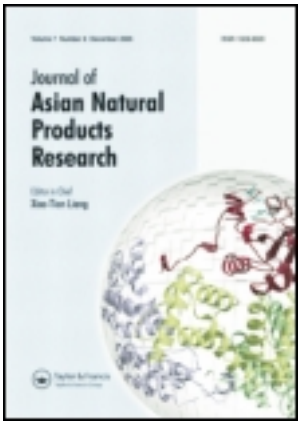

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Matrine inhibits matrix metalloproteinase-9 expression and invasion of human hepatocellular carcinoma cells

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Matrine inhibits matrix metalloproteinase-9 expression and invasion of human hepatocellular carcinoma cells

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Matrine is the major active component of the traditional Chinese medicine *Sophora flavescens*, but the molecular mechanisms of matrine on tumor invasion inhibition remain unclear. The aim of this study is to elucidate the effects of matrine on invasion ability of human hepatocellular carcinoma (HCC) cells, matrix metalloproteinase-9 (MMP-9), and nuclear factor (NF)-kappa B expression. The expression activity of MMP-9 was measured by reverse transcription polymerase chain reaction, Western blot, and gelatin zymography analysis. The expression of NF-kappa B was measured by the Western blot analysis. Matrine significantly inhibited MMP-9 expression of SMMC-7721 cells. NF-kappa B inhibitor PTDC induced a marked reduction in MMP-9 expression, and it suggested that NF-kappa B could play an important role in MMP-9 expression. Furthermore, matrine significantly suppressed NF-kappa B expression and the invasion of SMMC-7721 cells. Our results showed that matrine inhibited MMP-9 expression and the invasion of human HCC cells. The inhibitory effects are partly associated with the downregulation of the NF-kappa B signaling pathway.

Keywords: matrine; NF-kappa B; MMP-9; invasion

1. Introduction

Hepatocellular carcinoma (HCC) was the sixth most common cancer and the third most common cause of death from cancer [1]. As a result of the early and extensive spread of HCC, the mortality rate of patients with HCC is very high. The mechanisms of HCC progression remain largely unknown and there is no effective therapeutic treatment. Therefore, there is a need to clarify the metastasis and invasion mechanisms of HCC, and to explore more effective anticancer drugs and therapeutic approaches.

The main physiologic barriers to the movement of cell into tissues are the invasion of malignant cancer cells into the extracellular matrix (ECM) and basement membrane (BM), so the degradation of ECM and BM is the critical step in the process of cancer invasion and metastasis [2]. Matrix metalloproteinases (MMPs) comprise a family of at least 28 secreted or transmembrane enzymes. These enzymes play critical roles in the maintenance and turnover of ECM macromolecules including the interstitial and basement collagen. Matrix metalloproteinase-9 (MMP-9) is an endopeptidase of the large MMPs family [3]. An enhanced expression of MMP-9 has been shown to be associated with the progression and invasion of tumors [4,5].

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Over expression of MMP-9 is associated with the capsular infiltration of HCC and the growth of small HCC was reported [6,7]. These researches indicate that MMP-9 plays an important role in tumor invasion and metastasis, and that inhibition of its expression may be an effective technique for the prevention and treatment of malignant tumors. So, inhibiting MMP-9 expression is necessary for the HCC therapy.

Nuclear factor-kappa B (NF-kappa B) is a transcription factor discovered by Sen and Baltimore [8], in the kappa light chain of immunoglobulins in B cells. NF-kappa B has been found to be a ubiquitously expressed transcription factor for genes involved in cell survival, cell adhesion, inflammation, differentiation, and growth [9]. Amplification and overexpression of NF-kappa B have been found in cervical, ovarian, gastric, and breast carcinomas [10–13]. A recent study has demonstrated that NF-kappa B played a main role in the tight regulation of survival/proliferation pathways exerted by quercetin and that inhibition of NF-kappa B provoked by the flavonoid induced SMMC-7721 death [14]. Overexpression of NF-kappa B is also able to upregulate the expression of MMP-9 and enhance the invasive capacity of tumor cells [15]. These observations suggested that, in HCC cells, the NF-kappa B signaling pathway might be critical for the spread of metastatic cell.

