Antitumor activity of tanshinone and its nanoparticles on U14 cervical carcinoma-bearing mice

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Abstract
In this study, tanshinone was extracted from Salvia miltiorrhiza. To improve the utilization and the dissolution of the drug, the tanshinone extractions were prepared at a pharmaceutical nanoscale and in the nanometer range of 100–200 nm. Then, the rate of tumor inhibition and the activity of antioxidant system and the thymus/spleen indices were investigated to find the antitumor effect of nanoparticles of tanshinone in cervical carcinoma-bearing mice. Our data suggest that tanshinone inhibits cervical tumor growth and the rates of tumor inhibition of all drug groups were more than 45%. The highest rate was 70.88% in the high dose of nanoscale tanshinone group. The activities of superoxide dismutase were higher in drug groups than in the model control group, and the concentrations of malondialdehyde were significantly lower. These findings suggested that tanshinone enhance the superoxide dismutase activity of the mice and decrease the malondialdehyde content. It may be one of the mechanisms of antitumor effect of tanshinone. The thymus index and spleen index were higher than normal control or model control. These data suggested that tanshinone also enhanced the immune system of mice.

Keywords
Tanshinone, antitumor, U14 cervical carcinoma-bearing mice, HPLC, TEM

Introduction
Being a complex disease, which is caused by alterations of both genetic and epigenetic factors, cervical cancer is the second most common cancer among women in the world. Despite advances in chemotherapy and radiotherapy, advanced and recurrent cervical cancer has a poor prognosis and few effective therapeutic options. Currently available chemotherapeutics yield only modest increases in the 5-year survival rate among those patients with advanced cervical cancer. The major cause of mortality associated with this disease is the decreased chemosensitivity of cervical cancer cells to chemotherapeutic drugs.

Salvia miltiorrhiza Bunge (Lamiaceae) is a well-known Chinese herb, and its roots have been widely used for the treatment of menstrual disorders and cardiovascular diseases and for the prevention of inflammation. Tanshinone (also known as total tanshinones (TT)) are fat-soluble compounds that include more than 10 monomers, which are mainly found in

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in *S. miltiorrhiza.* The main compounds include cryptotanshinone, tanshinone-I (TII), tanshinone-I A, tanshinone-I B, and so on. Recent research suggested that TT has antitumor, blood circulation, promote wound healing, and many other effects, there are no obvious side effects for long-term using.

However, tanshinone insolubility decreased its utilization in vivo. To improve the utilization and the dissolution of the tanshinone extractions, pharmaceutical nanoscale of the drugs was worth to be tried.

This work aims to examine the antitumor activity of nanoparticles of tanshinone (called NT) on U14 cervical carcinoma-bearing mice and to find the molecular action of tanshinone tumor cells. The present study examined the antitumor effect of TT and NT on U14 cervical carcinoma-bearing mice and attempted to elucidate the mechanism behind it.

**Experiment**

**Materials and chemicals**

*S. miltiorrhiza* roots and tanshinone standard (cryptotanshinone (CT), TI, and tanshinone-I A) were purchased from Xian Senzhuo Bio-Technology Co., Ltd (Xi’an, China). The *Salvia* roots were ground into powders by a grinder. Cyclophosphamide (CTX) was purchased from the First Hospital (Hebei, China). The kits of superoxide dismutase (SOD) and malondialdehyde (MDA) detection were purchased from Nanjing Jiancheng Bio-Technology Co., Ltd (Nanjing, China). All other chemical reagents were of analytical grade.

**Tanshinone extraction and preparation of nano-tanshinone**

The *Salvia* powder was extracted for tanshinones with 4:1 methanol/dichloromethane (approximately 100 mg/mL), and the extract was then evaporated to dryness and redissolved in 9:1 methanol/dichloromethane. Tanshinone content in the extract solution was determined by high-performance liquid chromatography (HPLC) using a C18 column, acetonitrile/water (11:9, v/v) as the mobile phase, and ultraviolet detection at 275 nm. Three tanshinone species, CT, TI, and tanshinone-I A (TIIA), were detected and quantified with authentic standards. The tanshinone peaks of samples were further confirmed by co-chromatography with the standards. The TT content is the sum of the three tanshinone species in the samples.

