

Original Article

Triptolide sensitizes oral squamous cell carcinoma cell to cisplatin via inhibition of NF- κ B activity in vitro

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Abstract: Objective: Triptolide, a bioactive ingredient extracted from Chinese medicinal plant, has been shown to inhibit cell proliferation and induce apoptosis in several cancers including oral squamous cell carcinoma (OSCC). Here, we demonstrate that Triptolide (TPL) sensitizes human OSCC cell lines to cisplatin mediated apoptosis. Methods: Human OSCC cell lines SCC9, Cal27 and SCC25 were treated with various concentration of TPL or/and in combination with cisplatin for various time. With MTT, flow cytometry and TUNEL methods to detect cell growth and cell apoptosis. With RT-PCR, western blot and EMSA assay to detect NF- κ B protein and NF- κ B activity. Results: TPL inhibited cell growth in a dose- and time dependent manner. This was accompanied by increased apoptosis and concomitant attenuation of NF- κ B in vitro. In addition, TPL sensitizes OSCC cells to cisplatin-inducing apoptosis via inhibition of NF- κ B activity. Conclusion: Our results demonstrated that TPL inhibits cell proliferation, induces cell apoptosis and sensitizes OSCC cells to cisplatin through inhibition NF- κ B activity and expression. We also suggest that TPL could be further developed as a potential therapeutic agent for the treatment of OSCC.

Keywords: Oral squamous cell carcinoma, triptolide, apoptosis, cisplatin, NF- κ B

Introduction

Oral squamous cell carcinoma (OSCC) constitutes a major proportion of head and neck squamous cell carcinoma in the world. Despite advances in multidisciplinary treatment modalities, no improvement in the 5-year survival rate has been achieved over the past 20 years [1]. The standard treatment for OSCC remains radical resection whenever feasible and concurrent chemoradiotherapy (CCRT) when the tumor is unresectable [2]. Unfortunately, the prognosis of unresectable OSCC treated with a nonsurgical approach is poor, median survival ranging from 2 to 12 months [3]. Cisplatin, one of the most potent platinum-based chemotherapeutic agents currently in use, is effective as a single agent or in combination with other drugs for the treatment of OSCC [4, 5]. Treatment with cisplatin-based chemotherapy has been found to improve the prognosis of patients with OSCC [6, 7]. However, one of the most important clinical problems for cisplatin-based OSCC chemo-

therapy is the intrinsic/acquired chemoresistance to cisplatin [8].

Triptolide is a diterpene triepoxide antibiotic compound that can be isolated from extracts of the medicinal plant, *Tripterygium wilfordii* Hook F, which has been used for a number of years in traditional Chinese medicine [9]. *Tripterygium wilfordii* Hook F and triptolide have immunosuppressive and anti-inflammatory properties [10, 11]. Triptolide causes apoptosis by inducing the activation of caspases [12]. Proliferation of rheumatoid synovial fibroblasts associated with rheumatoid arthritis has also been shown to decrease with triptolide treatment through a mechanism of increasing caspase-3 activity [13]. Recently, it has found that TPL could inhibit the growth of various cancer cells by inducing apoptosis through a mechanism involves NF- κ B inhibition [14-16].

Many studies have demonstrated that the transcription factor NF- κ B (NF- κ B) is activated by chemotherapy and by irradiation in some

cancer cell lines, including OSCC cells [17-22]. Furthermore, inhibition of NF- κ B in vitro leads to enhanced apoptosis in response to a variety of different stimuli [19-22]. These results show that the activation of NF- κ B in response to chemotherapy is a principal mechanism of inducible tumor chemoresistance, and establish the inhibition of NF- κ B as a new approach to adjuvant therapy in cancer treatment.

In the present study, we investigated the role and mechanism (s) by which TPL may lead to the attenuation of NF- κ B, thereby inhibiting the growth and induced apoptosis of OSCC cells in vitro. Also, TPL could sensitize OSCC cells to cisplatin through inhibition NF- κ B activity and expression. We suggest that TPL could be further developed as a potential therapeutic agent for the treatment of OSCC.

Materials and methods

Ethics statement

This study was approved by the *first affiliated hospital of Zhengzhou University*, and all animal experiments were conducted in accordance with the guidelines of the *first affiliated hospital of Zhengzhou University Animal Experimentation Committee*. Animal experiments were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals of Jinling Hospital.