Matrine (**1**) is the major active component of the traditional Chinese medicine *Sophora flavescens* (Figure 1). It has been widely used in China for the treatment of viral hepatitis, hepatic fibrosis, arrhythmia, atherosclerosis, and skin diseases [16–19]. Recently, interest has been generated in the antitumor activity of **1**. Studies have indicated that **1** could inhibit tumor cell proliferation and induce cell differentiation and apoptosis [20].

In this report, we demonstrate that the treatment with **1** inhibits MMP-9 gene expression, and its mechanisms of action may be mediated via the inhibition of the NF-kappa B signaling pathway in cultured human HCC cells.

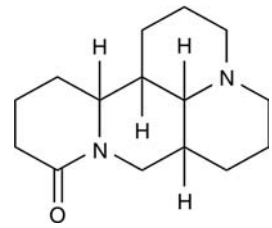

Figure 1. Chemical structure of **1**. The molecular formula of **1** is $C_{15}H_{24}N_2O$ and its molecular weight 248.36.

2. Results

2.1 Effects of 1 on the growth inhibiting rate of SMMC-7721 cells

The *in vitro* cell growth assay showed that **1** increased the growth inhibiting rate of human HCC cell line SMMC-7721 in dose-dependent manners after the cells were treated with **1** at 0–200 μM for 24 h, respectively (Figure 2).

2.2 Effect of 1 on MMP-9 activity

We conducted an reverse transcription polymerase chain reaction (RT-PCR) analysis to investigate the effect of **1** on MMP-9 mRNA expression in human hepatoma SMMC-7721 cells. The treatment of cells with **1** inhibits MMP-9 mRNA expression, as demonstrated in Figure 3. We used a specific kinase inhibitor pyrrolidine dithiocarbamate (PDTC) to examine the important role of NF-kappa B signaling pathway in the regulation of MMP-9 expression. We found the NF-kappa B specific inhibitor PDTC at 100 μM could significantly suppress MMP-9 mRNA expression in RT-PCR analysis ($p < 0.05$). At the same time, MMP-9 protein expression was obviously inhibited by PDTC ($p < 0.05$) according to Figure 4. Our results from zymography indicated that **1** could significantly suppress MMP-9 activity in a dose-dependent manner according to Figure 5.

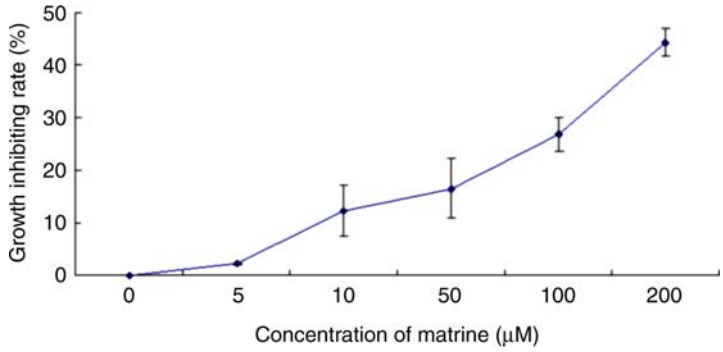

Figure 2. *In vitro* effects of **1** on the growth inhibiting rate of SMMC-7721 cells.

It suggested that the expression of MMP-9 was partly regulated by the NF-kappa B signaling pathway. Meanwhile, **1** inhibited MMP-9 expression ($p < 0.05$).

2.3 Effect of 1 on NF-kappa B activation

Transcription of several MMPs, including MMP-1, MMP-3, and MMP-9, is regulated by NF-kappa B. To further elucidate the effect of **1** on NF-kappa B activity, the human hepatoma SMMC-7721 cells were treated with **1** and the expression of NF-kappa B was measured by Western blot analysis. As shown in Figure 6, **1** significantly inhibited NF-kappa B activation in human hepatoma SMMC-7721 cells. This result suggests that NF-kappa B is probably involved in the inhibitory effect of **1** on MMP-9 expression.

2.4 Effect of 1 on human hepatoma SMMC-7721 cells invasion

Our results showed that **1** significantly inhibited HCC cell invasion (Figure 7).