Tanshinone and poly(lactic-co-glycolic acid) (1:10) were dissolved in acetone (approximately 1.5 mg/mL) to form an organic phase. Polyvinyl alcohol was dissolved in water (1.5%) to form an aqueous phase. The organic phase was added slowly to the aqueous phase (1:20) at 40°C. Save pressure rotary evaporation to remove the organic solvent, and then the solution was filtered through 0.8 mm Millipore filter (Billerica, Massachusetts, USA). The solution was dropped on a carbon grid and then detected by transmission electron microscope (TEM).

**Cell lines and animals treatment**

Murine U14 cell line was purchased from Chinese Medicine Beijing Tumor Cell Bank (Beijing, China).

Kunming mice (grade II, 6 weeks old) weighing 18–22 g, female, were acclimatized for 1 week before being used for the experiment. Before and during the experiment, the mice were housed under controlled environmental conditions of temperature (20 ± 2°C) at a 12-h light/12-h dark cycle and were fed with a standard pellet diet and provided with water ad libitum.

The mice were starved for 12 h before the establishment of tumor model, and 0.2 mL of cancer cell suspension (1 × 10⁶ cells/mL) was inoculated through subcutaneous injection at the right axilla of each mouse to establish a solid tumor model.

After 24 h, mice were randomly divided into six groups (eight mice per group) as follows: two doses of TT (50 and 100 mg/kg/day) groups, two doses of NT (50 and 100 mg/kg/day) groups, U14-bearing group (model control group), and positive control group. The mice in the model control group received an equal volume of double distilled water in the same way once daily, and the positive control group was treated with CTX (25 mg/kg/day, intraperitoneal injection), which was considered as the standard reference drug. All groups were continuously treated for 14 days. The animals’ living conditions were observed every day, and the changes of tumor volume and body weight were monitored. Twenty-four hours after the last administration, all the mice were killed. The kidney, liver, spleen, thymus, and tumor were immediately dissected and weighed.

**Histopathological and morphological examination of liver and kidney**

The kidney and liver of treated and control mice were excised and fixed in 4% formalin, embedded in paraffin, and cut into 4 μm sections for histological study.

**Determination of rate of tumor inhibition, viscera indices, and antioxidant indices**

The rate of tumor inhibition was calculated by the following formula: (the mean tumor weight of model control group – the mean tumor weight of treated group)/the mean tumor weight of model control group × 100.

Thymus and spleen weight was expressed in milligrams per gram of body weight. The viscera indices for thymus and spleen were calculated by the following formula: the mean viscera weight/the body weight without tumor × 10.

All animals were subjected to collect blood samples via retro-orbital eye bleed prior to killing. The blood samples were kept at 4°C for 1 h and centrifuged at 956 × g for 5 min at room temperature to prepare serum. Liver tissue of mice was washed with cold saline, weighed, and then made into 10% homogenate by adding normal saline in the ratio of 1:9. Homogenate was then centrifuged at 2500 r/min for 10 min
at 4°C to prepare the supernatant. SOD activity and MDA content were measured directly using commercial kits.\textsuperscript{14}

**Statistics**

Data were expressed as mean ± SD. Statistical analysis was performed by one-way analysis of variance, and differences between means were tasted using Duncan’s multiple range tests. The values of \( p \) less than 0.05 were considered statistically significant.

**Result**

**TT extraction and NT preparation**

The results of the TT extracts examined by HPLC test are shown in Figure 1. Then the extracts were prepared to nanotanshinone, and the results of TEM are also shown in Figure 1.

![Figure 1](image)

*Figure 1.* Results of TT extraction and NT preparation. (a) The retention time of tanshinone standards is 10.132 min (CT), 11.206 min (TI), and 18.979 min (TIIA). (b) Three tanshinone peaks at 10.089 min (CT), 11.162 min (TI), and 18.875 min (TIIA). (c) The TEM result (scale length 200 nm). TT: as total tanshinones; NT: nanoscale tanshinone; CT: cryptotanshinone; TI: tanshinone; TIIA: tanshinoneIIA.

