An Effective Inhibitory of Triptolide on Ovarian Cancer

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Abstract This study explores the inhibitory effect of triptolide on ovarian cancer cells through *in vitro* cell and *in vivo* animal model experiments. We use the CCK-8 survival rate experiment to determine appropriate concentration of triptolide and the type of cell line including SF9, HEK293, A2780 and Skov-3. Furthermore, the cell invasion and migration of A2780 and Skov-3 cells shows that triptolide had a strong inhibitory effect on ovarian cancer cell line. In addition, flow cytometry is used to detect the apoptosis and cycle of A2780 cells. The results prove that the proliferation of ovarian cancer cell lines is significantly inhibited by triptolide at a 200 nmol/L concentration. Finally, *in vivo* animal model shows a strong inhibitory effect of triptolide with a 200 nmol/L concentration on ovarian cancer. However, HE staining of animal liver, kidney and ovarian tissue section reveals that there is a stronger effect of 200 nmol/L triptolide on the damage of living organs coming from its toxicity. The result offers a chance for reasonable safety use of triptolide.

Keywords Triptolide, Ovarian cancer, Inhibitory, Toxicity

Introduction

Ovarian cancer is a serious threat to women's reproductive health, and the mortality is the first one of various gynecological tumors. The treatment of ovarian cancer with traditional Chinese medicine and western medicine has been focused by many reports. Among them, traditional Chinese medicine has a lot of strategies for controlling ovarian cancer to reduce chemoresistance, metastasis and enhance the quality of life of patients.^[1,2] For example, Liu *et al.* believes that Chinese medicine can improve the quality of patients with ovarian cancer by promoting *Qi* and *Blood* for the apoptosis of cancer cells.^[3]

Triptolide (TP), a diterpenoid obtained from *Tripterygium wilfordii* Hook.f.,^[4] is one natural active product possessing a variety of significant pharmacological activities such as anti-tumor, anti-inflammatory and immunosuppression activity, etc.^[5-7] In recent years, triptolide has shown its antitumor activities in the anti-tumor community of traditional Chinese medicine. For example, it has been reported that triptolide inhibits the growth of ovarian cancer cells *in vitro* and suppresses tumor growth *in vivo*. Triptolide could also sensitize ovarian cancer cells to cisplatin. Additionally, the role of triptolide in the treatment of ovarian cancer has attracted much attention in the treatment of multidrug resistance (MDR) in ovarian cancer, which is a major cause of post-treatment relapses, metastasis, and even death. Furthermore, the nano-drug delivery of triptolide has also been studied to provide a broader prospect for the treatment of ovarian cancer against their toxicity and the short half-life.^[8]

Moreover, there are more important researches about the anti-ovarian cancer mechanism of triptolide including inducing apoptosis of ovarian cancer cells, interfering in the cell cycle,

and suppressing cell metastasis.^[9-11] For example, it has been reported that triptolide inhibited proliferation of nasopharyngeal carcinoma (NPC) cells effectively.^[12] Triptolide has been shown to induce apoptosis by inhibiting NF- κ B in human degenerative thyroid cancer cells.^[13,14] Furthermore, triptolide can modulate the sensitivity of K562/A02 cells to adriamycin by regulating miR-21 expression.^[15] In addition, triptolide has been shown to enhance the tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) mediated lung cancer cell apoptosis by regulating the enhancement of reactive oxygen species (ROS) in cancer cells by NF- κ B regulation.^[16] Triptolide can also induce the production of ROS, leading to apoptosis of human adrenal carcinoma (NCI-H295) cells.^[17] Although some certain NF- κ B-regulated genes (including Bcl-2) play a major role in regulating the number of ROS in cells, ROS have multiple inhibitory and stimulatory effects on NF- κ B signaling.^[18,19] Although triptolide has a good anti-tumor activity and clear mechanism, it is a challenge to reveal the relationship between triptolide concentration and its pharmacological activity/toxicity. In this paper, a triptolide concentration-dependent inhibition of ovarian cancer cells is observed in order to improve its pharmacological activity, reduce its toxicity, and clarify its mechanism.