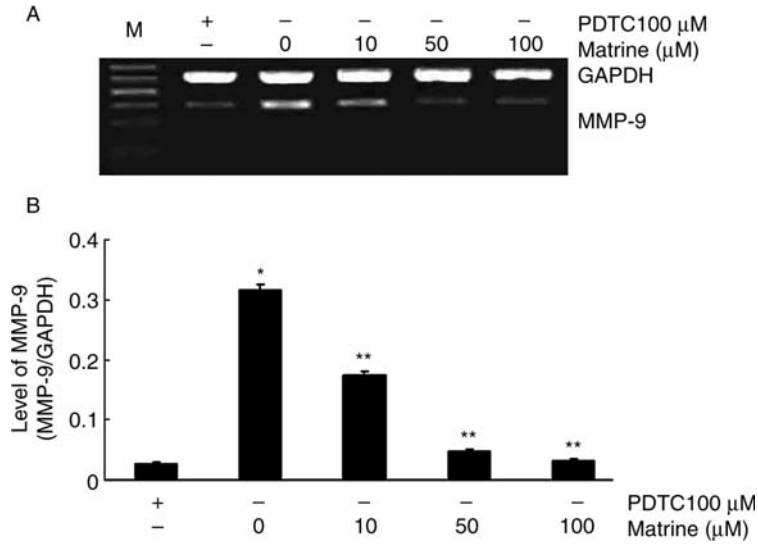

Figure 3. Effect of **1** on MMP-9 mRNA expression in human hepatoma SMMC-7721 cells. After 24 h treatment of PDTC and **1**, total RNA of hepatoma SMMC-7721 cells was isolated and RT-PCR analysis was performed. Levels of detection represent the expression of MMP-9 mRNA in hepatoma SMMC-7721 cells, and are noted in (A). Densitometric analysis is shown in (B). $*p < 0.05$, $**p < 0.01$ versus control. All the data shown are representative of three independent experiments.

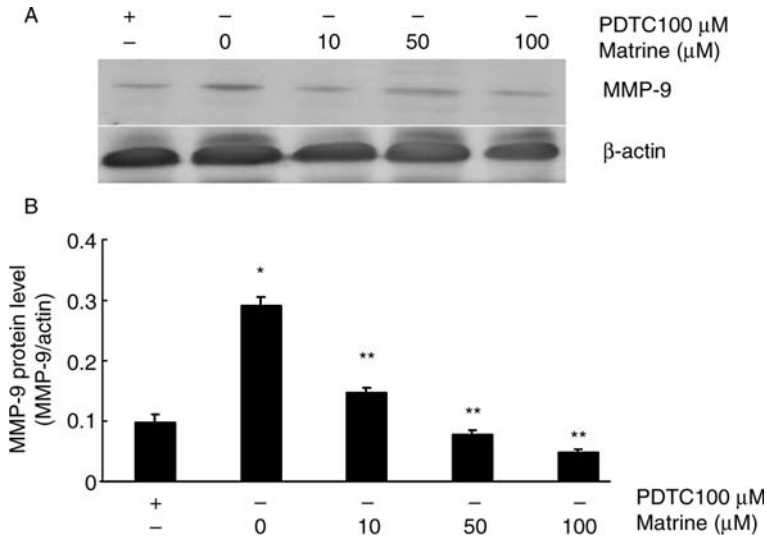

Figure 4. Effect of **1** on MMP-9 protein expression in human hepatoma SMMC-7721 cells. After 24 h treatment of **1**, the total SMMC-7721 cells lysates were blotted with anti-MMP-9 antibody by Western blot assay. Immunoreactive bands are noted in (A). Densitometric analysis is shown in (B). $*p < 0.05$, $**p < 0.01$ versus control. All the data shown are representative of three independent experiments.

