Therapeutic effects of tyroserleutide on lung metastasis of human hepatocellular carcinoma SK-HEP-1 and its mechanism affecting ICAM-1 and MMP-2 and -9

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Background: Tyroserleutide (YSL) inhibits the growth and metastasis of human hepatocellular carcinoma (HCC). This paper studied the effect of YSL on metastasis of human HCC and investigated its mechanisms.

Methods: In vivo, experimental lung metastasis models of human HCC SK-HEP-1 cells in nude mice were established, and in vitro, the proliferation, adhesion and invasion of SK-HEP-1 cells were detected.

Results: In vivo, YSL significantly inhibited the metastasis of human HCC. In vitro, YSL significantly inhibited the proliferation, adhesion and invasion of SK-HEP-1 cells. Through analyses with reverse transcription PCR (RT-PCR) and Western blot, we observed that YSL significantly inhibited the expressions of ICAM-1 in SK-HEP-1 cells. Through RT-PCR, Western blot and zymography methods, YSL was discovered to decrease the mRNA level, protein expression and activity of MMP-2 and -9 in SK-HEP-1 cells.

Conclusion: We concluded that YSL could inhibit tumor growth and metastasis of human HCC SK-HEP-1 cells.

Keywords: proliferation, adhesion, invasion, ICAM-1, MMP-2, MMP-9

Introduction

Tumor metastasis is the essential feature of biological behavior of malignant tumors. Human hepatocellular carcinoma (HCC) is one of the most common human carcinomas, with a high mortality rate. HCC is the fifth most common human cancer and the second cause of cancer-related death worldwide.1 Thus, it is crucial to find effective, low-toxicity drugs. Tyroserleutide (YSL), as a tripeptide compound, which is made up of L-tyrosine, L-serine and L-leucine, has been approved by the Chinese Pharmacopeia Commission. Its structural formula is C16H27N3O6, and the formula weight is 381.42. In our early research, YSL was found to have a significant therapeutic impact on HCC.

In vivo, YSL significantly inhibited the metastasis of human HCC. In vitro, YSL significantly inhibited the proliferation, adhesion and invasion of SK-HEP-1 cells. Through analyses with reverse transcription PCR (RT-PCR) and Western blot, we observed that YSL significantly inhibited the expressions of ICAM-1 in SK-HEP-1 cells. Through RT-PCR, Western blot and zymography methods, YSL was discovered to decrease the mRNA level, protein expression and activity of MMP-2 and -9 in SK-HEP-1 cells.

We concluded that YSL could inhibit tumor growth and metastasis of human HCC SK-HEP-1 cells. In this paper, we systematically studied the antitumor metastasis characteristics of YSL in both in vivo and in vitro models and investigated its antitumor metastasis mechanisms to provide the scientific basis for the clinical application.
Materials and methods
Reagents and animals
The YSL peptide used in this paper was produced by Shenzhen Kangzhe Pharmaceutical Co., Ltd. All cell-culture media were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Unless otherwise specified, chemical reagents were obtained from Sigma-Aldrich (Sigma-Aldrich Co., St Louis, MO, USA).

HCC SK-HEP-1 cell line was purchased from the Chinese Medical Academy of Science (Beijing, China) and was cultured in minimum Eagle’s medium (MEM) containing 10% FBS at 37°C, 5% CO₂.

293T cell line was purchased from the Chinese Medical Academy of Science and was cultured in DMEM containing 10% FBS at 37°C, 5% CO₂.

The female nude mice (BALB/c nu/nu) of 6–8 week old were purchased from the Chinese Academy of Military Medical Sciences (Beijing, China). The mice were housed in specific-pathogen-free conditions. Animal studies were carried out according to the standards established by the guidelines of Tianjin Medical University and were approved by the ethics committee of Tianjin Medical University.

Experimental lung metastasis model
In all, 293T cells growing in the log phase were planted in cell-culture dishes. The cells were incubated overnight at 37°C, 5% CO₂. According to the Lipofectamine™ 2000 reagent protocol, we co-transfected pLV-Luc plasmid and pMD2G plasmid into 293T gag-pol cells to get retrovirus expressing luciferase gene. After transfection for 48 hours, the homogenized cell suspension was harvested by centrifugation at 500×g for 10 minutes. The titer of virus was detected. Stable Luc-positive cell lines were generated and screened by transduction of Retro-Luc in human HCC cell line SK-HEP-1.