Cell culture

The human OSCC cell lines SCC9, Cal27 and SCC25 were purchased from American Type Culture Collection (Shanghai, China). SCC9 and SCC25 were maintained in DMEM-F12 (Gibco). Cal27 were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Hangzhou, China). All cell lines were supplemented with 10% fetal bovine serum and maintained at 37°C in a 5% CO₂ humidified incubator.

Drug exposure

Cells were exposed to different concentrations of TPL (0, 12.5 nM, 25 nM and 50 nM) or vehicle control for 72 hours. To determine the effect of cisplatin on NF- κ B activity and expression, cells were treated with various concentrations (0, 5, 10 and 20 μ M) of cisplatin for 24 h and 5 μ M cisplatin for various time lengths (0, 6, 12,

18 h). To determine the effect of TPL on cisplatin-induced NF- κ B activity and expression, cells were treated with 25 nM TPL 6 hs before cisplatin treatment (5, 10 and 20- μ M/ml). To determine the effect of TPL on cisplatin-induced apoptosis, cells were treated with 12.5 nM TPL 6 hs before cisplatin treatment (5, 10 and 20- μ M/ml).

MTT assay

Cell proliferation was measured using MTT assay. Cells in the logarithmic phase of growth were seeded into 96-well culture plates at 1×10⁴ cells per well for 24 hours. After drug exposure at various time, 100 μ l of MTT solution (1 mg/ml) were added to each well, and the cells were further incubated at 37°C for 4 hours. The number of cells was measured after 1, 2 and 3 days of drug exposure with the MTT-assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma-Adrich). The cells were allowed to reduce MTT into formazan (2 h at 37°C) the amount of which was measured spectrophotometrically at a wave length of 550 nm against background (650 nm) after lysing the cells in DMSO.

Flow cytometric assay

After the exposure to the drugs at various time, both detached and attached cells were collected and apoptotic cells were detected by using an Annexin V-FITC Apoptosis Detection Kit (Beckman coulter). The cells were stained with Annexin V-FITC and propidium iodide (PI) according to the manufacturer's instructions. The apoptotic fraction was identified as Annexin V-positive and PI-negative cells using a FACS Calibur (Becton Dickinson, Beijing, China). All assays were performed in quadruplicate.

TUNEL assay

After exposure to TPL or/and cisplatin, cells were trypsinized, fixed in 1% paraformaldehyde in PBS on ice for 15 min, suspended in ice cold ethanol (70%) and stored overnight at -20°C. Cells were then washed twice in PBS and incubated with 50 μ l of solution containing TdT and FITC-conjugated dUTP deoxy nucleotides 1:1 (Roche Diagnostic GmbH, Mannheim, Germany) in a humidified atmosphere for 60 min at 37°C in the dark. Samples were then washed in PBS containing 0.1% Triton X-100, counterstained

