

# Tanshinone II-A inhibits invasion and metastasis of human hepatocellular carcinoma cells *in vitro* and *in vivo*

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## ABSTRACT

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**Aims and background.** Tanshinone II-A is an alcohol extract of the root of the traditional Chinese medicinal plant *Salvia miltiorrhiza Bunge*, whose effects and mechanism in tumor metastasis are still unclear. The aim of this study was to investigate the effects of tanshinone II-A on tumor invasion and metastasis in human hepatocellular carcinoma (HCC) and its possible mechanism of action.

**Methods and study design.** The HCC cell lines HepG2 and SMMC-7721 were treated with tanshinone II-A at different doses. Invasion and metastasis of tumor cells were examined by *in vitro* and *in vivo* assays. The molecular mechanisms of tanshinone II-A for inhibiting invasion and metastasis of HCC cells were investigated by Western blot and gelatin zymography.

**Results.** Treatment with tanshinone II-A had inhibitory effects on the migration and invasion of HCC cells. Increasing doses resulted in enhanced inhibitory effects. At 0.5 mg/L, the inhibitory effect was noticeable. At 1 mg/L, the inhibitory rate was 53.15%. The inhibitory effect became stronger with time; among 24, 48, 72 and 96 hours of treatment, the most significant effects were observed at 72 hours. Tanshinone II-A also significantly inhibited *in vivo* metastasis of HepG2 cells. Tanshinone II-A inhibited *in vitro* and *in vivo* invasion and metastasis of HCC cells by reducing the expression of the metalloproteinases MMP2 and MMP9 and by blocking NF-kappa B activation.

**Conclusions.** Tanshinone II-A effectively inhibited invasion and metastasis of HCC cells *in vitro* and *in vivo*, partly by inhibiting the activity of MMP2 and MMP9, and partly via the NF-kappa B signal transduction pathway.

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## Introduction

Liver cancer is the sixth most common cancer worldwide and the third most common cause of cancer-related mortality. It is an important public health problem throughout the world, particularly in developing countries<sup>1</sup>. In China, hepatocarcinoma is one of the most common causes of malignancy-related death<sup>2</sup>. Even in Western countries, the morbidity of liver cancer is steadily increasing<sup>3,4</sup>. Until now, systemic treatment of human hepatocellular carcinoma (HCC) has not been effective in most cases, and its clinical therapy is a major challenge. Despite efforts to improve its prognosis, the overall survival of HCC patients is still unsatisfactory<sup>1</sup>. Increasing evidence shows that traditional Chinese medicines can be considered potential drugs for cancer treatment. For example, Ginsenoside Rg3 inhibits angiogenesis in Lewis lung carcinoma<sup>5,6</sup>, invasion and metastasis in intestinal adenocarcinomas and B16 melanoma<sup>7,8</sup>, and proliferation of prostate cancer cells<sup>9,10</sup>. Recently, reports showed that tanshinone II-A could reverse malignant phenotypes and inhibit the growth of the HCC cell line SMMC-7721<sup>11,12</sup>.

Tanshinone II-A is an alcohol extract of the root of *Salvia miltiorrhiza Bunge*, a traditional Chinese medicine that is known to have antiinflammatory, antioxidative and

**Key words:** metastasis, tanshinone II-A, metalloproteinase, liver cancer.

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cytotoxic activities<sup>2,13</sup>. It was shown that tanshinone II-A could induce differentiation of a human cervical carcinoma cell line (ME180) and leukemia cells (NB4, HL60 and K562), and reverse the malignant phenotype of the HCC cell line SMMC-7721<sup>2,11,14-15</sup>. However, there have been no studies on the inhibition of invasion and metastasis by tanshinone II-A in hepatocarcinoma cells. We used *in vitro* and *in vivo* methods to investigate the effect of tanshinone II-A on the invasion and metastasis of the human hepatocarcinoma cell lines HepG2 and SMMC-7721.

