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β-elemene enhances anticancer and anti-metastatic effects of osteosarcoma of ligustrazine *in vitro* and *in vivo*

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Abstract. The present study aimed to determine the anticancer effects of the combination of β-elemene and ligustrazine *in vitro* as well as in *in vivo*. Following evaluation using an MTT assay, β-elemene, ligustrazine and the β-elemene-ligustrazine combination treatments all exhibited the capacity to inhibit the growth of OS-732 cells, with inhibitory rates of 43.3, 54.4, and 75.0%, respectively. Using a flow cytometry assay, it was determined that the β-elemene-ligustrazine combination possessed the highest apoptotic rate (30.6%). Furthermore, β-elemene-ligustrazine combination treatment resulted in the highest downregulation of G protein-coupled receptor 124, vascular endothelial growth factor, matrix metallopeptidase (MMP)-2 and MMP-9 mRNA, and protein expression levels. In addition, the combined treatment led to an increase in the mRNA and protein expression of endostatin, TIMP metallopeptidase inhibitor (TIMP)-1 and TIMP-2 in OS-732 cells. Additionally, β-elemene-ligustrazine caused a decrease in nuclear factor-κB, interleukin-8, C-X-C motif chemokine receptor 4 and urokinase-type plasminogen activator mRNA expression, as well as an increase in caspase-3, caspase-8, and caspase-9 mRNA expression. *In vivo*, the β-elemene-ligustrazine combination was able to reduce the weight and the bulk of the tumor in BALB/c-nu/nu nude mice compared with any other group. All the results described above regarding changes to mRNA and protein expression were further confirmed *in vivo* in the tumor tissue of mice. The results of the present study have suggested that the combination of β-elemene-ligustrazine exhibits greater anticancer effects compared with β-elemene- or ligustrazine-alone treatment.

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Introduction

Elemene is a new drug first extracted in China using the herbs that activates blood circulation such as Curcuma Wenyujin in recent years. It has the broad-spectrum antineoplastic, immune protection and other effects (1). The main active component is β-elemene. The current research shows that β-elemene has the effects to induce cell apoptosis and differentiation, with reverse multiple drug resistance of tumor, and enhance sensibilization of combined radiotherapy and chemotherapy etc (2). Moreover, its toxic and side effects are very few (2). At present, it has been widely applied in the clinical treatment of liver cancer, lung cancer, breast cancer, cervical cancer, gastrointestinal tumor, carcinoma of urinary bladder, brain tumor and other superficial tumors, such as bladder cancer and gland cancer (3). The research shows that β-elemene can prevent NSCLC-H460 cell of human lung cancer, Hep-2 cell of human laryngocarcinoma, A2780 cell of human ovarian cancer, U251 cell of human cerebral glioma and HXO-RB 44 cell of human retinoblastoma entering phase G2/M from phase S (4). Besides, it also can reduce mitosis, suppress tumor growth and induce cancer cell apoptosis (5). Meanwhile, the research also finds that β-elemene can inhibit the hematogenous metastasis and lymphatic metastasis of tumor. It plays the role of down-regulation for the protein level of VEGF-C and VEGFR-3 in cell SPC-A-1 of human pulmonary carcinoma, which shows that β-elemene also can inhibit the hematogenous metastasis and lymphatic metastasis by reducing vascular growth factor and its receptors (6). In the experimental research on nude mouse model to find the cure of human laryngocarcinoma, it is found that β-elemene can inhibit the expression of VEGF-C and VEGFR-3 to block the growth of cell Hep-2 in laryngocarcinoma and its hematogenous metastasis accordingly (7).