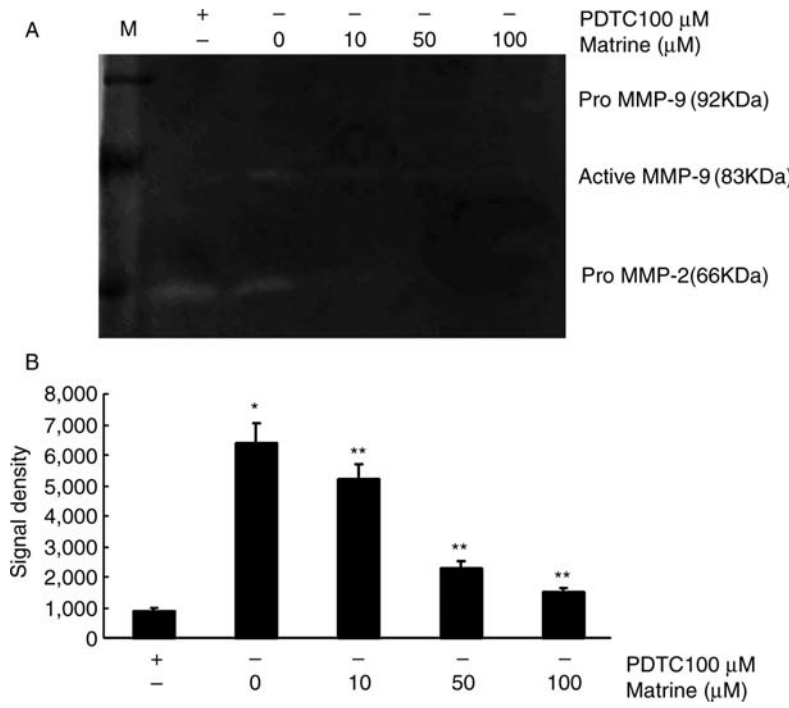

Figure 5. Effect of **1** on MMP-9 activity in human hepatoma SMMC-7721 cells. After 24 h treatment of **1**, conditioned medium was collected and loaded into gelatin-containing zymogram gel. Levels of detection represent the zymogen expression of MMP-9 in SMMC-7721 cells, and MMP-9 bands are noted in (A). Densitometric analysis is shown in (B). $*p < 0.05$, $**p < 0.01$ versus control. All the data shown are representative of three independent experiments.

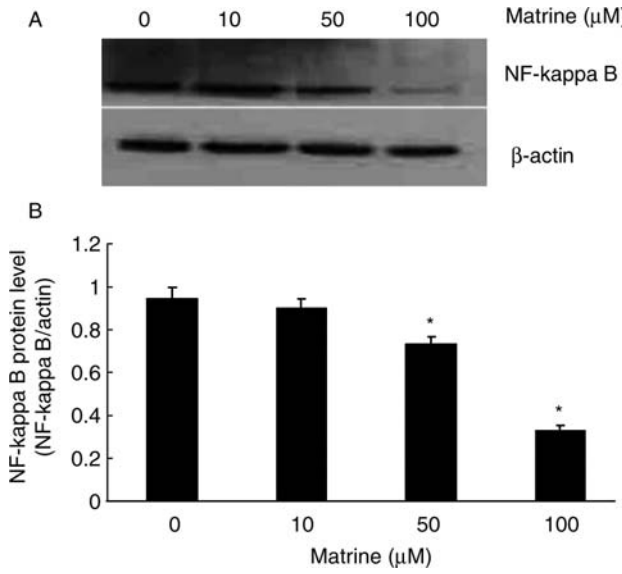

Figure 6. Effect of **1** on NF-kappa B expression in human hepatoma SMMC-7721 cells. After 24 h treatment of **1**, the total SMMC-7721 cells lysates were blotted with anti-NF-kappa B antibody by Western blot assay. Immunoreactive bands are noted in (A). Densitometric analysis is shown in (B). $*p < 0.05$ versus control group. All the data shown are representative of three independent experiments.

These results suggested that **1** could not only inhibit MMP-9 activity but also prevent the invasion of HCC cells. Therefore, **1** could function as a chemotherapeutic and therapeutic agent to prevent and suppress the spread of HCC cells.

3. Discussion

This study demonstrates that **1** inhibits MMP-9 expression in human hepatoma SMMC-7721 cells and that its inhibitory action may be mediated through the suppression of the NF-kappa B signaling pathway.