Triptolide sensitizes oral squamous cell carcinoma cell to cisplatin

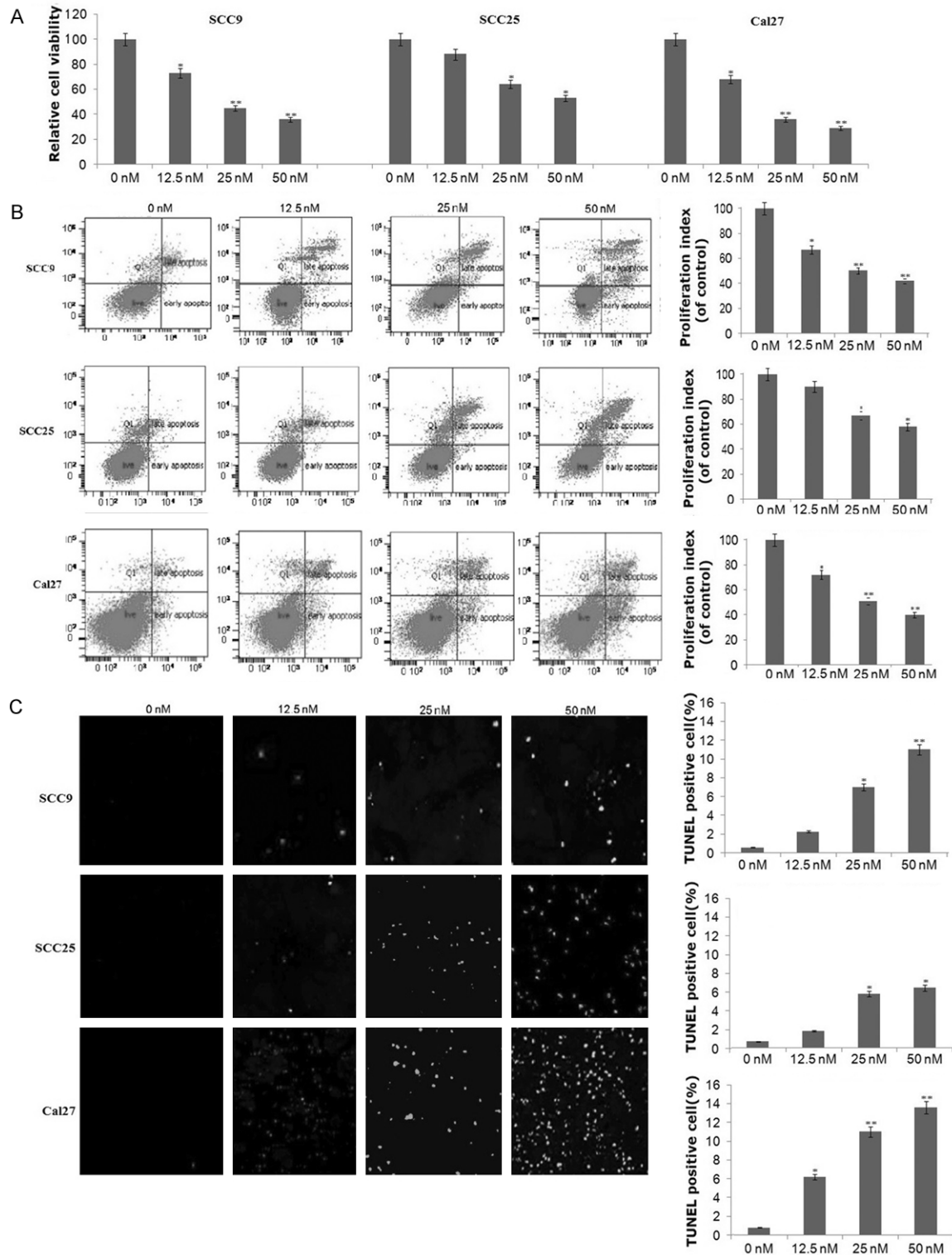


Figure 1. TPL inhibits growth and induces apoptosis in OSCC cells. A. TPL inhibits cell proliferation at indicated doses by MTT assay. B. TPL induces apoptosis at indicated doses by FCM assay. C. TPL induces apoptosis indicated doses by TUNEL assay. Data represented is the means $\pm$ SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$ compared with the control group.

with 3 $\mu\text{g/ml}$ of propidium iodide (Sigma Aldrich) and 10 Kunits/ml of RNase (Sigma-Aldrich) for

30 min at 4°C in the dark. Apoptosis of the cells was evaluated on the basis of the TUNEL assay