The concentration of log-phase SK-HEP-1 cells was adjusted to 5×10⁶/mL in 10% FBS MEM. To establish the lung metastasis model, 1×10⁵ SK-HEP-1 cells were implanted into nude mice via tail vein injection. Animals were distributed into three groups by random, 12 in each group. They received daily injections of YSL (320 and 640 µg/kg/day) and saline only (0.2 mL/day) from the day after tumor implantation. Drugs or saline were injected intraperitoneally for 60 days. Light flux value of SK-HEP-1-Luc cells in mice was measured by bioluminescence imaging system, when the substrate D-luciferin was intraperitoneally injected. The mice were euthanized on day 61. The lungs were obtained and fixed in 10% formalin. The number of metastatic lung nodules was calculated using the anatomical microscope. The specimen of tumor tissue was embedded in paraffin and routinely stained with H&E. Histopathology analysis was performed with a light microscope (Olympus, Tokyo, Japan), and the numbers of metastatic lung nodules were also calculated. The rate of metastasis inhibition (%)=(number of metastatic nodules of drug group/the number of metastatic nodules of the control)×100.

Human HCC cells’ proliferation assays in vitro
The concentration of log-phase SK-HEP-1 cells was adjusted to 1×10⁵/mL in 10% FBS MEM. The cells were planted in 96-well plates at 100 µL/well and cultured for 24 hours at 37°C with 5% CO₂. Supernatants were discarded after centrifugation. YSL (100 µL/well) at different doses was added. The final doses of the drug were 0.2, 0.4, 0.8, 1.6 or 3.2 mg/mL, and plain MEM was the negative control. Six parallel wells were designed to each group. The cells were cultured for 24, 48 and 72 hours at 37°C with 5% CO₂. Dimethylthiazol-carboxymethoxyphynyl-sulphophenyl-tetrazolium inner salt (MTS) was added, and then, the plates were incubated for an extra 2 hours. By using a microplate reader (Thermo Scientific), the OD of each well at a wavelength of 490 nm was detected. The inhibition rate of growth (%)=(1–OD value of drug group/OD value of the control)×100%.

Cell adhesion assay with Matrigel in vitro
HCC SK-HEP-1 cells were treated with YSL (0.2 and 0.4 mg/mL) for 24, 48 and 72 hours. Tumor cells were adjusted to 5×10⁵ cells/mL in 0.1% BSA MEM. Plain MEM was the negative control. 1×10⁴ SK-HEP-1 cells were planted in wells coated with 0.2 mg/mL Matrigel (BD Biosciences, San Jose, CA, USA). Six parallel wells were designed to each group. The cells were incubated for 1 hour at 37°C with 5% CO₂, and then, tumor cells, not bind to Matrigel, were rinsed out with PBS. OD values were detected using MTS assay. The inhibition rate of adhesion (%)=(1–OD value of drug group/OD value of the control)×100%.

Invasion analysis with Matrigel in vitro
Effect of mice lung extract on the invasion of human HCC cells
The female BALB/c mice (8–10 weeks old) were euthanized. Under aseptic conditions, the lungs were removed and washed with the MEM and then cut into 1 cm² pieces. The pieces were ground in extraction buffer (0.05 M Tris-HCl pH 7.4, 0.5 M NaCl, 5 mM EDTA, gentamicin 50 mg/L, 1:100 diluted cocktail, 1 mM 2-mercaptoethanol) under ice bath. The supernatants were harvested after centrifugation.
at 15,000×g for 15 minutes. Protein concentrations were detected using BCA protein assay kit and stored at −20°C.