Experimental

Materials

Triptolide (purity > 97.8%) was purchased from Gade Chemical Co., Ltd. Selected experimental ovarian cells of SF9, human embryonic kidney stem cells of HEK293, and human ovarian cancer cells of A2780 and Skov-3 were purchased from Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). The absolute ethanol and

dimethyl sulfoxide (DMSO) used in the experiment were purchased from Shanghai Titan Technology Co., Ltd. Culture media of Dulbecco's modified Eagle's medium (DMEM), PBS, FBS and insulin were purchased from Gibco (Carlsbad, California) Biotechnology Co., Ltd. The apoptosis kit was purchased from BD Biotechnology Co., Ltd. DAPI staining solution and CCK-8 analysis kit were used for macroscopic analysis and cell survival rate determination, respectively. They were purchased from Shanghai Beyotime Biotechnology Co., Ltd. HE staining kit for observing tissue lesions was purchased from Shanghai Beyotime Biotechnology Co., Ltd.

Cell viability

Four kinds of experimental cells including SF9, HEK293, A2780 and Skov-3 were selected to establish *in vitro* experiments. Cells (5,000 per well) were seeded in 96-well plates, and each of cells was re-welled in five, and incubated at 37 °C, 5% CO₂ for 24 h before the next treatment. The control group was cultured under normal conditions, and the experimental group was cultured under gradient concentration of triptolide such as 1, 100, 200, 300 and 400 nmol/L. The experimental group and the control group were cultured under the same conditions. Subsequently, 10 µL of CCK-8 stain was added to each well. After co-incubation in an incubator at 37 °C, 5% CO₂ for 4 h, the cell proliferation levels of four different cells and different triptolide concentrations were measured using a microplate reader at 450 nm. The cell survival rate was calculated using an average of five independent measurements.

Cell cycle experiment

A2780 and Skov-3 cells were cultured and passaged separately with similar densities. The cells in the control group were cultured normally, and those of the experimental group were cultured in medium containing 200 nmol/L triptolide besides the other same culture conditions. After cultured for 24 h, the cells were digested with trypsin without EDTA for 5 min. The cells were collected, washed them 3 times with cold PBS, fixed with cold absolute ethanol for 24 h. Then, the cells were redispersed in the HEPES buffer after removing the fix solution, and adjusted the concentration in each group to 10⁶–10⁷ per mL. The cell cycle was detected by the flow cytometry test after adding 10 µL of propidium iodide (PI) staining solution stain for 10 min at 4 °C in the dark.

Invasion detection

An invasion assay was used to study the cell migration ability in the 3D gel. Specially, Skov-3 and A2780 cells were treated with triptolide at a 100 nmol/L concentration for 24 h. And then, 3 × 10⁴ per mL cells were seeded into the upper chamber of 8 µm well Transwell. To check the invasion through the Matrigel barrier, 100 µL of culture medium was added to the upper chamber of each well. After being allowed cells to migrate for 24 h, the migrated cells were fixed with 4% paraformaldehyde fixative solution (FPS), stained and washed 3 times. Finally, the cells were counted from six random fields, from which the average number was taken.

In vivo experiment

In order to study the therapeutic effect of triptolide on ovarian cancer *in vivo*, we conducted a comparative study on the efficacy of inhibiting ovarian tumors. Sixteen female SPF grade nude mice with 4-week-old were chosen in the experiment. After being fed for 1 week, these mice were divided into 2 groups (one is a control group and another is an experimental group) with each group of 8 mice. The experimental group was inoculated with about 1 × 10⁷ (0.2 mL × 5 × 10⁷/mL) A2780 cells with the same injection location

(axillary) and method (subcutaneous tumor). Furthermore, the living environment conditions were the same between the experimental group and the control group except that the experimental group and the control group was fed with 0.2 mL, 200 nmol/L triptolide solution and PBS (pH = 7.4) daily, respectively. The tumor was observed, taken pictures, and recorded by their volume on 1, 3, 5, 7, 9, 11 ... 29 and 31 d. In the final observation period, the experimental nude mice were euthanized and their tumors were dissected. Meanwhile, the liver, kidney and ovary tissues of each experimental nude mice were dissected and fixed with 4% FPS for 72 h. The damage of these tissues and organs was evaluated using HE immunohistochemical staining.