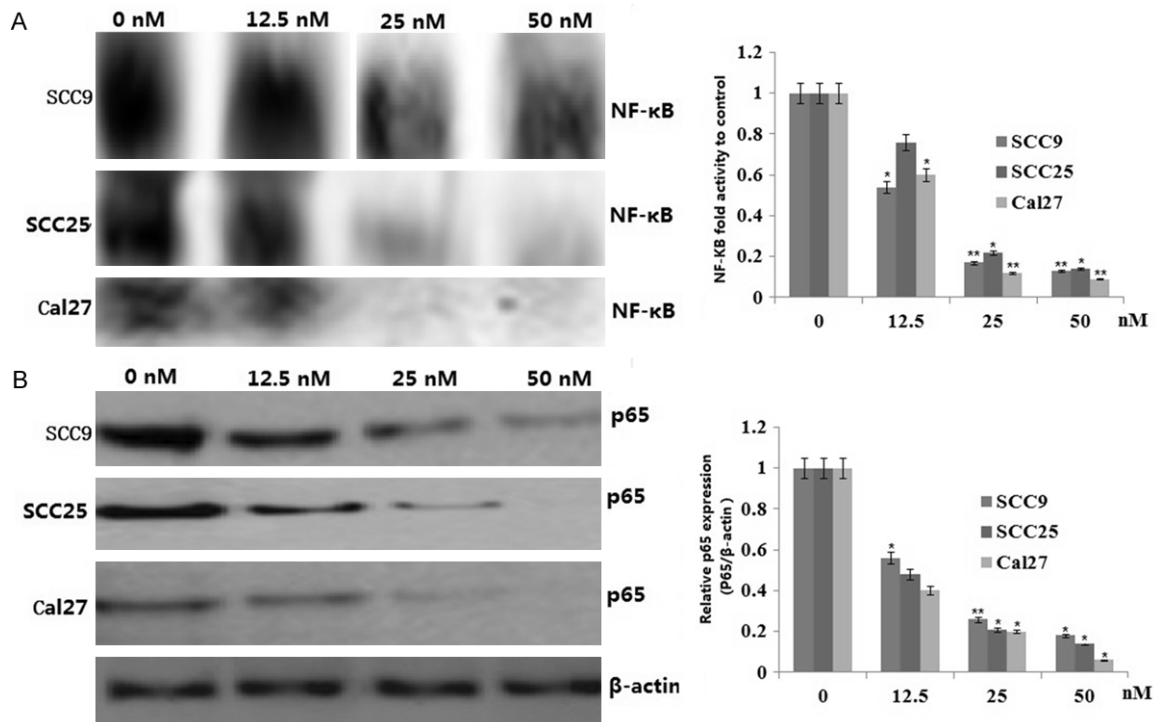


Figure 2. TPL inhibits NF- κ B activity in OSCC cells. SCC9, Cal27 and SCC25 cells were exposed to 12.5, 25 and 50 nM TPL for 24 hs. A: NF- κ B activity was detected by EMSA assay in the three cells. B: NF- κ B p65 protein was detected by western blot assay in all the three cells. * $P < 0.05$, ** $P < 0.01$ compared with the control group.

using the Dead End Fluorometric TUNEL System (Promega, Madison, WI) according to the manufacturer's instructions. All assays were performed in quadruplicate.

Western blot analysis

Cells were lysed and protein was analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The following antibodies were applied: anti-NF- κ B p65 and anti- β -actin. Secondary antibodies (dilution 1:20,000) were horseradish peroxidase-conjugated (Beijing, China).

RT-PCR assay

RT-PCR was used to detect NF- κ B p65 mRNA expression after drug exposure. We performed the reactions according to standard protocols, using the following gene expression assays. All of the oligonucleotide primers were designed using PerlPrimer software and synthesized commercially (Sangon Biotechnology, Shanghai, China). NF- κ B p65: 5'-GCG AGA GGA GCA CAG ATA CC-3' and 5'-CTG ATA GCC TGC TCC AGG TC-3'; GAPDH: 5'-GGTCTCTCTGACA-3' and 5'-AGCCAAATTCGTAC-3'.

Electrophoretic mobility-shift assay (EMSA)

After exposure to TPL or/and cisplatin at various time, nuclear extracts were prepared from cells, and aliquots of extracts were used for EMSA assays. EMSA was performed using the Light Shift Chemiluminescent EMSA Kit (Pierce Biotechnology, Rockford, IL) according to the manufacturer's instructions. In brief, 100 fmol of biotin end-labeled probe was incubated with 15 μ g of nuclear extract, and then electrophoresed on a native polyacrylamide gel. The DNA was transferred to a nylon membrane, UV cross-linked, probed with the streptavidin-horseradish peroxidase conjugate, and chemiluminescent substrate was used for detection. 5'-biotin labeled NF- κ B oligo, 5'-AGTTGAGGGG-ACCTTCCAGGC-3', was purchased from Sigma-Aldrich. For competition experiments, 100-fold molar excess of unlabeled oligo was used.

Statistical analysis

Statistical analyses were performed with SPSS for Windows 17.0 (SPSS Inc., Chicago, IL, USA). All data values were expressed as means $\pm$ SD.