## Materials and methods

### Cell culture and materials

The HCC cell lines HepG2 and SMMC-7721 were provided by the Shanghai Institute of Cancer Research<sup>2,16</sup>. The cells were maintained on cell plates at 37°C, 5% CO<sub>2</sub> in DMEM (GIBCO) supplemented with 10% FBS, 100 units/mL penicillin and 0.1 mg/mL streptomycin.

### Drugs and treatment

Tanshinone II-A was obtained from the Institute of Traditional Chinese Medicine at a concentration of 96%. It was dissolved in DMSO to a final concentration of 0.2 mL/L. The solution was filtered through a 0.22- $\mu$ m micropore filter and stored at 4 °C, then diluted further in cell culture medium<sup>2</sup>. HepG2 and SMMC-7721 cells were seeded in flasks or dishes. The tanshinone II-A group was treated with different doses (0.25, 0.5, 1.0, 1.5, 2 mg/L) for 24, 48, 72 and 96 hours. The negative control group was treated with an equal concentration of DMSO. The cells were measured over 96 hours of treatment.

### *In vitro* invasion assay

Cell invasion assays were performed as described previously<sup>17</sup> using Transwells (8- $\mu$ m pore size, Corning Costar Corp.). A 50- $\mu$ g aliquot of matrigel solution was placed on the lower surface of a polycarbonate filter and incubated at 37 °C for 2 hours to produce an artificial basement membrane. After rinsing with PBS, the filters were placed into wells, and 600  $\mu$ L of DMEM containing 10% bovine serum was added to the lower compartment. HepG2 or SMMC-7721 cells, either with or without tanshinone II-A at different doses, were added to the upper compartment of the chamber (100  $\mu$ L serum-free DMEM containing 5 $\times$ 10<sup>4</sup> cells), and 600  $\mu$ L of conditioned medium was added to the lower chamber. After 24-hour, 48-hour, 72-hour and 96-hour incubations, cells were removed from the upper surface of the filter with a cotton swab; the cells that had invaded into the bottom surface of the filter were fixed with methanol and stained with hematoxylin. The invasive ability was determined as the number of penetrating cells seen under a microscope at 200 $\times$  magnification in 10 random

fields per well. Each experiment was performed in triplicate.

### *In vitro* migration assay

The *in vitro* migration assay was performed as previously described<sup>17</sup> using Transwells (8- $\mu$ m pore size, Corning Costar Corp.) without matrigel, and was similar to the invasion assay. HepG2 or SMMC-7721 cells were suspended at 2 $\times$ 10<sup>4</sup>/mL. The incubation time was 24 hours. Each experiment was performed in triplicate.

### Wound-healing assay

For monolayer wound-healing assays, a total of 2 $\times$ 10<sup>5</sup> cells were collected and plated in a 12-well plate. At 100% confluence, 2 parallel wounds of 1 mm were made using a pipette tip. Wound size after 24 hours was measured using Zeiss LSM Image Browser software, version 3.1, in 3 independent experiments.

### *In vivo* orthotopic implantation in nude mice

Male athymic BALB/c nu/nu mice were obtained from the Shanghai Institute of Materia Medica, Chinese Academy of Science. Nude mice were handled using best humane practices and were cared for in accordance with the NIH Animal Care and Use Committee guidelines. Human HCC tumor models were established by orthotopic inoculation as described previously<sup>18,19</sup>. A left upper abdominal pararectal incision was made under anesthesia. The left lobe of the liver was exposed and part of the liver surface mechanically injured with scissors. A piece of HepG2 tumor tissue (size, 2 mm  $\times$  2 mm  $\times$  2 mm) was fixed within the liver tissue, the wounds were closed primarily and the abdominal wall was closed. Nude mice bearing orthotopic xenografts were randomized into a control group C0 (0 g/kg/d) and intervention groups C1 (1.5 g/kg/d), C2 (4.5 g/kg/d) and C3 (13.5 g/kg/d), with stepwise increased doses of tanshinone II-A. Daily intragastric administration of tanshinone II-A was performed for 5 consecutive weeks, beginning 24 hours after orthotopic implantation. Five weeks later, the mice were sacrificed. The lung tissues were observed visually, and the number of visible tumors on the lung surface were counted. Lung tissues were prepared as serial sections before being HE dyed and observed under a light microscope. Each experimental group contained 10 mice.