### Table 1. Effect of tanshinone on tumor growth in vivo.a

| Group | Dose (mg/kg/day) | Animal number | Tumor weight (g) | Inhibition rate (%) |
|-------|-----------------|---------------|----------------|-------------------|
| Model control | – | 10 | 9 | 3.64 ± 0.52 |
| CTX | 25 | 8 | 8 | 1.14 ± 0.19*** 68.77 |
| TT | 50 | 8 | 8 | 2.00 ± 0.03*** 45.11 |
| 100 | 8 | 8 | 1.45 ± 0.28*** 60.21 |
| NT | 50 | 8 | 8 | 1.27 ± 0.13*** 65.03 |
| 100 | 8 | 8 | 1.06 ± 0.12*** 70.88 |

**TT** total tanshinones; **NT**: nanoscale tanshinone; **CTX**: cyclophosphamide.

*aResults are expressed as mean ± SD. Significant differences compared with the model control.*

**\(*p < 0.01.**

**\(***p < 0.001.**
We showed that the retention time of the three peaks in Figure 1(b) is similar to that of the three peaks in Figure 1(a) as the reference standard (Figure 1(a) and (b)). CT, TI, and TIIA account for approximately 90% of TT. Figure 1(c) shows the result of TEM with the bar in the lower right corner representing 200 nm. NT particles are round, smooth, and dispersed, and the average size is 100–200 nm.

**Acute toxicity studies and effect of tanshinone on kidney and liver**

Acute toxicity studies revealed that mice treated with TT and NT did not show mortality nor any obvious symptom of toxicity or any significant changes in general behavior. To determine whether TT or NT have any side effects on kidney and liver, pathological examination of kidney and liver tissues was conducted. There was no obvious pathological change in the kidney and liver tissues of treated mice (not shown).

**Effect of tanshinone on tumor growth in vivo**

To evaluate the antitumor activity of NT in vivo, we evaluated its effect on the growth of allografted U14 tumors in Kunming mice. After daily intraperitoneal (i.p.) administration of CTX (25 mg/kg) and intragastric administration (i.g.) administration of TT and NT (two doses for each group) for 15 days, a significant tumor regression (Table 1) was observed in a dose-dependent manner compared with the model control group (p < 0.05, p < 0.01 or p < 0.001). Consistent with the antitumor activity, these two indexes increased to maximum at the dose of 100 mg/kg of NT group. Compared with thymus index (< 0.05), spleen index was significantly higher (p < 0.001) than normal and model control groups. However, the immune organ indices in CTX-treated group were lower than that of the model control (p < 0.01 or p < 0.001), indicating the suppressing effect of CTX on immune system as reported previously. The body weight (without tumor) in CTX-treated group was lower than that of model control (p < 0.01), indicating the strong side effect to the body (Table 2).

**Effect of tanshinone on antioxidant system in vivo**

The SOD activity and MDA content are determined in serum and liver tissues of all groups of mice (Table 2).

The activity of SOD in liver in the CTX, TT, and NT groups was not significantly higher than the model control. While the activity of SOD in serum in CTX group was significantly higher than the model control (p < 0.001). In all treated groups, the mean values of MDA were significantly lower than in the model control group (p < 0.05; p < 0.01 or p < 0.001), decreasing in dose-dependent manner with TT or NT. This result shows that tanshinone may inhibit the generation of MDA in the serum and the liver of tumor-bearing mice.

It suggests that both TT and NT enhance the antioxidant ability of the mice serum and liver tissues.

**Effect of tanshinone on the viscera indices in vivo**

The thymus and spleen indices were determined in all groups of mice (Table 3). The relative spleen and thymus weights of the TT and NT groups were higher than those of the model control groups (p < 0.05). Consistent with the antitumor activity, these two indexes increased to maximum at the dose of 100 mg/kg of NT group. Compared with thymus index (p < 0.05), spleen index was significantly higher (p < 0.001) than normal and model control groups. However, the immune organ indices in CTX-treated group were lower than that of the model control (p < 0.01 or p < 0.001), indicating the suppressing effect of CTX on immune system as reported previously. The body weight (without tumor) in CTX-treated group was lower than that of model control (p < 0.01), indicating the strong side effect to the body (Table 2).