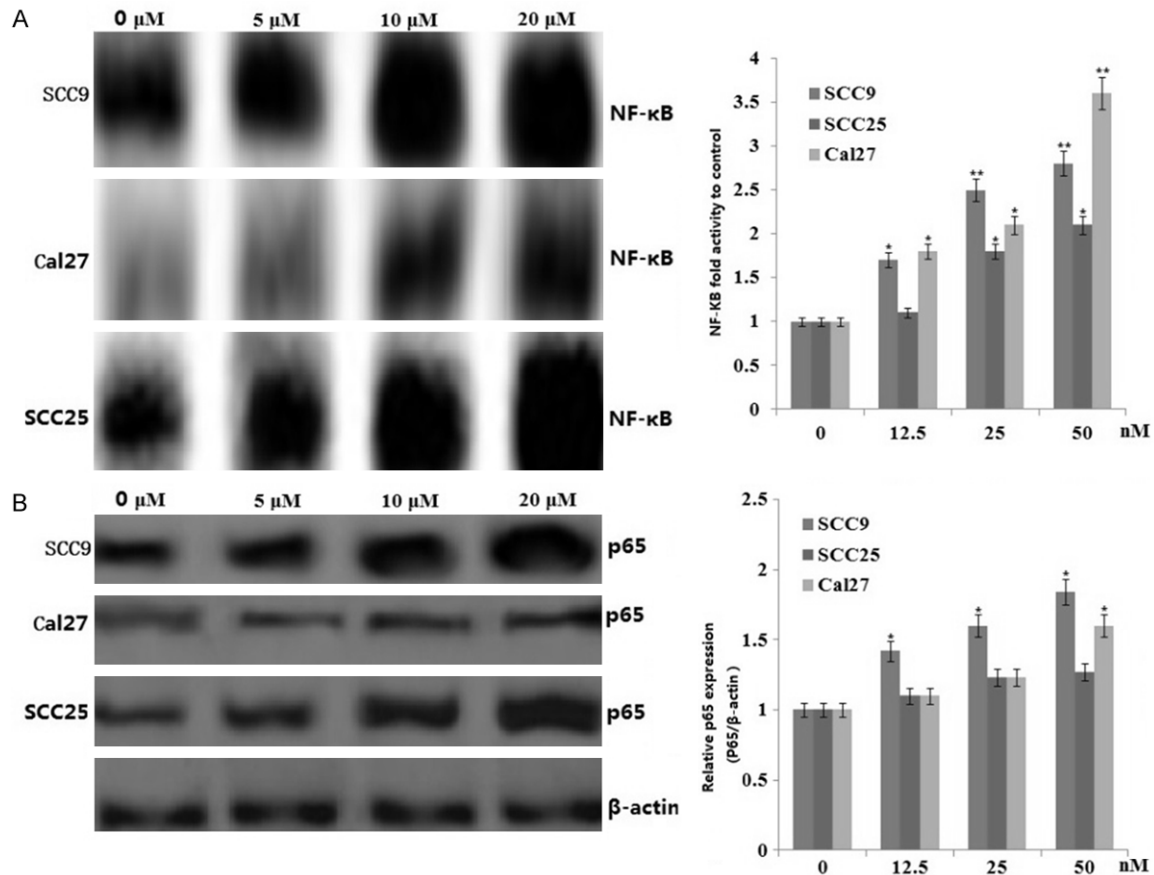


Figure 3. Effect of cisplatin of NF-κB activity and expression in OSCC cells. SCC9, Cal27 and SCC25 cells were treated with 0, 5, 10, 20 μM of cisplatin for 24. A: NF-κB activity was detected by EMSA assay; B: NF-κB protein expression was detected by western blot assay. * $P < 0.05$, ** $P < 0.01$ compared with the control group.

Comparisons of means among multiple groups were performed with one-way ANOVA followed by *post hoc* pairwise comparisons using Tukey's tests. A two-tailed $P < 0.05$ was considered statistically significant in this study.

Results

TPL inhibits cell growth and induces apoptosis in OSCC cells

To determine the effect of TPL on OSCC cell lines, the rate of proliferation was evaluated by the MTT assay. SCC9, Cal27 and SCC25 cell lines were treated with different amounts of TPL (12.5, 25 and 50 nM) for 72 h. As shown in **Figure 1A**, TPL reduces the proliferation of all the cell lines studied in a dose response manner. Data were confirmed by 3H-thymidine incorporation and by cell count (data not shown).

To determine whether TPL is able to induce cell death in OSCC cells, apoptosis analysis was performed. Cells were treated or not with TPL (12.5, 25 and 50 nM) and cultured for 72 hs. At the end of incubation, cells were stained with propidium iodide (PI) and analysed by flow cytometry to evaluate the DNA content. **Figure 1B** shows that all the 3 cell lines undergo apoptosis induction with a different susceptibility, which has the same results as MTT assay. TUNEL assay showed that cell apoptosis rate was also increased with the TPL treatment like the flow cytometry assay (**Figure 1C**).