### Gelatin zymography

Gelatin zymography analysis was performed as described<sup>20</sup>. HepG2 cells were grown in SFCM for the required time period. To obtain conditioned SFCM containing matrix metalloproteinases MMP2 and MMP9 as standards, HepG2 cells were grown in SFCM for 24 hours. The culture supernatant was collected by centrifugation. Gelatinases were separated from the SFCM

using Gelatin Sepharose 4B beads and shaking overnight at 4 °C. The beads were washed 3 times with Tris-buffered saline with 0.02% Tween-20 (TBST) and suspended in 50 mL of 1X sample buffer (0.075 g Tris, 0.2 g SDS in 10 mL water, pH 6.8) for 30 minutes at 37°C. The extract was then subjected to zymography on 7.5% by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) co-polymerized with 0.1% gelatin. Gels were washed in 2.5% Triton-X-100 for 30 minutes to remove SDS and incubated overnight in reaction buffer (50 mM Tris-HCl pH 7, 4.5 mM CaCl<sub>2</sub>, 0.2 M NaCl). After incubation, gels were stained with 0.5% Coomassie Blue in 30% methanol and 10% glacial acetic acid. The bands were visualized by destaining the gel with 30% methanol and 10% glacial acetic acid.

#### Preparation of nuclear extract

After treatment, cells were harvested and washed twice with ice-cold PBS, and resuspended in 1 mL of the same buffer. Nuclear extracts were prepared on ice as previously described<sup>21</sup>. After centrifugation at 13,000 rpm, the cell pellet was suspended in ice-cold buffer (10 mM HEPES, 1.5 mM MgCl<sub>2</sub>, 0.2 mM KCl, 0.2 mM phenylmethylsulphonylfluoride, 0.5 mM dithiothreitol), vortexed for 10 seconds and centrifuged at 13,000 rpm for 5 minutes. The nuclear pellet was washed in 1 mL buffer (20 mM HEPES, 25% glycerol, 0.42 M NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA), resuspended in 30 mL buffer, rotated for 30 minutes at 4 °C, then centrifuged for 20 minutes. The supernatant was used as a nuclear extract. The nuclear extracts were analyzed for protein content using the Bradford assay.

#### Western blot analysis

Protein extraction and immunoblot analyses were performed as described<sup>17</sup>. Cells were washed twice with Hanks's balanced salt solution and lysed in RIPA buffer [50 mM Tris-HCl pH 7.4, 1% (v/v) Triton X-100, 1 mM EDTA, 1 mM leupeptin, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 1mM Na<sub>3</sub>VO<sub>4</sub>]. The lysates were centrifuged at 14,000 rpm for 30 minutes at 4 °C and the supernatants were collected. To detect the expression of secreted, active MMPs in the supernatant, 10 mL of conditioned medium was concentrated 100-fold in 10 kDa microcentrifuge concentrators (Millipore). Cell lysate (60 µg) or supernatant proteins (10 µg) were separated by SDS-PAGE, blotted onto nitrocellulose membranes and incubated with the following primary antibodies: mouse monoclonal anti-MMP2 and anti-MMP9 (diluted 1:300; Santa Cruz Biotechnology), anti-NF-κB p65 (diluted 1:1000, Santa Cruz Biotechnology). and mouse monoclonal anti-β-actin (diluted 1:5000; Sigma Chemical Co.) overnight at 4 °C. After repeated washing, the membranes were incubated with horseradish-peroxidase-conjugated anti-mouse secondary antibody (Santa Cruz Biotechnology) diluted 1:2000. The bands were

visualized using the enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech). Each experiment was performed in triplicate.