It was suggested that active constituents of traditional Chinese medicines may inhibit the invasiveness and metastasis of malignancies effectively through one or more of the following mechanisms of tumor metastasis: the inhibition of the degradation effect of proteolytic enzymes on the BM and ECM inhibition of the adhesion of tumor cells to the matrix component; the inhibition of tumor angiogenesis; the regulation of the expression of tumor metastasis-associated genes and tumor metastasis-suppressive genes; and the regulation of the tumor metastasis-associated signal transduction pathway. In this study, we investigated the effect of **1** on invasion, and levels of MMP-9 mRNA and protein of SMMC-7721 cells to determine the possible mechanisms of its antimetastatic activity.

Since MMPs play a major role in promoting angiogenesis and tumor metastasis [21], agents inhibiting that expression may prove useful in treating such diseases. An enhanced expression of MMP-9 was shown to be associated with the progression and invasion of tumors [22,23]. Therefore, in this study, we investigated the effect of **1** against MMP-9 expression. We found that **1** efficiently suppressed MMP-9 expression in human hepatoma SMMC-7721 cells.

At the same time, MMP-9 promoter consists of NF-kappa B binding sites, and it was plausible to investigate the important roles of NF-kappa B signaling pathways in

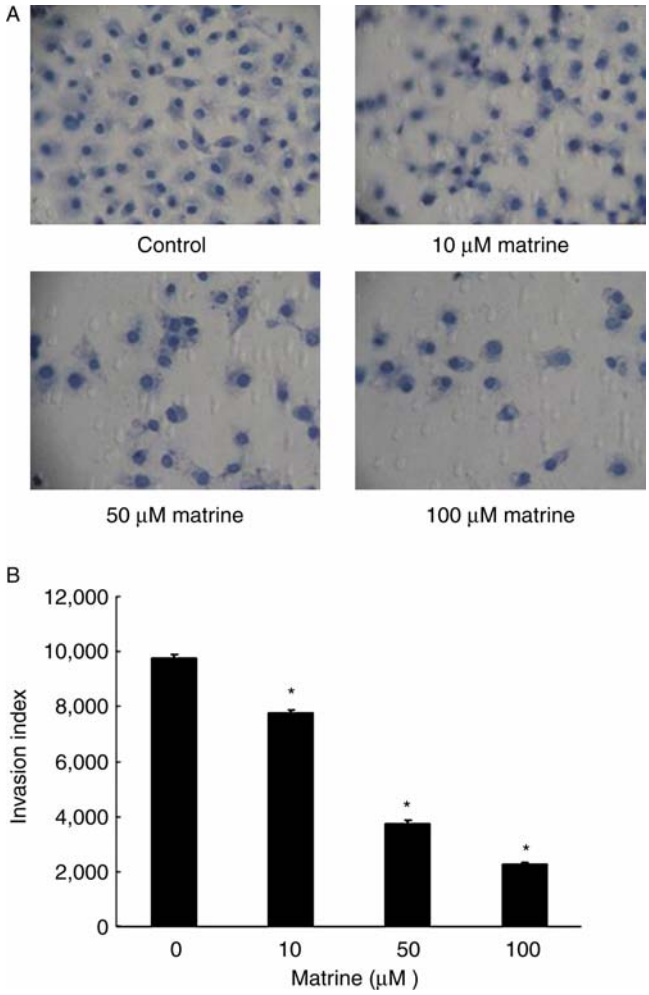

Figure 7. Effect of **1** on SMMC-7721 cells invasion. After incubation with **1** for 24 h, cells were stained and the number of cells invading the lower side of the filter was measured as invasive activity. (A) Microphotographs of invasive SMMC-7721 cells. (B) The number of invasive cells. $*p < 0.05$ versus control. The data shown are representative of three independent experiments.

MMP-9 activation. NF-kappa B signaling pathway was shown to correlate with MMP-9 expression and tumor invasion, and the NF-kappa B sites are essential for MMP-9 expression [24]. In this study, we found that **1** markedly suppressed the transcriptional activity of NF-kappa B.