TPL inhibits NF-κB activity in OSCC cells

When the cell lines SCC9, Cal27 and SCC25 cell were exposed to 12.5, 25 and 50 nM TPL for 24 hs, NF-κB activity as well as NF-κB p65 protein was detected by EMSA and western blot assay. NF-κB activity (**Figure 2A**) as well as

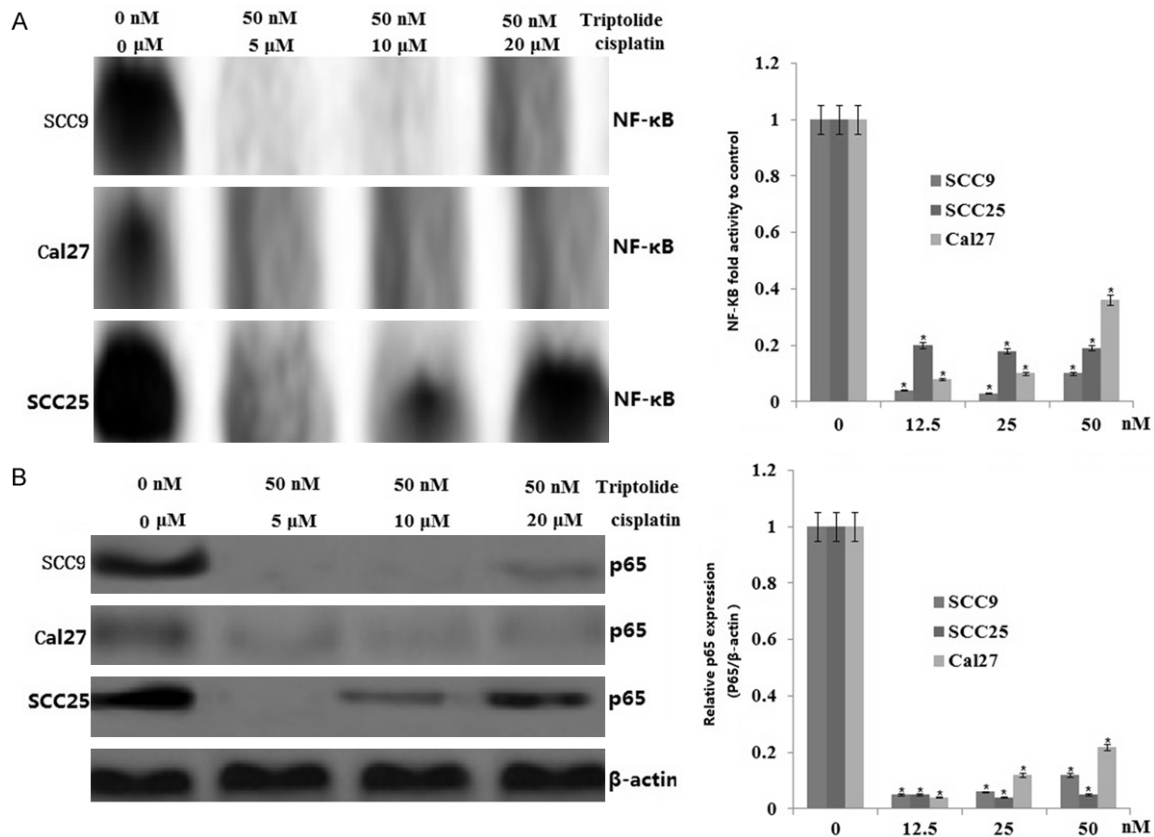


Figure 4. Effect of TPL on cisplatin-induced NF-κB activity and expression in OSCC cells. SCC9, Cal27 and SCC25 cells were treated with 50 nM TPL for 6 hs, then treated with 5, 10, 20 μM of cisplatin for 24 hs. NF-κB activity was detected by EMSA assay (A); NF-κB p65 protein was detected by western blot assay (B). **P* < 0.01, compared with the control group.

NF-κB p65 protein (**Figure 2B**) was inhibited in a dose-dependent manner in all the 3 cells.