#### Statistical analysis

Statistical analysis was performed with the Kruskal-Wallis rank test, and the Mann-Whitney U-test was used to calculate *P* values and compare differences in immunohistochemistry data. Assays for characterizing cell phenotypes were analyzed by Student's *t*-test. The SPSS statistical software package (SPSS Inc., Chicago) was used to analyze data. Differences were considered statistically significant at *P* < 0.05.

## Results

#### Tanshinone II-A inhibits *in vitro* invasion and metastasis of HCC cells

The HCC cell lines HepG2 and SMMC-7721 are known to have invasive abilities, as demonstrated by penetration of a matrigel-coated transwell in an *in vitro* invasion assay. They metastasize *in vivo* to the lung rather than other organs after orthotopic implantation in nude mice. Since invasive and migratory potentials are common features in the process of tumor metastasis, we investigated the influence of tanshinone II-A on the invasive and migratory abilities of HCC cells using an *in vitro* invasion and migration assay. We found that tanshinone II-A had inhibitory effects on the invasive and migratory ability of HepG2 and SMMC-7721 cells in a dose- and time-dependent manner (Tables 1 and 2). As shown in Figure 1, tanshinone II-A induced marked,

**Table 1A - Number of invasive HepG2 cells after treatment with tanshinone II-A**

Time (h)	Tanshinone II-A (mg/L)					
	0	0.25	0.5	1	1.5	2
24	26 ± 6	23 ± 4	18 ± 4	16 ± 5	13 ± 4	11 ± 3
48	38 ± 4	35 ± 9	27 ± 6	17 ± 5	16 ± 5	14 ± 6
72	57 ± 9	50 ± 11	35 ± 8	26 ± 8	20 ± 8	17 ± 6
96	62 ± 5	58 ± 8	39 ± 6	30 ± 5	26 ± 7	21 ± 5

**Table 1B - Number of invasive SMMC-7721 cells after treatment with tanshinone II-A**

Time (h)	Tanshinone II-A (mg/L)					
	0	0.25	0.5	1	1.5	2
24	31 ± 7	27 ± 6	21 ± 3	19 ± 4	17 ± 6	14 ± 4
48	48 ± 6	43 ± 7	36 ± 6	27 ± 7	22 ± 4	19 ± 7
72	71 ± 6	66 ± 5	41 ± 9	36 ± 9	27 ± 9	23 ± 5
96	79 ± 8	75 ± 13	50 ± 12	40 ± 7	31 ± 7	25 ± 4

**Table 2A - Number of migratory HepG2 cells after treatment with tanshinone II-A**

Time (h)	Tanshinone II-A (mg/L)					
	0	0.25	0.5	1	1.5	2
24	56 ± 9	50 ± 6	43 ± 7	30 ± 5	25 ± 5	20 ± 6
48	78 ± 6	74 ± 9	68 ± 5	41 ± 11	35 ± 6	28 ± 6
72	97 ± 12	91 ± 8	76 ± 9	49 ± 9	42 ± 9	32 ± 8
96	115 ± 11	103 ± 14	84 ± 6	58 ± 6	49 ± 9	37 ± 5

**Table 2B - Number of migratory SMMC-7721 cells after treatment with tanshinone II-A**

Time (h)	Tanshinone II-A (mg/L)					
	0	0.25	0.5	1	1.5	2
24	71 ± 8	64 ± 5	55 ± 8	38 ± 9	33 ± 4	27 ± 9
48	98 ± 7	92 ± 12	77 ± 11	46 ± 7	42 ± 7	34 ± 7
72	121 ± 13	109 ± 14	88 ± 15	55 ± 7	50 ± 9	37 ± 9
96	134 ± 10	126 ± 8	95 ± 10	61 ± 9	54 ± 6	41 ± 5