The upper side of filter membranes was coated with 0.2 mg/mL Matrigel, and the underside of filter membranes (containing 8 µm pores) was coated with fibronectin. The membranes were air dried and inserted in the transwell cell-culture chambers. The concentration of log-phase SK-HEP-1 cells was adjusted to 2×10^5/mL in 0.1% BSA MEM. A 100 µL aliquot of tumor cell suspensions was seeded into the upper compartment, and 600 µL of lung tissue extracts (0.04, 0.2, 1 and 5 mg/mL) was added into the lower compartment of chamber. SK-HEP-1 cells were incubated for 24 hours at 37°C in 5% CO₂. The cells from the upper surface of the membranes were wiped away. Tumor cells migrating to the lower surface of the membranes were fixed and stained with H&E. The numbers of cells across the filters were counted under the microscope (×400 magnification).6,7

Effect of YSL on the invasion of human HCC cells

The pretreatment of transwell chamber is the same as earlier. SK-HEP-1 cells pretreated with 0.2 or 0.4 mg/mL YSL or MEM for 72 hours were collected in 0.1% BSA MEM. In all, 100 µL cell suspensions (2×10^6 cells/mL) were seeded into the upper compartment. The lower compartments were filled with 600 µL of lung tissue extracts at a concentration of 1 mg/mL. The 0.1% BSA MEM in the lower chamber wells was used as the control. The cells were cultured for 24 hours at 37°C, 5% CO₂. The method for counting the number of invasive cells is the same as earlier. The inhibition rate of invasion (%)=(1−the number of invasive cells in drug group/the number of invasive cells in lung extract control group)×100%.

Western blot

After SK-HEP-1 cells were treated with YSL (0.2 or 0.4 mg/mL) or MEM as the control for 72 hours, protein was extracted through lysing cells for 20 minutes in ice bath using RIPA (50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, and protease inhibitor cocktail). The concentrations of proteins were detected using BCA assay. Proteins were separated using denaturing SDS-PAGE and then transferred onto the polyvinylidene difluoride (PVDF) membrane. The membranes were blocked with tris-buffered saline (50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 0.1% Tween 20 and 5% nonfat dry milk) overnight at 4°C with shaking and then incubated in 1:250 ICAM-1 antibody (Chemicon, Temecula, CA, USA), 1:200 MMP-2 antibody (Chemicon), 1:500 MMP-9 antibody (Abcam, Cambridge, MA, USA) or 1:6,000 β-actin antibody for 2 hours at room temperature. After rinsing with tris-buffered saline-tween 20 for three times, membranes were probed with 1:6,000 horseradish peroxidase-conjugated mouse IgG (KPL, Gaithersburg, MD, USA). The combined antibodies were detected using Lumiglo Chemiluminescent Substrate Kit (KPL). The relative expression of proteins was determined as MMP/β-actin densitometric ratio through using TotalLab software.8

Reverse transcription PCR (RT-PCR) and real-time RT-PCR

After SK-HEP-1 cells were treated with YSL (0.2 or 0.4 mg/mL) or MEM as the control for 72 hours, total RNA extracts were prepared by using TRIzol reagent. The cDNA synthesis reaction was conducted using 2 µg RNA with Reverse Transcriptase M-MLV (Takara, Tokyo, Japan) and 500 ng oligo(dT), following the manufacturer’s instructions. The mRNA expression levels of ICAM-1 and MMP-2/-9 were detected by quantitative RT-PCR using SYBR Green Master Mix and ABI Prism 7500. The list of gene primers was as follows: ICAM-1 forward, 5′-ACCGTGAAATGCTCTCCC-3′ and ICAM-1 reverse, 5′-CGCTGGCGTTATAGAGGTAC-3′; MMP-2 forward, 5′-CCGTCGCCCAATCATCAAGT-3′ and MMP-2 reverse, 5′-CTGTCGAGTCCAAGG-3′; MMP-9 forward, 5′-GTGCCTGGGCTGCTGTTGCTG-3′ and MMP-9 reverse, 5′-GTCGCCCTCAAGTTTG GAAT-3′ and β-actin forward, 5′-GGCTGATATCCCC TCCATCG-3′ and β-actin reverse, 5′-CCAGTGGG TAAACAATGCTGT-3′. The amplifying condition was as follows: 95°C for 1 minute, followed by 38 cycles at 95°C for 15 seconds; 58°C for 15 seconds and 72°C for 45 seconds, using β-actin as an internal reference. The fold changes in expression of target genes were calculated using the ΔCt method.