Cisplatin induces NF-κB activity in OSCC cells

We treated the SCC9, Cal27 and SCC25 cells with various concentrations (0, 5, 10, 20 μM) of cisplatin for 24 h. NF-κB activity levels (**Figure 3A**) and NF-κB expression (**Figure 3B**) was increased in a dose- dependent matter in the SCC9, Cal27 and SCC25 cells. We then treated cells with 5 μM cisplatin for various time lengths (0, 6, 12, 18 h). NF-κB p65 protein levels and NF-κB activity was also increased in a time-dependent matter in the cells (data not shown).

TPL abrogates cisplatin-induced NF-κB upregulation in the OSCC cells

SCC9, Cal27 and SCC25 cells were treated with 50 nM TPL for 6 hs, then treated with 5, 10, 20 μM of cisplatin for 24 hs. NF-κB activity and NF-κB p65 protein was detected by EMSA and

western blot assay. The results showed that NF-κB activity (**Figure 4A**) and NF-κB p65 protein (**Figure 4B**) was not increase compared to the cisplatin alone-treated cells, which suggested that TPL inhibited cisplatin-induced NF-κB activity.

TPL enhanced cisplatin-induced OSCC cell apoptosis and growth inhibition in OSCC cells

The three cell lines were treated with 5, 10, 20 μM of cisplatin for 72 hs. SCC9 and SCC25 were resistant to cisplatin. Treatment with 5, 10, 20 μM of cisplatin for 72 hs did not significantly inhibit cell growth and increase apoptosis in SCC9 and SCC25 cells (**Figure 5A**). However, cell apoptosis was significantly increased in the Cal27 cells in a dose-dependent manner (**Figure 5B, 5C**). The cisplatin resistance may be associated with the *endogenous* NF-κB activity in the OSCC cells. The three cell lines were exposed to 12.5 nM TPL for 6 hs, then