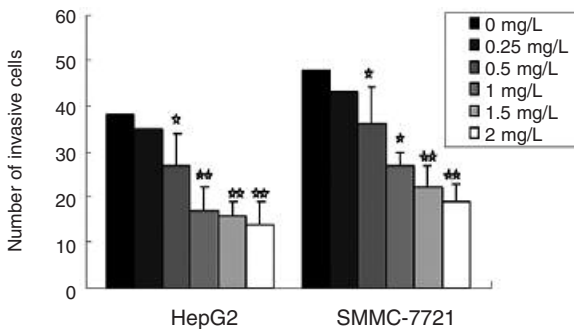
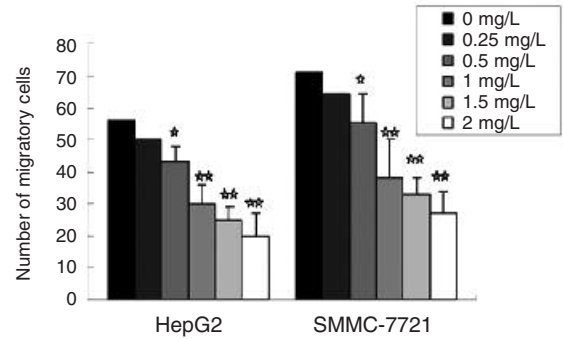


Figure 1 - Effects of tanshinone II-A on invasion by hepatocarcinoma cells. A representative experiment of 3 with similar results is shown. After 48 hours of incubation with 0.25, 0.5, 1.0, 1.5 and 2 mg/L tanshinone II-A, the invasive ability was evaluated by counting cells that invaded through a transwell with matrigel and an 8- $\mu$ m pore membrane. \* $P < 0.05$  and \*\* $P < 0.01$  vs cells without treatment.

dose-dependent inhibition of the invasion abilities of HepG2 and SMMC-7721 cells. At 0.5 mg/L, an inhibitory effect was noticeable, while at 1 mg/L the inhibitory rate was 53.15%, with an average inhibition rate of 48.15% compared with the control group at 48 hours. Similar results were observed in *in vitro* migration assays. Tanshinone II-A significantly inhibited migration of HepG2 and SMMC-7721 cells in a Boyden chamber assay without matrigel, with an average inhibition rate of 48.15% compared to the control group at 24 hours. The results were also similar in the wound-healing assay (Figure 2A and 2B). The inhibitory effect became gradually stronger over time, with time points taken at 24, 48, 72 and 96 hours. The most significant effect was observed at 48 hours (Table 2). Both *in vitro* invasion and

A



B

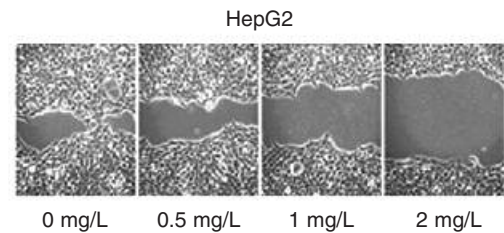


Figure 2 - Effects of tanshinone II-A on migration by hepatocarcinoma cells. A representative experiment of 3 with similar results is shown. A) After 24 hours of incubation with 0.25, 0.5, 1.0, 1.5 and 2 mg/L tanshinone II-A, the migratory ability was evaluated by counting cells that migrated through an 8- $\mu$ m pore membrane in a transwell. \* $P < 0.05$  and \*\* $P < 0.01$  vs cells without treatment. B) The migratory ability of HepG2 was evaluated by a wound-healing assay after 24 hours of incubation with 0, 0.5, 1.0, and 2 mg/L tanshinone II-A.

migration assays suggested that tanshinone II-A had the potential to inhibit HCC metastasis.

#### Tanshinone II-A inhibited *in vivo* metastasis of HCC cells

An *in vivo* orthotopic implantation assay in nude mice was used to examine the inhibiting effects of tanshinone II-A on *in vivo* metastasis of HepG2 cells. Compared to the control group, tanshinone II-A significantly inhibited lung metastasis of liver cancer after orthotopic implantation of HepG2 tumor tissue (Table 3). The

**Table 3 - Number of visible tumors on the lung surface of mice treated with different doses of tanshinone II-A**

Group	Dose (g/kg/d)	Number of lung metastases	$P$ value
C0	0	14 ± 5	
C1	1.5	12 ± 4	$P > 0.05$
C2	4.5	9 ± 4	$P < 0.05$
C3	13.5	6 ± 3	$P < 0.01$

Tanshinone IIA treatment for 5 weeks at the indicated dose. The lung tissues were observed visually, and the number of visible tumors on the lung surface were counted.

tumor inhibition rates in the C2 and C3 groups were 35.71% and 57.14% (Figure 3, both  $P < 0.05$ ). Both the *in vitro* invasion assay and the *in vivo* nude mice assay suggested that tanshinone II-A could inhibit hepatocellular carcinoma metastasis.

