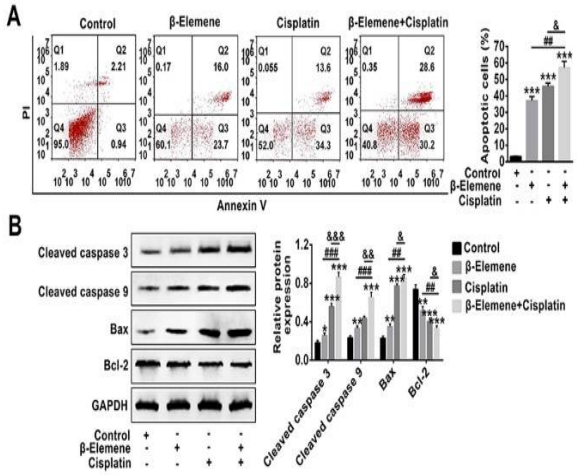

Figure 3: β-Elemene enhances induction of cell apoptosis by cisplatin (A and B). The 5-8F cells were treated with 40 μg/mL β-elemene or 10 mM cisplatin or their combination for 48 h at 37 °C (A). The apoptosis ratio of 5-8F cells was determined by flow cytometric analyses. Bars are mean ± SEM (n = 3). $^{***}p < 0.001$ *vs* control, $^{\#\#}p < 0.01$, $^{\&}p < 0.05$, by ANOVA. (B) Immunoblotting analysis of apoptosis associated proteins. Histograms represent the relative densitometry values of indicated proteins relative to GAPDH. Data are mean ± SEM (n = 3). $^{*}P < 0.05$ *vs* control, $^{**}p < 0.01$ *vs* control, $^{***}p < 0.001$ *vs* control, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, $^{\&}p < 0.05$, $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$, by ANOVA. GAPDH = glyceraldehyde 3-phosphate dehydrogenase

β-Elemene enhances cisplatin-induced apoptosis of 5-8F cells via ER stress pathway

To investigate the signaling pathway responsible for the enhancement of β-elemene on the cytotoxicity induced by cisplatin, the protein expression levels of ER stress pathway proteins were determined. Western blotting analysis revealed that the protein levels of PERK, IRE1α, ATF6, ATF4, and CHOP were significantly increased in the combined treatment with β-elemene and cisplatin group, when compared to those of the single treatment group (Figure 4).

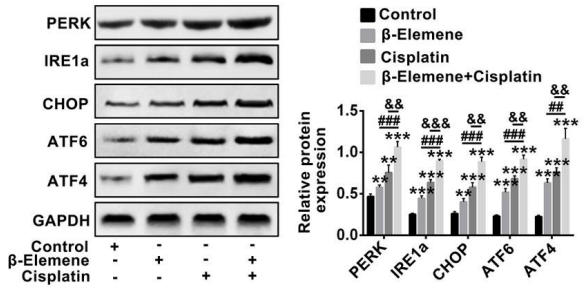

Figure 4: β-Elemene enhances cisplatin-induced apoptosis of 5-8F cells via ER stress pathway. Immunoblotting analysis was performed to evaluate the expression levels of ER stress pathway proteins, including PERK, IRE1α, ATF6, ATF4, and CHOP. GAPDH served as a reference protein. Histograms represent the relative densitometric values of indicated proteins relative to GAPDH. Data are mean ± SEM (n = 3); $^{**}p < 0.01$ *vs* control, $^{***}p < 0.001$ *vs* control, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$, by ANOVA. GAPDH = glyceraldehyde 3-phosphate dehydrogenase

DISCUSSION

In Southeast Asia, NPC is a very common head and neck cancer, but it is rare in Europe and the USA [1]. Nasopharyngeal carcinoma has been shown to be highly radiosensitive, and concurrent cisplatin-radiotherapy is the standard treatment for advanced NPC [1,3,19]. However, cisplatin radiotherapy has relatively severe toxic effects, and is easy to develop radioresistance [1,3,4,20]. It has been reported that NPC can develop a cisplatin-resistant phenotype [20]. Therefore, cisplatin-based combination chemotherapy could be useful strategy. β-Elemene is a traditional Chinese herbal medicine which is reported to possess anticancer activity [5-10]. However, the involved molecular mechanism remains poorly understood. The present work showed that β-elemene have a cisplatin chemo-sensitizing effect on NPC 5-8F cells.

The cytotoxicity of β-elemene on NPC 5-8F cells displayed concentration and time dependence, which were consistent with the studies in other cancer phenotypes [6,9,21]. β-Elemene sensitized NPC 5-8F cells to cisplatin. Thus it promoted cell apoptosis induced by cisplatin through induction of cell cycle arrest, inhibition of cyclin D1, and activation of cyclin-dependent kinase (CDK) inhibitors p21 and p27. The close cooperation of cyclins and CDKs is necessary for cellular function and cell cycle control, indicating their indispensable impact on cellular pathophysiology [22]. It has been suggested that loss of p21 and p27 can lead to a drug-resistant malignancy. thus these may become the targets

for drug resistance mechanism studies [23]. Western blotting analysis revealed that β-elemene and cisplatin synergistically downregulated cyclin D1 and enhanced the expression levels of p21 and p27, indicating that their combination could be a promising strategy for cisplatin-resistant NPC.

The ER is one of the most important intracellular organelles regulating Ca^{2+} homeostasis, protein synthesis, and protein folding [24]. Extensive studies indicated that ER stress plays a critical role in cell fate [14-17].β-elemene regulates ER stress and induces cell apoptosis through the PERK/IRE1α/ATF6 pathway [25]. This study showed that β-elemene promoted cytotoxicity of NPC 5-8F cells by cisplatin via activation of the ERs-related proteins, PERK, IRE1α, ATF6, ATF4, and CHOP. Endoplasmic-reticulum stress and the UPR, as well as the mitochondria appear to play major roles in cell fate decisions. Moreover, ER and mitochondrial functions are associated with these processes [24]. The membranes of endoplasmic-reticulum and mitochondria serve as a platform for their communication by providing various stress signals, such as the altered Ca^{2+} levels[24]. β-Elemene and cisplatin synergistically upregulate expressions of cleaved caspase 3, cleaved caspase 9, and Bax, and inhibit Bcl-2 expression. These results suggest that β-elemene enhances cisplatin cytotoxicity of NPC 5-8F cells by cisplatin through activation of the ER stress pathway, resulting in mitochondria-dependent cell apoptosis. The present work investigated the antitumor activity of cisplatin combined with β-elemene in NPC 5-8F cells. This study for the first time showed that the combination of β-elemene and cisplatin exhibited synergistic effects on NPC 5-8F cell apoptosis, which involved the ER stress pathway.

CONCLUSION

The findings of this study show that β-elemene enhances the antitumor activity of cisplatin in NPC since cisplatin therapy often develop radioresistance which largely restrains its therapeutic efficacy, β-elemene may serve as a novel candidate treatment approach in the clinical management of NPC in the future.

DECLARATIONS

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Conflict of interest

The authors declare that no conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the researchers listed in this article. All liabilities related with the content of this article will be borne by the authors. Hanlin Lv and Zezhang Tao designed all the experiments and revised the paper. Yan Wang formed the experiments, while Rui Yang wrote the paper.

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