Statistical analysis

The results were expressed as the mean ± standard deviation (SD). Statistical analysis was done using a Student's *t* test. Differences were considered significant at a *P* value of < 0.05.

Results and Discussion

The inhibitory effect of triptolide on four kinds of cells was shown in the survival rate experiments. As shown in Figure 1, there are two different inhibitory trends from the four types of cells. The cell livability of both ovarian cancer cells (A2780 and Skov-3) decreases linearly, showing about 53% and 58% of cell livability at medium drug concentration of 200 nmol/L triptolide, respectively, which suggests that their inhibition rate is about 50%. Furthermore, the IC₅₀ of triptolide for A2780 and Skov-3 cells is 257 and 284 nmol/L, respectively. The result reveals that the inhibition of ovarian cancer cells is triptolide concentration-dependent. The higher the drug concentration, the lower the survival rate. Compared to that of A2780 and Skov-3 cells, the livability of SF9 and HEK293 cells decreases slowly before 200 nmol/L triptolide. There is about 90% and 88% of cell livability at 200 nmol/L triptolide. However, their cell livability falls about 40% quickly from 200 to 250 nmol/L of triptolide. Moreover, IC₅₀ of triptolide for SF9 and HEK293 cells is 339 and 336 nmol/L, respectively. The result implies that the low concentration of drugs less affects the cell viability of SF9 and HEK293, which can give an appropriate triptolide administration concentration for our later experiments.

Figure 1 Effect of triptolide on four kinds of cells by CCK-8.

To understand the triptolide inhibition mechanism of ovarian cancer A2780 and Skov-3 cells, we used flow cytometry to identify the cell cycle stages. After incubating the two ovarian

cancer cells of A2780 and Skov-3 with triptolide at the concentration of 200 nmol/L for 24 h, the cell population of G0/G1, S and G2/M were measured (Figure 2a), whose statistical results are shown in Figure 2b. Compared with DMSO control cells ($15.8 \pm 2.6\%$), triptolide significantly increases the S-phase percentage of A2780 cells to $56.83 \pm 4.30\%$ and decreases that of the G2/M and G0/G1 phase, which implies that A2780 cells blocked by S phase cannot enter G2/M phase ($p < 0.05$). Similarly, triptolide increases the percentage of Skov-3 cells in S phase from $10.14 \pm 2.24\%$ to $59.98 \pm 6.32\%$ and decreases the compensation of G2/M and G0/G1 phase cells, which suggests that Skov-3 cells are also blocked by the S phase to enter the G2/M phase ($p < 0.05$). The above results indicate that both of the ovarian cancer A2780 and Skov-3 cells can be blocked in S phase by 200 nmol/L of triptolide, which leads to an increasing S-phase accumulation by inhibiting their progression from G2/M to S-phase in the cell cycle. Furthermore, the result contributes to the inhibition of the ovarian cancer cells proliferation.

Figure 2 Effect of 200 nmol/L triptolide on A2780 and Skov-3 cells by flow cytometry. (a) Flow cytometry of cell cycle stages before/after triptolide treatments and (b) their statistical results.

To study the invasive properties of ovarian cancer cells affected by triptolide, we used a Matrigel transmembrane invasion assay, whose migration of cells across a membrane toward a source of serum attractant was monitored. 100 nmol/L of triptolide was performed in different ovarian cancer cells of A2780 and Skov-3 at the same culture conditions. Migration of ovarian cancer cells is analyzed by staining as shown in Figure 3a, whose statistical analysis is shown in Figure 3b. After 24 h of invasion through matrigel, about the number of 180 untreated invasive A2780 and SKOV3 cells are enumerated. Furthermore, the number of migrated A2780 and SKOV3 cells treated by 100

Figure 3 Effect of 100 nmol/L triptolide on invasion of two kinds of ovarian cancer cells. (a) Transwell assays of the ovarian cancer cells and (b) their statistical analysis.

nmol/L of triptolide is about 68 ± 8 and 75 ± 10 cells, respectively, which implies that the invasive ability of triptolide-treated A2780 cells and Skov-3 cells is decreased by 69% ($p < 0.05$) and 58% ($p < 0.05$). Interestingly, A2780 cells migration is similar to SKOV3 cells' for the two cell lines.