#### Regulation of MMP2 and MMP9 by tanshinone II-A in invasion by HCC

Extracellular matrix (ECM) degradation is an essential step in tumor invasion and metastasis, and is mainly mediated by metalloproteinases such as MMP2 and MMP9<sup>22,23</sup>. We therefore examined the effect of tanshinone II-A on the expression of MMP2, MMP9 and uPA in HCC cells. Western blotting and gelatin zymography showed that expression of MMP2 and MMP9 proteins in the cytoplasm (inactive form, 92 kDa) and supernatant (active form, 86 kDa) of HepG2 cells was downregulated

by tanshinone II-A in a time- and dose-dependent manner (Figure 4). Levels of the  $\beta$ -actin loading control were similar for all samples, and SMMC-7721 cells gave similar results. Taken together, these results suggested that the inhibiting effect of tanshinone II-A on metastasis of HCC cells was at least partially mediated by downregulation of MMP2 and MMP9 proteins, which normally cause ECM degradation.

#### Involvement of NF- $\kappa$ B in the effects of tanshinone II-A on HCC

Previous studies showed that expression of MMPs can be induced during hyperglycemia via the activation of transcription factors such as NF- $\kappa$ B<sup>24</sup>. The phosphorylation of p65, a major subunit of NF- $\kappa$ B, leads to translocation to the nucleus, which is required for transcriptional activation. We investigated p65 levels in the nu-

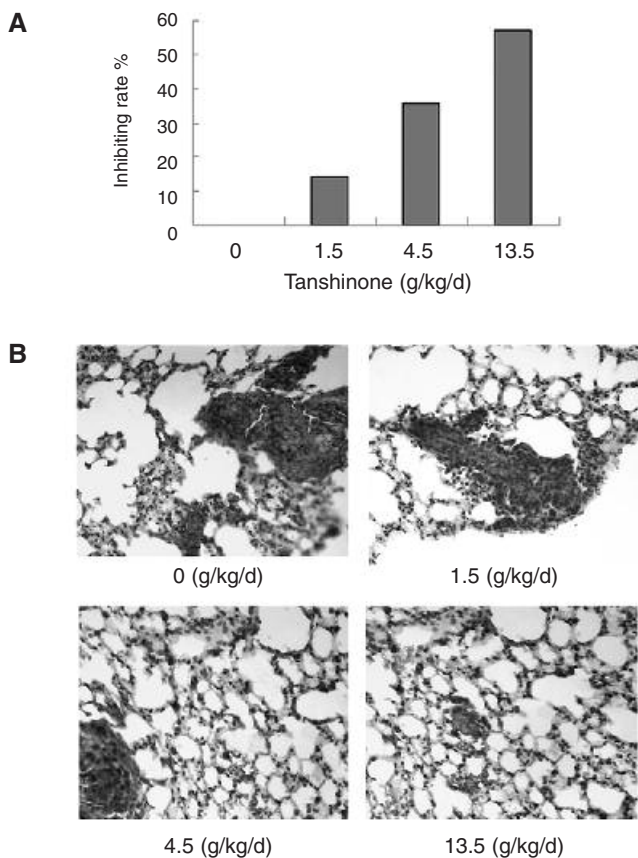


Figure 3 - Effects of tanshinone II-A on the metastatic ability of HepG2 cells in nude mice. A) Inhibition by tanshinone II-A of metastasis of HepG2 cells in nude mice. An *in vivo* metastasis model was made by orthotopic implantation of HepG2 tumor tissue into the livers of nude mice. Experimental and control groups consisted of 10 mice each. Groups were treated with tanshinone II-A at 0 g/kg/d, 1.5 g/kg/d, 4.5 g/kg/d and 13.5g/kg/d, respectively. Four weeks later, the mice were sacrificed. The number of visible tumors on the lung surface was counted. \* $P < 0.05$  vs the control group without tanshinone II-A. B) Serial sections of lung tissues were HE dyed and observed under a light microscope.