**Discussion**

Tanshinones including CT, TI, and TIIA have been known to be the major diterpenes isolated from *S. miltiorrhiza* Bunge (Danshen). It was reported to show cytotoxic effects on various human carcinomas such as the colon, ovary, lung, and mouth. In this study, we found that attenuation of tumor burden by TT and NT has dose-dependent effect. For example, the tumor inhibition rate of TT-50, TT-100, NT-50, and NT-100 group was 45.11%, 60.21%, 65.03%, and 70.88%, respectively. We also noticed that combined use of TT or NT was far more effective than use of single ones.
and all the tumor inhibition rate of single factor treatment group was lower than 30%. It suggested combination treatment of TT or NT plays a synergy as antitumor agents. Some studies have demonstrated that TI can inhibit tumor cell proliferation by blocking cell cycle. Kim’s study found that treatment of TI to colorectal cancer cells can induce cyclin D1 proteasomal degradation.17 TIIA may induce cell apoptosis though Jun amino-terminal kinases and caspase pathway.18 These studies may provide a novel mode of treatment for using TT or NT in mice.

Reactive oxygen species is playing a key role in oxidative damage accumulating during the life cycle, which induces the development of age-dependent diseases such as cancer.19 SOD is an essential enzyme that scavenges oxygen-free radicals and protects cells from oxidative damage. MDA, the end product of the lipid peroxidation caused by attacking on biofilm polyunsaturated fatty acids, is known as a biological marker of free radicals in the body. Our results showed that compared with the model control group, the activities of SOD in serum increased (p < 0.05), while the contents of MDA in serum and liver decreased (p < 0.01 or p < 0.001) significantly in the NT-100 group. We deduced that nanoparticles of TT may enhance the level of SOD to eliminate excessive free radicals, which resulted in reducing the lipid peroxidation, suggesting that NT exerts an anticervical cancer effect probably through enhancing the activity of antioxidant system of tumor-bearing mice.

**Conclusion**

In our study, we found that the inhibition rate of U14 cervical tumor-bearing mice was significantly increased after tanshinone treatments. We also found that the antitumor effect of nanoparticles of tanshinone was much higher than the normal tanshinone extracts. The viscera indices especially spleen index were also significantly increased, and MDA in the serum and the liver of tumor-bearing mice was decreased at the same time. It may be the mechanisms of antitumor effect of tanshinone.

**Declaration of conflicting interests**

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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### Table 3. Effect of tanshinone on spleen indices and thymus indices in U14-bearing mice.

| Group      | Dose (mg/kg/day) | Body weight increased (g) | Thymus index (mg/10 g) | Spleen index (mg/10 g) |
|------------|------------------|---------------------------|-----------------------|------------------------|
| Normal     | –                | 6.18 ± 0.53               | 62.15 ± 5.95          | 52.17 ± 2.51           |
| Model control | –            | 2.94 ± 0.28               | 66.58 ± 4.48          | 80.27 ± 7.94*         |
| CTX        | 25               | 1.84 ± 0.30*              | 35.26 ± 2.35**##     | 44.80 ± 2.35***###    |
| TT         | 50               | 3.77 ± 0.52**             | 53.09 ± 8.18          | 80.12 ± 5.11***##     |
|            | 100              | 5.22 ± 0.41***            | 48.88 ± 10.34*##     | 145.29 ± 6.75*##      |
| NT         | 50               | 4.29 ± 0.10*              | 55.84 ± 3.51***###   | 133.64 ± 9.41*##      |
|            | 100              | 6.02 ± 0.54***            | 60.25 ± 6.75***###   | 189.31 ± 12.19*#####  |

TT: total tanshinones; NT: nanoscale tanshinone; CTX: cyclophosphamide

*Results are expressed as mean ± SD. Significant differences compared with the normal control. Significant differences compared with the model control.

*p < 0.05.

**p < 0.01.

***p < 0.001.

*p < 0.05.

##p < 0.01.

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