SDS-PAGE gelatin zymography

Gelatin zymography is a technique to detect enzymatic activity of MMP-2 and -9. Conditioned media were harvested from tumor cells pretreated with YSL (0.2 and 0.4 mg/mL) or MEM as the control for 72 hours. In all, 2 mg of protein samples was subjected to 10% SDS-PAGE electrophoresis with 1 mg/mL gelatin. Following electrophoresis, the gels were rinsed twice with 2.5% Triton X-100, three times in activation buffer (50 mM Tris-HCl, pH 7.5; 15 mM CaCl₂; 50 mM NaCl) and then incubated overnight at 37°C. Gels were stained with Coomassie blue R-250 and destained with 4% methanol and 8% acetic acid. Areas corresponding to gelatinolytic activity showed clear white zones against the blue-stained background. The areas were
quantified by a phosphoimager (Amersham Biosciences, Uppsala, Sweden).6

Statistical analyses
Data are reported as mean±SD. One-way ANOVA and a Student–Newman–Keuls posttest were applied. P-value of <0.05 was considered to indicate statistical significance.

Results
Effect of YSL on experimental metastasis of SK-HEP-1 cells
To determine the antitumor metastasis effect of YSL on human HCC, the tumor cell suspension was injected into a tail vein of each nude mouse, and YSL was delivered for 60 days. YSL (640 and 320 µg/kg/day) significantly inhibited lung metastasis of SK-HEP-1 cells. There were significant differences in the fluorescence value between the drug and the control group (P<0.05; Figure 1).

YSL (640 µg/kg/day) significantly inhibited lung metastasis lesions of SK-HEP-1 cells. The number of metastatic lung lesions of the YSL-treated group was smaller than that in the saline-treated group. The difference between YSL (640 µg/kg/day) group and saline-treated group was significant (P<0.05). The inhibition rate of metastasis of YSL (640 µg/kg/day) is 36.102 (Figure 2).

The results of H&E-stained lung tissues of nude mice showed that YSL (640 µg/kg/day) decreased the number and size of SK-HEP-1 cell clumps at metastatic sites and vascular tissues compared to those in the saline group, with the metastasis inhibition rate of 28.12% (P<0.05). The focal immune infiltrates are shown (Figure 3).

Cytotoxic function of YSL in vitro
After treatment with YSL (0.2, 0.4, 0.8, 1.6 and 3.2 mg/mL) for 24, 48 or 72 hours, respectively, the proliferations of SK-HEP-1 cells were significantly inhibited compared to the negative control (P<0.05) in a dose- and time-dependent manner. The highest inhibition rate of YSL on SK-HEP-1 cells at 3.2 mg/mL was 32.24% (Figure 4).

Inhibitory effect of YSL on adhesion in vitro
Matrigel, rich in laminin and collagen IV, is extracted from mouse Engelbreth-Holm-Swarm (EHS) sarcoma. It could

![Figure 1](image1.png)

YSL inhibited the experimental metastasis of SK-HEP-1 cells (in vivo imaging system).

Notes: Nude mice (12 in each group) took an injection via tail vein of SK-HEP-1 liver carcinoma cells (1×10⁶ cells/mouse) and were imaged 60 days later. The results are presented as mean±SD. (A) Effect of YSL on lung metastasis of SK-HEP-1 cells. Bars indicate SD. *P<0.05 compared to the saline group. (B) YSL 640 µg/kg/day. (C) YSL 320 µg/kg/day. (D) Saline group. Magnification ×1.

Abbreviations: ROI, region of interest; YSL, tyroserleutide.
recombine the basement membrane (BM), and the number of tumor cells adhering to Matrigel can reflect the adhesive capability of cells. After the treatment by YSL (0.2 and 0.4 mg/mL) for 24, 48 or 72 hours, respectively, the capabilities of SK-HEP-1 cells to adhere to Matrigel were inhibited. The OD level of each YSL-treated group was significantly smaller than that of the negative control ($P < 0.05$), with a dose- and time-dependent inhibitory effect. The highest inhibition rate of adhesion was 28.67% (Figure 5).

**Inhibitory effect of YSL on invasion in vitro**

**Effect of mice lung extract on the invasion of human HCC cells**

Using fibronectin as a chemokine, the number of SK-HEP-1 cells crossing the porous filter coated with Matrigel in transwell chambers represented the invasion ability of the cells. The results showed that lung extract (0.2, 1 or 5 mg/mL) could significantly promote the invasion of SK-HEP-1 cells. The dose–effect curve of lung extract on SK-HEP-1 cell invasion showed the bell shape. The number of invasive cells was the highest in the 1 mg/mL lung extract group (Figure 6).