Ligusticum wallichii, also known as Xiongqiong, is an artemisia plant in umbrelliferae family of Chinese traditional medicine, which is mainly produced in Sichuan, Yunnan, Guizhou, Guangxi, Hubei and other places in China. Ligustrazine, one of the effective components of *Ligusticum wallichii*, belongs to amides alkaloid, whose chemical structure is tetramethylpyrazine (TMP) and contains many biological functions as well, including promoting blood circulation, removing blood stasis and dredging veins, etc (8). Modern researches have further proven that it has various pharmacological effects and

can be used in the treatment for many deadly diseases such as cardiovascular diseases, pulmonary hypertension, chronic renal failure, liver cirrhosis, and with radioactive treatment of pulmonary fibrosis. Latest research suggests TMP has significant protective effects on spinal cord injury. Meanwhile, we come to know that TMP also can inhibit the apoptosis of spinal cord injury cells (9) as well as can help in the production of endothelin, which can dilate vessels and increase the effect of blood supply in the ischemic area (10). It can inhibit the aggregation of platelet thus, reducing blood viscosity simultaneously improve microcirculation and regulate the expression of interleukin in order to inhibit inflammation (11).

Angiogenesis might be a condition for the growth of primary and metastatic tumors. The recent research finds that the increase of GPR124 expression is closely related to angiogenesis, which plays an important part in in its significant role of promoting tumor invasion, metastasis and further in poor prognosis (12). At present, the limited research related to GPR124 function is mostly focused on the angiogenesis of central nervous system. The research related to its specific mechanism of action is very few. A prior research, suggests that GPR124 in endothelial cells may bind with PDZ region of hDIg through PDZ binding motif at the terminal of its carboxyl, by regulate as well as control cell proliferation, and adhesion through regulating, controlling the anchorage of hDIg which further affect the angiogenesis (13).

When chemotherapy is combined with elemene to treat patient with gastric cancer, the result shows that chemotherapy drug can remarkably increase the chemotherapy sensitivity to gastric cancer, which has effectively reversed the patient tolerance with advanced gastric cancer for chemotherapy drug (14). The research also finds that β-elemene can effectively inhibit the growth of gastric cancer cell after get combined with fluorouracil drug. Its mechanism is closely related to the induction of cell apoptosis. The development of bone sarcoma is rapid (15). The prognosis is poor and the death rate, high. It is a kind of malignant disease severely life threatening. In order to reduce the toxic side effects of drug and effectively kill the sarcoma cell, we choose to combine the low-concentration of β-elemene and ligustrazine to apply them into the cell of bone sarcoma to research the combined effect which reduces the drug toxicity so as to realize the sufficient anti-sarcoma effect of drug (16). In this research, we observe the external cell and the experiment on animal body, illustrating the mechanism through the experimental technique of molecular biology, and making a sufficient research for the combined effect of β-elemene and ligustrazine.

Materials and methods

Cell line. OS-732 human osteosarcoma cells was purchased from ATCC (American Type Culture Collection, Manassas, VA, USA). The cells were cultured using RPMI1640 medium (Biosera, Nuaille, France) with 10% FBS at 37˚C in 5% CO_2 incubator (MCO-20AIC; SANYO, Tokyo, Japan).

MTT assay. Culture solution was added to adjust the concentration of OS-732 cells in logarithmic growth phase added to $2x10^4$/dish, then transferred to the 96-well culture plate with 50 μl per well, and placed in incubator for 5% CO_2 at 37˚C for 24 h. β-elemene-ligustrazine (Zhengzhou Cheuk-Fung Pharmaceutical Co., LTD) were added into 96-well plate having 50 μl per well. In the meantime 50 μl culture solution was added to the control group and cultured in CO_2 incubator for 48 h. Subsequently, the control group was added with MTT solution after the removal of supernatant thereafter incubated for 4 h. In the blank control group 100 μl DMSO was added after removal of the supernatant and shocked for 30 min, the enzyme standard instrument were used to detect at 570 nm (680 Microplate reader, Bio-Rad, CA, USA) (17).