Triptolide sensitizes oral squamous cell carcinoma cell to cisplatin

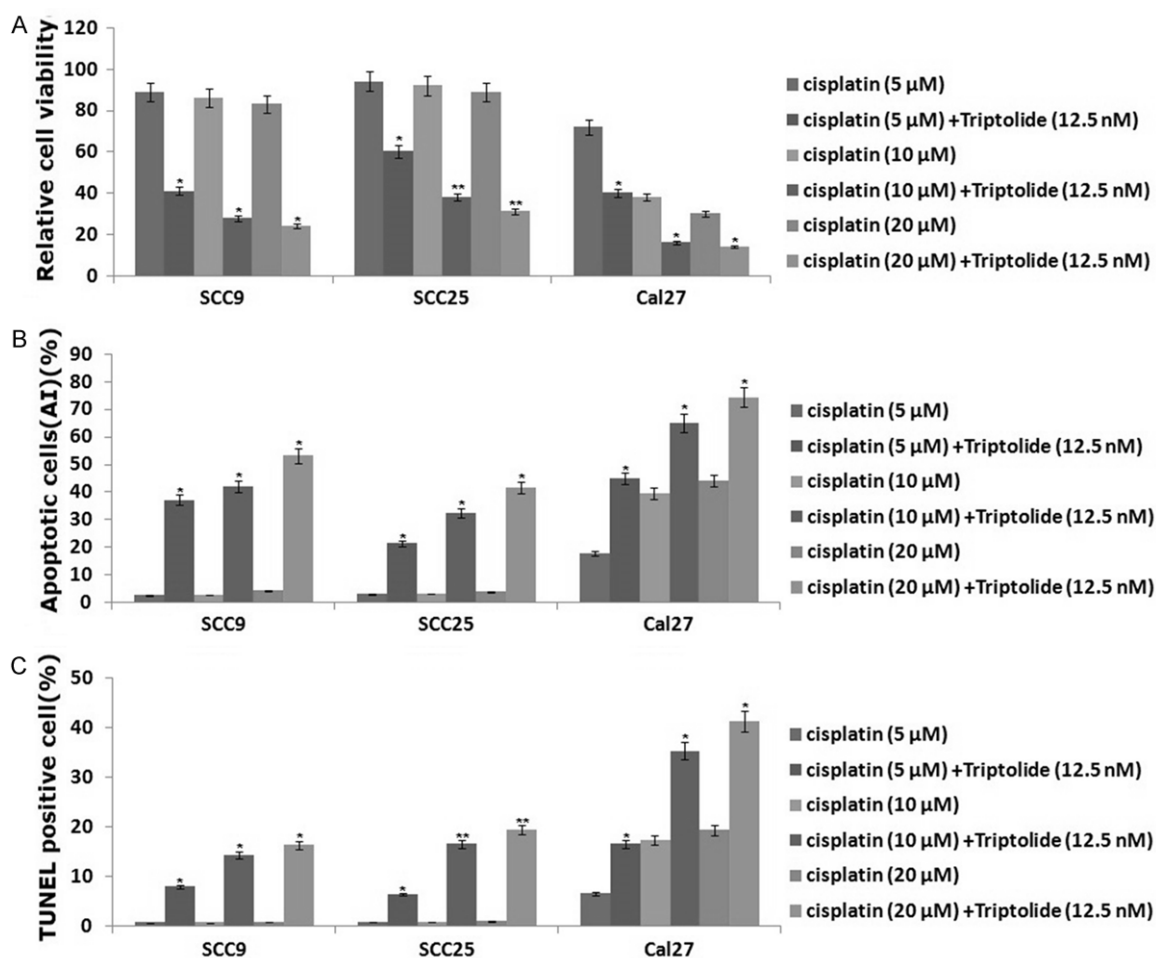


Figure 5. Effect of TPL in combination with cisplatin on apoptosis and cell growth in SCC9 cell. A. Combined treatment inhibits SCC9 cell proliferation by MTT assay; B. Combined treatment induces SCC9 cell apoptosis by FCM assay; C. Combined treatment induces SCC9 cell apoptosis by TUNEL assay; Data represented is the means $\pm$ SD of three independent experiments. * $P < 0.05$.

treated with 5, 10, 20 μ M of cisplatin for 72 hs, cell survival and cell apoptosis was detected. The results showed that combined treatment significantly induces SCC9 cell apoptosis and inhibited growth in all the three cells (**Figure 5A-C**).

Discussion

Resistance to anticancer drugs represents a major problem for the long-term effectiveness of chemotherapy. Deregulation of various genetic pathways drives drug resistance in cancer cells. Recent advances in tumor biology have identified nuclear translocation of NF- κ B as a suppressive mechanism for programmed cell death. Additionally, inhibition of NF- κ B nuclear transport enhances apoptosis [23-25]. In the

present study, we found that SCC9 and SCC25 cells which have active NF- κ B signaling, were resistant to cisplatin treatment, and Cal27 cells which has less active NF- κ B, was sensitive to cisplatin treatment. It is suggested that *endogenous* NF- κ B activity could drive cisplatin resistance in OSCC cells. Thus, novel therapeutic interventions that prevent drug resistance are needed.

Recently, the interest in exploiting traditional Chinese medicine (TCM) for prevention or treatment of cancer has been greatly increased [26]. Among TCMs, the well-known herbal medicines triptolide (TPL) has been found to exhibit anticancer potential both *in vitro* and *in vivo* [26-30]. A wealth of data indicates that TPL, with its broad-spectrum anticancer activity, can

be used as a single agent to treat different tumors. In the present study, TPL demonstrated strong anti-tumor effect no matter in cisplatin-resistant OSCC cells or cisplatin-sensitive OSCC cells. The effect of which is mediated by apoptosis induction via inhibition of NF- κ B activity, in part, which provide new insight into the mode of action of the traditional Chinese medicine TPL in cancer therapy.

Recently, many evidences have been demonstrated indicating the possibility of using TPL in combination with other anticancer drugs to improve efficacy. TPL has also been found to enhance the action of other anticancer agents or therapies, such as idarubicin [31], sorafenib [32] and 5-FU [33], making the combination superior to mono-therapy alone. In this study, we found the cisplatin could activate NF- κ B activity in all the three cells, and combination treatment with TPL and cisplatin decreases cellular viability and enhances OSCC cell death via apoptosis in all the three cells as demonstrated by inhibition of NF- κ B activity and expression. It is conceivable that NF κ B activity plays a role in chemoresistance in the OSCC cells, suggesting that the combination of TPL and cisplatin may possess clinical potential for OSCC, and further studies are clearly warranted.

Conclusion

In summary, we presented experimental evidence, which strongly supports the antitumor effects of TPL in OSCC in vitro. In addition, we believe that TPL could sensitize OSCC cells to cisplatin-induced antitumor effects via inactivation of NF- κ B, resulting in the inhibition of cell growth and induction of apoptosis. Our study suggests that TPL combined with cisplatin represent a promising method that should be developed for the treatment of OSCC especially for the cisplatin-resistant OSCCs.

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Disclosure of conflict of interest

None.

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