**Inhibitory effect of YSL on the invasion of human HCC cells**

To study the inhibitory effect of YSL on the invasion of SK-HEP-1 cells, we used the lung extract at the concentration at 1 mg/mL. The results showed that the number of invasive cells is higher in the lung extract group than in the control group. After treatment with YSL (0.2 or 0.4 mg/mL) for 72 hours, the invasion of SK-HEP-1 cells was inhibited.

There are significant differences in the number of invasive cells between the YSL-treated group and lung extract control group ($P < 0.05$). The inhibition rates of invasion of YSL (0.2 and 0.4 mg/mL) were 19.33% and 33.70%, respectively (Figure 7).

**Inhibitory effect of YSL on the expression of ICAM-1**

ICAM-1 (CD54) is a member of the immunoglobulin superfamily. As an adhesion molecule, ICAM-1 could indicate the potential capability of tumor invasion and metastasis on some level. After treatment by YSL (0.2 and 0.4 mg/mL) for 72 hours, the ICAM-1 protein expression of SK-HEP-1 cells was significantly inhibited compared to that of the negative control. The RT-PCR assay of ICAM-1 showed

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**Figure 2** YSL inhibited the experimental metastasis of SK-HEP-1 cells (anatomical microscope).

**Notes:** Nude mice (12 in each group) took an injection via tail vein of SK-HEP-1 liver carcinoma cells ($1 \times 10^6$ cells/mouse) and were sacrificed 60 days later. The lungs were obtained and fixed. The number of metastatic lung nodules was calculated using the anatomical microscope. The results are presented as mean±SD. 

(A) Effects of YSL on the numbers of lung metastatic nodules of SK-HEP-1 cells. Bars indicate SD. *$P < 0.05$ compared to the saline group. (B) YSL 640 µg/kg/d. (C) YSL 320 µg/kg/d. (D) Saline group. Magnification x1.

**Abbreviation:** YSL tyroserleutide.
Figure 3  YSL inhibited the experimental metastasis of sK-heP-1 cells (H&E staining).
Notes: Nude mice (12 in each group) took an injection via tail vein of sK-heP-1 liver carcinoma cells (1×10^6 cells/mouse) and were sacrificed 60 days later. The specimen of tumor tissue was embedded in paraffin and routinely stained with H&E. The results are presented as mean±SD. (A) Impact of YSL on the number of lung metastasis nodules of sK-heP-1 cells (H&E staining). Bars indicate SD. *P<0.05 compared to the saline group. (B) H&E, YSL 640 μg/kg/day, 40×. (C) H&E, YSL 320 μg/kg/day, 40×. (D) H&E, saline group, 40×.
Abbreviation: YSL, tyroserleutide.

Figure 4 Effect of YSL on the sK-heP-1 proliferation in vitro.
Notes: sK-heP-1 cells (1×10^5/mL) were pretreated with different concentrations of YSL for 24, 48 and 72 hours. (A) The OD value was detected by MTS. (B) The inhibition rate of growth (%)=[(1−OD value of drug group/OD value of the control)]×100%. *compared to the control, P<0.05 and n=6.
Abbreviations: MTS, dimethylthiazol-carboxymethoxyphnyl-sulfophenyl-tetrazolium inner salt; YSL, tyroserleutide.
that treatment with YSL (0.2 and 0.4 mg/mL) for 72 hours significantly decreased ICAM-1 mRNA levels in SK-HEP-1 cells ($P<0.05$; Figure 8).

**Inhibitory effect of YSL on the activities, protein expressions and mRNA levels of MMP-2 and -9**

The upregulation of the levels of MMP-2 and -9 is positively correlated to the improved tumor growth and metastasis. Gelatin zymography analysis demonstrated that YSL (0.2 and 0.4 mg/mL) significantly reduced the activities of MMP-2 and -9 of SK-HEP-1 cells with treatment for 72 hours ($P<0.05$). The results also showed that YSL significantly inhibited protein and mRNA expressions of MMP-2 and -9 in SK-HEP-1 cells ($P<0.05$; Figure 9).