Flow cytometry assay. Centrifugation of single cell suspension should be done in order to remove stationary liquid and washed by 3 ml PBS twice, followed by centrifugation of 5 min; added with 1 ml PI staining solution and incubated in refrigerator at 4˚C for 30 mins while keeping it in a dark place as to prohibit sunlight exposure; and then filtered by 500-hole copper mesh; flow cytometry detection (Accuri C6, BD Biosciences, La Jolla, CA, USA) and argon ion laser with 15 mA excitation light source with 488 nm wavelength was used for testing, and 630 nm band-pass filter to receive the light. Selection of 10,000 cells were taken place using FSC/SSC scattered point diagram method, we also used gating technology to eliminate adhesive cells and cell debris, to analyze the percentage of apoptotic cells in PI fluorescence histogram (17).

Real-time quantitative PCR assay. Whole RNA of cancer cells and mice tumor tissues were extracted using RNAzol, and DNase RNase-free was adopted to digest total RNA at 37˚C for 15 min, and then RNase kit to purify RNA in order to adjust its concentration to 1 μg/μl. The 2 μg RNA was used as the template to synthetize cDNA by reacting it with reverse transcriptase at 37˚C for 120 min, at 99˚C for 4 min, and at 4˚C for 3 min. Followed by, reverse transcription-polymerase chain reaction method was adopted to amplify the gene expression of GPR124, TIMP-1, TIMP-2, MMP-2, MMP-9, VEGF, endostatin, NF-κB, IL-8, CXCR4, uPA, caspase-3, caspase-8 and caspase-9 to determine the transcription level of mRNA, and β-actin was used as the housekeeping genes of internal control group (StepOne, Applied Biosystems, Alameda, CA, USA) (18).

Mice experiment. 7 weeks (n=60) old male BALB/c-nu/nu nude mice were purchased from Beijing University; they were sustained in a temperature-controlled facility at 23±1˚C and relative humidity of 50±5% with a 12-h light/dark cycle. The experiment of this study was performed following the protocols approved by the Animal Ethics Committee of Beijing University (Beijing, China).

In vivo experiment. Cultivate OS-732 cells into 100 ml culture solution of RPMI-1640. The cell grows rapidly and vigorously, one passage grows within three to four days, in total there were four passages. Collect OS-732 cells in logarithmic phase, and wash once with PBS. Then adjust the cell concentration to make it as $5x10^7$/ml cell suspension. Carry out a 0.2 ml subcutaneous inoculation in the posterior axillary of the right forelimb of nude mouse under aseptic condition, and subcutaneously inoculating about $1x10^7$ oncocyte for each mouse. After the inoculation, we observe the general condition and

local wound status of nude mouse each day. From the first day, the β-elemene, ligustrazine and β-elemene-ligustrazine combination group mice were treated with 1.2 mg/kg β-elemene, 1.2 mg/kg ligustrazine and 0.8 mg/kg β-elemene + 0.4 mg/kg ligustrazine respectively by intravenous injection. From the eighth day after the operation, we weigh the nude mouse with electronic balance after every four days. Measure the short diameter (a) and the long diameter (b) of tumor with vernier caliper. According to the formula, $V=0.5xa^2xb$. calculation of relative tumor volume and draw growth curve was done. According to the formula, RTVn=Vn/V 8, we able calculate the relative growth rate of tumor at the 28th days. At the 28th day after the inoculation, we sacrificed the nude mouse by the method of cervical vertebra dislocation to get the tumor tissue followed by weigh the tumor (19).

Statistical analysis. The experiments data were expressed as mean ± standard deviation (SD). The significant difference ($P<0.05$) of data of different groups were calculated using Duncan's multiple range test using SPSS Statistics 22.0 (IBM, Armonk, NY, USA).

Results

The growth of β-elemene and ligustrazine in OS-732 cells. Using MTT assay, after treated with different concentrations of β-elemene (1, 5, 10, 50, 100, 500 μg/ml) for 24 and 48 h, the IC_{50} of OS-732 cells were 41.36±1.89 and 19.12±0.88 μg/ml respectively. Meanwhile, after the treatment of different concentrations of ligustrazine (0.1, 0.5, 1, 5, 10, 50 μg/ml) for 24 and 48 h, the IC_{50} of OS-732 cells were 9.42±0.33 and 3.98±0.28 μg/ml respectively. The 20 μg/ml β-elemene and 10 μg/ml ligustrazine were used as a combination treatment for next part of the experiments. The 30 μg/ml β-elemene, 30 μg/ml ligustrazine and β-elemene-ligustrazine combination (20 μg/ml β-elemene and 10 μg/ml ligustrazine) showed the inhibitory effects at 59.3, 72.6 and 87.8%, respectively (Table I).