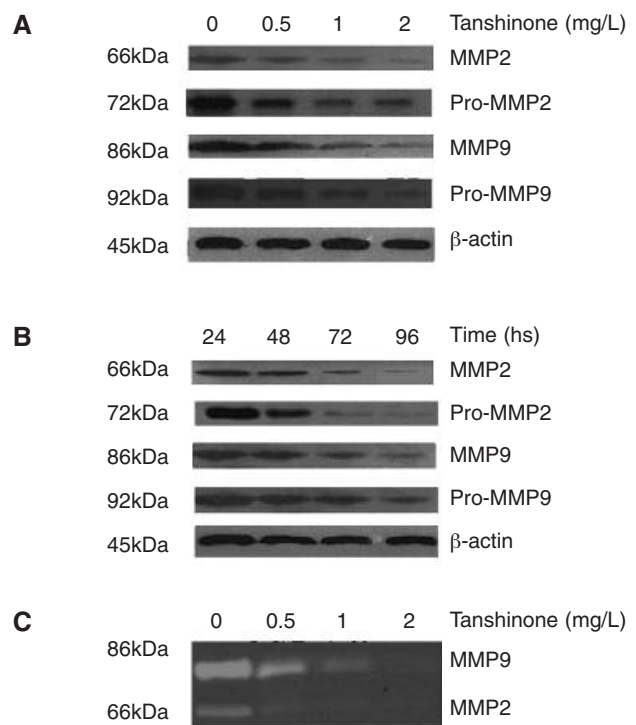


Figure 4 - The effect of tanshinone II-A on MMP2 and MMP9 in hepatocarcinoma cell lines. A representative experiment of 3 with similar results is shown. The expression of MMP2 and MMP9 in the cytoplasm, and the active forms of MMP2 and MMP9 in the supernatant were evaluated by Western blot.  $\beta$ -actin was used as an internal control. A) After 48 hours of incubation with 0, 0.5, 1.0 and 2 mg/L tanshinone II-A, the levels of MMP2 and MMP9 in hepatocarcinoma cells decreased dose-dependently compared to cells without treatment ( $P < 0.05$ ), as indicated by the ratio of MMP2/actin and MMP9/actin. B) After 24, 48, 72 and 96 hours of incubation with 1 mg/L tanshinone II-A, the levels of MMP2 and MMP9 in hepatocarcinoma cells decreased time-dependently ( $P < 0.05$ ).  $\beta$ -actin was used as internal control. C) HepG2 cells were pre-cultured in serum-free medium treated with tanshinone II-A (0, 0.5, 1, 2 mg/L) for 24 hours. The activities of MMP2 and MMP9 were determined by gelatin zymography on the cell supernatants.

cleus of HCC cells after treatment with tanshinone II-A. As shown in Figure 5, translocation of NF- $\kappa$ B in the nuclear fractions of HepG2 was significantly and dose-dependently decreased by treatment with tanshinone II-A.

## Discussion

HCC is a malignant tumor with a high worldwide death rate. Fifty-five percent of HCC cases are in China alone. In Europe, the mortality from primary liver cancer increased 4-fold for men and 2-fold for women between 1979 and 1994<sup>4,24</sup>, and there was a 41% increase in the mortality rate over the past 2 decades in the United States<sup>3</sup>. HCC is one of the most prevalent cancers in Asia and Africa<sup>25</sup>. More than 70% of patients are not candidates for surgical treatment at the time of HCC diagnosis, and systemic treatment is often not effective. Even in patients undergoing curative resection, postoperative metastasis and recurrence remain major obstacles for further survival<sup>26</sup>. Most patients are of middle or advanced age when diagnosed. Therefore, finding a new therapy for mid- and late-stage hepatocarcinoma from traditional Chinese medicine may expand our understanding of this disease and develop new therapeutic drugs.