**Discussion**

Tumor metastasis is the most essential characteristic of malignant tumors and the common cause of cancer-related deaths. Metastasis does not happen at random. The occurrence of metastases has a natural tendency, which includes hematogenous spread, transcoelomic spread and lymphatic spread. There is a trend for a particular tumor to seed in certain organs. Many cancers, such as HCC, tend to metastasize to the lung. In this study, by building an experimental lung metastasis model of human HCC SK-HEP-1 cells in nude mice, we investigated the effect of YSL on lung metastasis of HCC. We constructed the stable Luc-positive SK-HEP-1 cell line. Intravenous tail vein injection mainly causes pulmonary metastasis. The experimental lung metastasis model represented the procedures that happened after SK-HEP-1 cells entered the blood circulation. YSL at a dose of 640 µg/kg/day led to a reduction in the number and size of metastatic lung lesions. Metastasis largely developed at the hilum of lung or pleural membrane.

Metastasis is a complex process involving many stages. At first, invasion demands tumor cells to lose cell–cell adhesion in order to obtain motility, following invading the adjacent tissues. Then, cancer cells infiltrate the endothelial tissue of blood vessels and migrate intravascularly to invade the circulation system. Finally, the cells penetrate the endothelial cell layer (extravasation). In the end, a small fraction of tumor cells from a primary tumor proliferate unlimitedly to develop into metastases. This shows that metastasis is closely related to many biological activities of tumor cells, such as growth, adhesion, invasion, hematogenous metastasis and lymphatic metastasis. Through analyzing the effect of YSL on proliferation and adhesive and invasive capacities of cancer cells, we investigated the mechanisms of anticancer metastasis of YSL. In vivo, with YSL treatment, the number of metastatic lung nodules decreased, and H&E staining showed that the size of tumor cell clusters was reduced. This indicated that the antimetastatic mechanism of YSL involves the direct interference of cancer cells’ growth. In vitro, cytotoxic function of YSL at doses ranging from 0.2 to 3.2 mg/mL was observed. The result showed that the proliferation of SK-HEP-1 cells was inhibited. It may be that YSL regulates cell reproductive cycle, inducing tumor cell apoptosis, and so decreases the survival capability of tumor cells.

Matrigel is the product derived from the mouse EHS sarcoma, consisting of type IV collagen, laminin and glycoproteins. Matrigel is the most widely used tumor microenvironment mimicking matrices for in vitro adhesion, invasion and capillary formation assays. YSL (0.2 and 0.4 mg/mL) significantly inhibited the adhesion of SK-HEP-1 to Matrigel.
Figure 6 Effect of different concentrations of lung extract on sK-heP-1 invasion in vitro (H&E ×100).

Notes: The sK-heP-1 cells (2×10^5/well) were placed into the upper chambers of the wells. The different doses of lung extracts (0, 0.04, 0.2, 1 or 5 mg/mL) were added into the lower chambers. The cells invaded to the lower side of the filter membrane were stained with H&E and counted using a microscope. (A) The effect of different doses of lung extracts on SK-HEP-1 invasion in vitro. Bars indicate SD. *Compared to the control, P<0.05 and n=3. (B) Medium group. (C) Lung extract (0.04 mg/mL) group. (D) Lung extract (0.2 mg/mL) group. (E) Lung extract (1 mg/mL) group. (F) Lung extract (5 mg/mL) group.
The reduction of adhesion of liver tumor cells by YSL can inhibit the adhesive ability of cells to surrounding tissues and blood capillaries, so interfering with the procedure of metastasis.

Adhesion is the first step of metastasis. Another critical step of metastasis is the invasion to adjacent tissues. Cancer cells penetrate the BM or extracellular matrix (ECM) to invade distant tissues. The organ specificity in cancer metastasis has been studied. SK-HEP-1 cells easily cross filter membranes coated with Matrigel by the induction of mice lung extracts, corresponding to that HCC tends to metastasize to the lung. An optimum

![Figure 7](image-url)
**Figure 8** Inhibitory effect of YSL on the protein expression and mRNA level of ICAM-1 in SK-HEP-1 cells. 