Sub-G1 content of OS-732 cells. Using the flow cytometry assay, β-elemene-ligustrazine combination treatment for OS-732 cells had the most apoptotic cells (41.3±2.6%, sub-G1 DNA content), β-elemene and ligustrazine when treated OS-732 cells separately also had many apoptotic cells (6.5±0.2 and 18.7±1.1%), these apoptotic cells were more than the control group cells (2.4±0.2%).

mRNA and protein expression of GPR124, VEGF and endostatin in OS-732 cells. The control group cells had the highest GPR124, VEGF mRNA expression and lowest expression for endostatin. β-elemene-ligustrazine combination could reduce GPR124, VEGF expression and raise endostatin expression significantly higher as compared with the other group cells (Fig. 1, Table II).

mRNA and protein expression of TIMP-1, TIMP-2, MMP-2 and MMP-9 in OS-732 cells. The control cells showed the lowest TIMP-1, TIMP-2 expression and highest MMP-2, MMP-9 expression (Fig. 2, Table III), and β-elemene, ligustrazine, β-elemene-ligustrazine combination treated OS-732 cells showed the higher TIMP-1, TIMP-2 expression and lower MMP-2, MMP-9 expression than control cells, β-elemene-ligustrazine combination showed the highest TIMP-1, TIMP-2 expression and lowest MMP-2, MMP-9 expression.

mRNA expression of NF-κB, IL-8, CXCR4 and uPA in OS-732 cells. β-elemene-ligustrazine combination could reduce the NF-κB, IL-8, CXCR4 and uPA expression as compared to control cells (Table IV), β-elemene and ligustrazine also could reduce these expression, but higher than β-elemene-ligustrazine combination treated cells.

mRNA expression of caspases in OS-732 cells. As shown in Table V, β-elemene-ligustrazine combination could increase the caspase-3, caspase-8 and caspase-9 expressions higher as compared to control OS-732 cells, and these expressions of β-elemene and ligustrazine treated OS-732 cells were also higher than the control cells.

Table I. Growth inhibitory effects of OS-732 human osteosarcoma cells by MTT assay.

Treatment	OD_{570} value	Inhibitory rate (%)
Control	0.482±0.006	/
β-elemene	0.196±0.007[a]	59.3±3.1[a]
Ligustrazine	0.132±0.005[a]	72.6±3.4[a]
β-elemene-ligustrazine combination	0.059±0.004[a]	87.8±2.8[a]

Results are presented as the mean ± SD of triplicate experiments. Differences between groups were statically analyzed using Duncan's new multiple-range test. [a]$P<0.01$ vs. the control group. β-elemene, 30 μg/ml of β-elemene; ligustrazine, 30 μg/ml of ligustrazine; β-elemene-ligustrazine combination, 20 μg/ml of β-elemene and 10 μg/ml of ligustrazine.

Table II. Quantitative analysis of GPR124, VEGF and endostatin mRNA expressions in OS-732 cells (Folds of control).

Treatment	GPR124	VEGF	Endostatin
Control	1.00±0.05	1.00±0.03	1.00±0.26
β-elemene	0.61±0.04[a]	0.55±0.04[a]	2.36±0.35[a]
P-value	(0.005)	(0.005)	(0.008)
Ligustrazine	0.43±0.05[a]	0.35±0.03[a]	4.57±0.39[a]
P-value	(0.006)	(0.007)	(0.005)
β-elemene-ligustrazine combination	0.22±0.03[a] (0.005)	0.18±0.03[a] (0.007)	7.03±0.42[b] (0.006)

Results are presented as the mean ± SD of triplicate experiments. Differences between groups were statically analyzed using Duncan's new multiple-range test. [a]$P<0.01$ vs. the control group. β-elemene: 30 μg/ml of β-elemene; ligustrazine, 30 μg/ml of ligustrazine; β-elemene-ligustrazine combination, 20 μg/ml of β-elemene and 10 μg/ml of ligustrazine.