We then evaluated the therapy performance of triptolide on A2780 ovarian cancer tumor bearing mice *in vivo*. Two groups of nude mice were injected with the equal volume of triptolide (200 nmol/L) or PBS *via* hypodermic injection into the tumor tissue. The triptolide treated group reveals the significant increasing growth inhibitory effect in comparison with PBS treated groups (Figure 4a). As shown in Figure 4b, the tumor growth of the experimental group become slowdown after the triptolide administration, which indicates that 200 nmol/L triptolide has a considerable growth inhibitory effect on the tumor. After treated for 31 d, the mice with tumor were sacrificed and tumors were excised. The mean tumor volume in the triptolide group (about 1600 mm³) is lower than that of PBS group (about 2300 mm³), indicating its considerably enhanced tumor inhibition activity. The result shows that the treatment with 200 nmol/L triptolide can enhance anti-tumor performance for a long time.

Furthermore, we estimated the injuries by triptolide to internal tissues of mice. Mice were sacrificed after treated with triptolide for 31 d, and then their internal tissues were stained hematoxylin and eosin (H&E). As depicted in Figure 5, some tissues of liver, kidney and ovary have been assessed. Compared to the control group, after being treated by 200 nmol/L of triptolide, the liver has several damage holes and fibrotic connective tissues, and the kidney also has many tissue fibrosis. However, many corpus luteum follicles in ovarian tissue could be observed in both experiment and control group, which shows that triptolide doesn't injury the ovulation. The result implies that 200 nmol/L of triptolide is harmful to the liver/kidney and it has no obvious effect on the ovarian.

Conclusions and Perspectives

An effective inhibitory of triptolide on ovarian cancer is studied. The cell viability, cycle, invasion assay and *in vivo*

Figure 4 A2780 ovarian cancer before/after triptolide treatment *in vivo* and quantitative analysis. (a) The photograph of the tumor and (d) their mean tumor volume in different groups.

Figure 5 H&E stained images of liver, kidney and ovary before/after triptolide treatment *in vivo*. Scale bar = 200 μm.

experiments have been performed. The survival results show that triptolide can inhibit the proliferation of A2780 and Skov-3 cells by a dose-dependent manner, and it has a small effect on normal cell proliferation within a certain concentration. Furthermore, 200 nmol/L of triptolide can inhibit strongly the proliferation of A2780 and Skov-3 cells. Transwell analysis shows that triptolide can significantly inhibit the invasive ability of A2780 and Skov-3 cells, which probably contributes to

prevent cells from developing from G2/M phase to S phase coming from invasion detection. *In vivo* experimental results show that 200 nmol/L of triptolide has a strong inhibitory effect on ovarian cancer accompanying by some organ damage from fibrosis. The result offers a chance for reasonable safety use of triptolide.

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Conflict of Interest

The authors declare no conflict of interest.