Notes: (A) Protein expression was determined by Western blot analysis using total protein extracted from SK-HEP-1 cells pretreated by YSL for 72 hours. The proteins were separated using SDS-PAGE, and then, Western blot analysis was conducted with the antibody against the target proteins. Representative figure shows analysis of ICAM-1 expression of SK-HEP-1 cells. (B) mRNA was assessed by real-time PCR in extracts from SK-HEP-1 cells pretreated by YSL for 72 hours. ICAM-1 mRNA level was calculated as $2^{-\Delta \Delta C_T}$ using $\beta$-actin as the internal reference. Bars indicate SD. *Compared to the control, $P<0.05$ and $n=3$. 
Abbreviation: YSL, tyroserluteide.

**Figure 9** Inhibitory effect of YSL on the activities, protein expressions and mRNA levels of MMP-2 and -9 of SK-HEP-1 cells. 

Notes: After treatment with YSL for 72 hours, (A) supernatants were collected and proteins were separated by SDS-PAGE. The gels were incubated, stained and destained. The activity of MMP-2 and -9 was quantified. (B) The proteins were separated using SDS-PAGE, and then, Western blot assay was conducted with the antibodies against the target proteins. (C) mRNA was assessed by RT-PCR. The mRNA levels of MMP-2 and -9 were calculated as $2^{-\Delta \Delta C_T}$ using $\beta$-actin as the internal reference. Bars indicate SD. *Compared to the control, $P<0.05$ and $n=3$. 
Abbreviations: RT-PCR, reverse transcription PCR; YSL, tyroserluteide.
dose of 1 mg/mL lung extract was chosen. YSL (0.2 and 0.4 mg/mL) significantly decreased numbers of SK-HEP-1 cells crossing the filters and reduced the invasiveness of cells. We inferred that YSL inhibited cells’ invasiveness and destruction of BM and ECM produced by the cancer cells and reduced a capability of cancer cells to infiltrate the capillary vessels and invade the target tissues, which inhibit distant metastases.

ICAM-1, as an adhesion molecule, is related to cell–cell adhesion and the adhesion of cancer cells to BM and ECM.\textsuperscript{19} It has been demonstrated that the expression of ICAM-1 is correlated with the malignancy and metastases potential of cancer cells. We previously reported that YSL significantly inhibited mRNA and protein expressions of ICAM-1 in mouse melanoma B16-F10 cells.\textsuperscript{19} In this paper, in gene expression analysis and Western blot analysis,\textsuperscript{20} we discovered that YSL (0.2 and 0.4 mg/mL) significantly inhibited the expressions of ICAM-1 of SK-HEP-1 cells. Downregulation of ICAM-1 could affect ICAM-1-dependent cell–cell adhesion between endothelial and tumor cells and the adhesion of SK-HEP-1 cells to BM and ECM, which can regulate the transmigration steps of metastatic cascades.

The degradation of natural barriers such as BM or ECM is a crucial step in the metastatic cascade of cancer cells. MMPs facilitate tumor cell invasion and metastasis. As an MMP superfamily member, MMP-2 and -9 can degrade the proteins of the ECM of cancer cells,\textsuperscript{21,22} and they can also promote metastatic tumor cells to penetrate the BM, leading to invasion and metastasis.\textsuperscript{23} YSL (0.2 and 0.4 mg/mL) significantly reduces the RNA levels, protein expressions and activities of MMP-2 and -9 in HCC SK-HEP-1 cells. Activation of MMP-2 and -9 is regulated by lots of factors. The downregulation of MMP-2 and -9 by YSL can interfere with migration of SK-HEP-1 cells crossing tissue barriers and may prevent the MMP-2 and -9 signaling pathways, which are correlated with tumor growth, invasion and metastases.

Conclusion

In vivo studies, YSL inhibited the metastasis of HCC SK-HEP-1 cells. Our in vitro studies came to the conclusion that YSL can inhibit the growth and adhesive and invasive capability of cancer cells to reduce their metastatic potential. In this study, YSL reduced the adhesion of SK-HEP-1 to Matrigel and the quantity of SK-HEP-1 cells invading ECM or BM. In addition, these effects are associated with the downregulation of the expression levels of ICAM-1, MMP-2 and MMP-9 of tumor cells, which have been found to play essential roles in cancer invasion and metastasis.

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Disclosure

The authors report no conflicts of interest in this work.

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