Table III. Quantitative analysis of TIMP-1, TIMP-2, MMP-2 and MMP-9 mRNA expressions in OS-732 cells (folds of control).

Treatment	MMP-2	MMP-9	TIMP-1	TIMP-2
Control	1.00±0.04	1.00±0.04	1.00±0.26	1.00±0.15
β-elemene	0.68±0.04[a]	0.77±0.06[a]	3.02±0.35[a]	3.45±0.25[a]
P-value	(0.003)	(0.005)	(0.005)	(0.008)
Ligustrazine	0.37±0.03[a]	0.45±0.05[a]	5.41±0.39[a]	7.12±0.21[a]
P-value	(0.006)	(0.005)	(0.003)	(0.005)
β-elemene-ligustrazine combination	0.15±0.02[a]	0.32±0.04[a]	6.89±0.42[a]	8.59±0.51[a]
P-value	(0.005)	(0.006)	(0.005)	(0.007)

Results are presented as the mean ± SD of triplicate experiments. Differences between groups were statically analyzed using Duncan's new multiple-range test. [a]P<0.01 vs. the control group. β-elemene, 30 μg/ml of β-elemene; ligustrazine, 30 μg/ml of ligustrazine; β-elemene-ligustrazine combination, 20 μg/ml of β-elemene and 10 μg/ml of ligustrazine.

Table IV. Quantitative analysis of NF-κB, IL-8, CXCR4 and uPA mRNA expressions in OS-732 cells (folds of control).

Treatment	NF-κB	IL-8	CXCR4	uPA
Control	1.00±0.03	1.00±0.03	1.00±0.03	1.00±0.03
β-elemene	0.82±0.03[a]	0.65±0.06[a]	0.76±0.06[a]	0.54±0.03[a]
P-value	(0.003)	(0.002)	(0.005)	(0.002)
Ligustrazine	0.52±0.05[a]	0.45±0.04[a]	0.48±0.06[a]	0.36±0.06[a]
P-value	(0.005)	(0.006)	(0.004)	(0.004)
β-elemene-ligustrazine combination	0.31±0.03[a]	0.33±0.02[a]	0.21±0.04[a]	0.18±0.04[a]
P-value	(0.008)	(0.007)	(0.005)	(0.006)

Results are presented as the mean ± SD of triplicate experiments. Differences between groups were statically analyzed using Duncan's new multiple-range test. [a]P<0.01 vs. the control group. β-elemene, 30 μg/ml of β-elemene; ligustrazine, 30 μg/ml of ligustrazine; β-elemene-ligustrazine combination, 20 μg/ml of β-elemene and 10 μg/ml of ligustrazine.

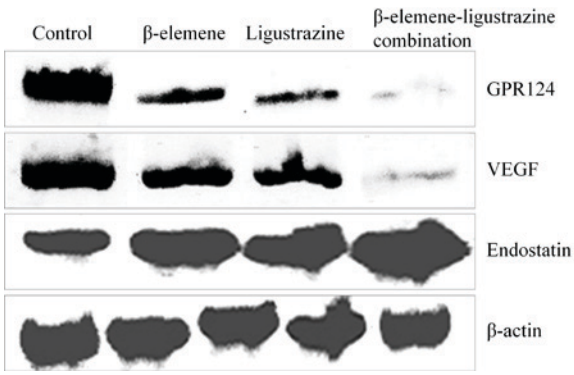

Figure 1. The protein expression of GPR124, VEGF and endostatin in OS-732 cells. Results are presented as the mean ± SD of triplicate experiments. β-elemene, 30 μg/ml of β-elemene; ligustrazine, 30 μg/ml of ligustrazine; β-elemene-ligustrazine combination, 20 μg/ml of β-elemene and 10 μg/ml of ligustrazine.