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References

- [1] Zhang, A. C.; Pei, L. L. Progress in diagnosis and treatment of ovarian cancer. *Chin. Family Planning Obstetrics Gynec.* **2013**, *5*, 5–8.
- [2] Engelberth, S. A.; Hempel, N.; Bergkvist, M. Development of nanoscale approaches for ovarian cancer therapeutics and diagnostics. *Crit. Rev. Oncog.* **2014**, *19*, 281–315.
- [3] Zhang, B. B.; Zhuang, L. W.; Chen, J. Progress in the treatment of ovarian cancer with traditional Chinese medicine and western medicine. *TMR Cancer* **2018**, *1*, 124–128.
- [4] Wong, K. F.; Yuan, Y.; Luk, J. M. Tripterygium wilfordii bioactive compounds as anticancer and anti-inflammatory agents. *Clin. Exp. Pharmacol. P.* **2012**, *39*, 311–320.
- [5] Lou, Y. J.; Jin, J. Triptolide down-regulates bcr-abl expression and induces apoptosis in chronic myelogenous leukemia cells. *Leuk Lymphoma.* **2004**, *45*, 373.
- [6] Cui, Y. Q.; Lu, L.; Wang, X. M. Neuroprotective effect and mechanism of triptolide and its derivatives. *J. Capit. Univ. Med. Sci.* **2011**, *32*, 559–564.
- [7] Xu, K. J.; Zhao, Y. K.; Gu, H.; Zhang, C.; Lu, A. P.; Li, J. Study on the pharmacology and toxicology mechanism of Tripterygium wilfordii based on network pharmacology. *J. Integr. Tradit. Chin. West. Med.* **2018**, *38*, 1006–1010.
- [8] Gu, Y. W.; Tang, X. M.; Yang, M.; Yang, D. S.; Liu, J. Y. Transdermal drug delivery of triptolide-loaded nanostructured lipid carriers: Preparation, pharmacokinetic, and evaluation for rheumatoid arthritis. *Int. J. Pharmaceut.* **2019**, *554*, 235–244.
- [9] Qiu, D. M.; Kao, P. N. Immunosuppressive and anti-inflammatory mechanisms of triptolide, the principal active diterpenoid from the Chinese medicinal herb Tripterygium wilfordii Hook. f. *Drugs R. D.* **2003**, *4*, 1–18.
- [10] Yang, H. J.; Chen, D.; Cui, Q. Z. C.; Yuan, Xiao.; Dou, Q. P. Celastrol, a triterpene extracted from the Chinese “Thunder of God Vine,” is a potent proteasome inhibitor and suppresses human prostate cancer growth in nude mice. *Cancer Res.* **2006**, *66*, 4758–4765.
- [11] Xi, C.; Peng, S. J.; Wu, Z. P.; Zhou, Q. P.; Zhou, J. Toxicity of triptolide and the molecular mechanisms involved. *Biomed. Pharmacother.* **2017**, *90*, 531–541.

- [12] Zhou, H.; Liu, Y.; Wang, C.; Liu, L. M.; Wang, H.; Zhang, Y. Q.; Long, C.; Sun, X. P. Triptolide inhibits Epstein-Barr nuclear antigen 1 expression by increasing sensitivity of mitochondria apoptosis of nasopharyngeal carcinoma cells. *J. Exp. Clin. Canc. Res.* **2018**, *37*, 192.
- [13] Zhang, W. Y.; Kang, M.; Zhang, T. T.; Li, B.; Liao, X. Y.; Wang, R. S. Triptolide combined with radiotherapy for the treatment of nasopharyngeal carcinoma via NF- κ B-Related mechanism. *Int. J. Mol. Sci.* **2016**, *17*, 2139.
- [14] Zhu, W. B.; Hu, H. Y.; Qiu, P. X.; Yan, G. M. Triptolide induces apoptosis in human anaplastic thyroid carcinoma cells by a p53-independent but NF kappaB related mechanism. *Oncol. Rep.* **2009**, *22*, 1397–1401.
- [15] Li, H.; Hui, L. L.; Xu, W. L.; Shen, H. L.; Chen, Q. Y.; Long, L. L.; Zhu, X. L. Triptolide modulates the sensitivity of K562/A02 cells to adriamycin by regulating miR-21 expression. *Pharm. Biol.* **2012**, *50*, 1233–1240.
- [16] Lee, K. Y.; Park, J. S.; Jee, Y. K.; Rosen, G. D. Triptolide sensitizes lung cancer cells to TNF-related apoptosis-inducing ligand (TRAIL) induced apoptosis by inhibition of NF kappaB activation. *Exp. Mol. Med.* **2002**, *34*, 462–468.
- [17] Wu, P. P.; Liu, K. C.; Huang, W. W.; Ma, C. Y.; Lin, H.; Yang, J. S.; Chung, J. G. Triptolide induces apoptosis in human adrenal cancer NCI H295 cells through a mitochondrial dependent pathway. *Oncol. Rep.* **2011**, *25*, 551–557.
- [18] Morgan, M. J.; Liu, Z. G. Crosstalk of reactive oxygen species and NF-kappaB signaling. *Cell Res.* **2011**, *21*, 103–115.
- [19] Kane, D. J.; Sarafian, T. A.; Anton, R.; Hahn, H.; Gralla, E. B.; Valentine, J. S.; Ord, T.; Bredesen, D. E. Bcl 2 inhibition of neural death: decreased generation of reactive oxygen species. *Science* **1993**, *262*, 1274–1277.

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