Tanshinone II-A is an alcohol-extracted product from the root of the traditional Chinese medicine plant *Salvia miltiorrhiza Bunge*. Although previous reports showed that it could induce apoptosis and inhibit the proliferation of some kinds of tumors<sup>2</sup>, its effect on metastasis of HCC and the mechanisms are not fully known. In the present study, we provide evidence that tanshinone II-A can effectively suppress invasion and metastasis of hepatocarcinoma in *in vitro* assays. Further, we examined the mechanisms of the inhibiting effects of tanshinone II-A. Previous studies showed that invasion and metastasis of solid tumors require the action of tumor-associated proteases, which promote the dissolution of the surrounding tumor matrix and the basement membrane<sup>27</sup>. MMPs play an important role in that process in carcinomas<sup>28-31</sup>. In this study, we observed the relationship between tanshinone II-A treatment and MMPs. We found that tanshinone II-A treatment could reduce the levels of MMP2 and MMP9 proteins. Downregulation by

tanshinone II-A of MMP2 and MMP9 could reduce degradation of the ECM and basement membrane and the subsequent mobilizing of growth factors that promote survival, cell migration and invasion<sup>32</sup>. Thus, *in vitro* evidence demonstrated that tanshinone II-A significantly inhibited invasion of HCC cells, at least partly by downregulating the protein expression of MMP2 and MMP9.

NF- $\kappa$ B exists in the cytoplasm bound to an inhibitory protein, I- $\kappa$ B. Upon stimulation, it acts as an inducible transcription factor by forming homodimers or heterodimers with proteins of the NF- $\kappa$ B family, including p65, p50/p105, p52/p100, RelB, and c-Rel<sup>21</sup>. Previous work showed that NF- $\kappa$ B activation is associated with the expression of MMPs and human aortic smooth muscle cell (HASMC) migration<sup>33</sup>. When NF- $\kappa$ B was activated by stimulating phosphorylation, I- $\kappa$ B was degraded<sup>34,35</sup>. Activated NF- $\kappa$ B is translocated into the nucleus, leading to transcriptional expression of genes associated with cellular metastatic properties<sup>21</sup>. In this study we evaluated the effect of tanshinone II-A on phosphorylation of the p65 subunit during NF- $\kappa$ B activation. We found that p65 phosphorylation was altered, which might lead to a decrease in translocation in a dose-dependent manner by treatment with tanshinone II-A. A limitation of this study is the relative high dosages of tanshinone II-A required to observe anticancer effects. This is just a preliminary study and further optimization of tanshinone II-A through trials determining adequate dosage in cell and animal models is required. It could be that this compound may serve as a lead compound to generate drugs that would work at lower doses, but only if it is chemically appropriate for this compound.

In summary, we found that tanshinone II-A could significantly inhibit *in vitro* invasion and metastasis of HCC cells, at least in part by downregulating the protein expression of MMP2 and MMP9. These activities may be partly mediated by blocking NF- $\kappa$ B activation. We believe that tanshinone II-A could be a prospective highly effective anticancer drug with low toxicity that could lead to a therapeutic drug for HCC.

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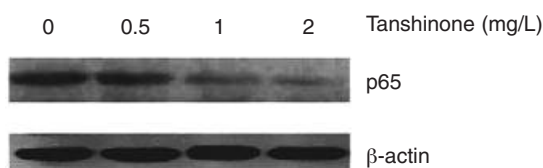


Figure 5 - The effect of tanshinone II-A on NF- $\kappa$ B p65 in hepatocarcinoma cell lines. A representative experiment of 3 with similar results is shown. The expression of p65 in the nucleus was evaluated by Western blot.  $\beta$ -actin was used as internal control.

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