To investigate whether MMP-9 upregulation is mediated through the activation of the NF-kappa B signaling pathway, we test our hypothesis by using a specific kinase inhibitor PDTC, an NF-kappa B specific inhibitor [25]. As shown in Figure 1, 100 μM PDTC could effectively block the expression of MMP-9. The result suggested that MMP-9 expression is sensitive to NF-kappa B signaling pathway. The activation of NF-kappa B signaling pathway played an important role in the regulation of MMP-9 expression.

Since MMP-9 played an important role in cellular invasion, we further examined the effects of **1** on human HCC cell invasion. **1** significantly inhibited cellular invasion in a dose-dependent manner.

Therefore, the effect of **1** on *in vitro* invasion inhibition was presumably correlated with its effect on MMP-9 inhibition.

In conclusion, the present study demonstrates that **1** can inhibit MMP-9 expression, and the mechanisms underlying its action may be mediated through the inhibition of the NF-kappa B signaling pathway. These findings suggested that **1** could be of therapeutic value in the prevention of MMP-9-related tumor invasion.

4. Materials and methods

4.1 Materials

Matrine (**1**) (98%), which was obtained from Sigma (St Louis, MO, USA), was dissolved in dimethylsulfoxide (DMSO) and used for the cell culture (1:1000 dilution). Dulbecco's modified eagle's medium (DMEM), trypsin, Trizol reagent, and fetal bovine serum (FBS) were obtained from Gibicol (Carlsbad, CA, USA). ExScriptTM RT reagent kit and Taq DNA polymerase were obtained from Invitrogen (Carlsbad, CA, USA). Goat monoclonal antihuman MMP-9 antibody was from NeoMarker (Westinghouse Dr Fremont, CA, USA). Rabbit polyclone antihuman NF-kappa B antibody was from Santa Cruz (Santa Cruz, CA, USA). Millicell invasion chambers were obtained from Millipore (Billerica, MA, USA). radioimmunoprecipitation assay (RIPA) buffer was purchased from Biotek Corporation (Thorold, CA, USA).

4.2 Cell culture and in vitro *cell growth assays*

Human hepatoma SMMC-7721 cells were purchased from Shanghai Institute of Cell Biology, Chinese Academy of Sciences, Shanghai, China. Human hepatoma SMMC-7721 cells were grown in DMEM with high glucose (4.5 g/L) and supplemented with 10% FBS, 100 units/ml of penicillin and 100 mg/ml of streptomycin, and sodium bicarbonate (2.0 g/L) at 37°C in 5% CO_2 incubator with saturated humidity. The *in vitro* assays were done according to the published methods. The cells in control group were treated with DMSO (0.1%, final concentration). The cells were, respectively, incubated in DMEM and DMEM medium supplemented with 10% FBS (in the case of *in vitro* assay) containing different concentrations of **1**. Cell viability was measured 24 h after the treatments using MTT assay kit. Each experiment was repeated three times.

4.3 Reverse transcription polymerase chain reaction

SMMC-7721 cells in the logarithmic growth phase were put into 25 ml culture bottles at the density of 2×10^5/ml. All the cells were cultured with DMEM containing 10% FBS for 24 h and with serum-free medium for another 24 h. At the end of culture, cells were divided into control group (serum-free DMEM) and experimental groups (medium containing 100 μM PDTC and **1** at concentrations of 10, 50, and 100 μM, respectively) and cultured for 24 h. Total cellular RNA was isolated by the Trizol reagent extraction at the indicated time. Total RNA (0.5 mg) was reversely transcribed into cDNA using the ExScriptTM RT reagent kit. The primers were designed and synthesized by Augct Biotechnology (Beijing, China). MMP-9 (328 bp) sense 5′-TCC CTG GAG ACC TGA GAA CC-3′ antisense 5′-CGG CAA GTC TTC CGA GTA GTT-3′; GAPDH (527 bp) sense 5′-TCC TGC ACC ACC AAC TGC TT-3′ antisense 5′-TCC ACC ACC CTG TTG CTG TA-3′. The reaction conditions were set as follows: 95°C for 5 min, 32 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s; followed by extension at 72°C for 7 min. PCR product analysis: MMP-9 PCR product and GAPDH PCR product from the same sample were observed with 1.5% agarose gel electrophoresis. The images

with gel imaging system were fixed and the target gene expression level with the semiquantitative analysis was measured. Relative content of mRNA = accumulative photon value of target gene band/accumulative photon value of internal standard GAPDH band.

4.4 Protein extraction and Western blotting assay

SMMC-7721 cells in the logarithmic growth phase were transferred into 25 ml culture bottles at the density of 2×10^5/ml. All the cells were cultured with DMEM of 10% FBS for 24 h and with serum-free medium for another 24 h. At the end of culture, cells were divided into control group (serum-free DMEM) and experimental groups (medium containing 100 μM PDTC and **1** at concentrations of 10, 50, and 100 μM, respectively) and cultured for 24 h. According to the instruction of RIPA buffer, cell lysates were collected by centrifugation at 12,000 rpm for 15 min at 4°C, and then transferred to clean microcentrifuge tubes. Protein concentration was determined by the bicinchoninic acid protein assay. Fifty micrograms of lysate protein from each group were separated by 10% SDS-polyacrylamide gel electrophoresis and then transferred to nitrocellulose membrane at 300 mA for 70 min. After blocking with 5% nonfat dried milk overnight, the membrane was probed with β-actin, NF-kappa B, and MMP-9 antibody, respectively, and incubated at 4°C overnight. The membrane was washed three times with 0.1% Tween 20-TBS and then incubated in the horseradish peroxidase-linked secondary antibody for 90 min at room temperature. After rinsing the membrane three times with 0.1% Tween 20-TBS, the immunoreactive bands were detected using enhanced chemiluminescent plus reagent kit. The band density for the target protein in each sample was measured with the image analysis software (Gene Genus, Gene Company, Hong Kong, China) and normalized to β-actin expression.

4.5 Gelatin zymography

SMMC-7721 cells in the logarithmic growth phase were transferred into 25 ml culture bottles at the density of 2×10^5/ml. All the cells were cultured with DMEM of 10% FBS for 24 h and with serum-free medium for another 24 h. At the end of culture, cells were divided into control group (serum-free DMEM) and experimental groups (medium containing 100 μM PDTC and **1** at concentrations of 10, 50, and 100 μM, respectively) and cultured for 24 h. Conditioned medium was collected and loaded into 10% polyacrylamide-SDS gel with 1 mg/ml of gelatin. The gel was incubated at room temperature for 2 h in the presence of 2.5% Triton X-100 and subsequently for 42 h in substrate buffer (50 mM Tris-HCl, 5 mM $CaCl_2$, 1.0 mM $ZnCl_2$ and 0.02% NaN_3, pH 7.5) at 37°C. The gel was stained for protein with 0.25% Coomassie blue and photographed on a light box. Proteolysis was detected as a white zone in a dark field. The band density for the target protein in each sample was measured with the image analysis software (Gene Genus, Gene Company, Hong Kong).

4.6 Invasion assay

The invasion of tumor cells was analyzed in Millicell invasion chambers with a polyvinyl/pyrrolidone-free polycarbonate filter of 12 μm pore size. Each filter was coated with 100 μl of a 1:10 diluted matrigel in cold DMEM to form a thin continuous film on the top of the filter. Then, the chambers were divided into control group and experimental groups. In control group, SMMC-7721 cells were cultured in DMEM (50,000 cells/well) without stimulation; in experimental groups, SMMC-7721 cells were stimulated with **1** at concentrations of 10, 50, and 100 μM, respectively.

Five hundred micrometer conditioned medium with 10% FCS were added to the lower compartment of the invasion chamber. The Millicell invasion chambers were incubated at 37°C in 5% CO_2. After incubation for 24 h, cells in 10 randomly selective fields were counted. The number of cells invading the lower side of the filter was measured as invasive activity (invasive index).

4.7 Statistical analysis

The results were expressed as mean ± SD. All the data were processed with the software SPSS13.0. One-factor variance analysis was used in the comparison of several groups, while *t*-test (student test) was used to compare two groups. $p < 0.05$ was considered statistically significant.

Acknowledgements

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