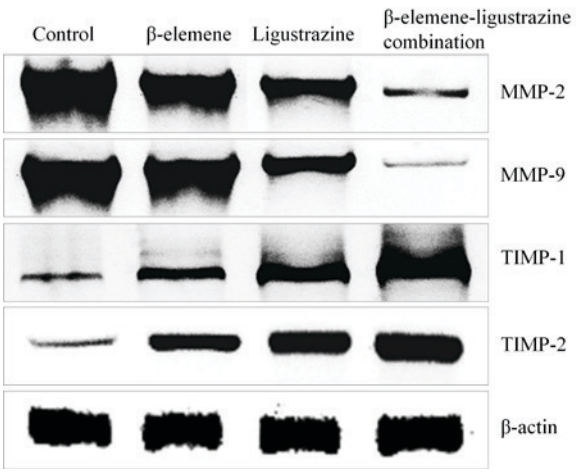

Figure 2. The protein expression of TIMP-1, TIMP-2, MMP-2 and MMP-9 in OS-732 cells. Results are presented as the mean ± SD of triplicate experiments. β-elemene, 30 μg/ml of β-elemene; ligustrazine, 30 μg/ml of ligustrazine; β-elemene-ligustrazine combination, 20 μg/ml of β-elemene and 10 μg/ml of ligustrazine.

Weight and bulk of tumor in mice. The untreated mice showed the heaviest weight and bulk of tumor (Table VI), β-elemene, ligustrazine and β-elemene-ligustrazine combination decreased the weight and bulk of tumor in mice, and β-elemene-ligustrazine combination showed the best effects.

mRNA and protein expression of GPR124, VEGF and endostatin in mice. The control mice showed the highest GPR124, VEGF

Table V. Quantitative analysis of caspase-3, caspase-8 and caspase-9 mRNA expressions in OS-732 cells (Folds of control).

Treatment	Caspase-3	Caspase-8	Caspase-9
Control	1.00±0.12	1.00±0.15	1.00±0.18
β-elemene	3.54±0.18[a]	2.97±0.22[a]	3.12±0.15[A]
P-value	(0.008)	(0.005)	(0.008)
Ligustrazine	6.12±0.45[a]	4.58±0.35[a]	4.77±0.29[a]
P-value	(0.006)	(0.007)	(0.005)
β-elemene-ligustrazine combination	8.41±0.32[a]	7.74±0.40[a]	7.52±0.25[a]
P-value	(0.007)	(0.007)	(0.006)

Results are presented as the mean ± SD of triplicate experiments. Differences between groups were statically analyzed using Duncan's new multiple-range test. [a]P<0.01 vs. the control group. β-elemene, 30 μg/ml of β-elemene; ligustrazine, 30 μg/ml of ligustrazine; β-elemene-ligustrazine combination, 20 μg/ml of β-elemene and 10 μg/ml of ligustrazine.

Table VI. Weight and bulk of tumor in mice.

Treatment	Weight of tumor (g)	Bulk of tumor (mm^3)
Control	3.42±0.24	1,839.47±245.12
β-elemene	2.71±0.17[a]	1,287.15±137.71[a]
Ligustrazine	1.79±0.12[b]	915.87±66.58[b]
β-elemene-ligustrazine combination	0.69±0.09[a]	435.21±44.29[b]

Results are presented as the mean ± SD of triplicate experiments. Differences between groups were statically analyzed using Duncan's new multiple-range test. [a]P<0.05, [b]P<0.01 vs. the control group. β-elemene, 1.2 mg/kg of β-elemene; ligustrazine, 1.2 mg/kg of ligustrazine; β-elemene-ligustrazine combination, 0.8 mg/kg of β-elemene and 0.4 mg/kg of ligustrazine.

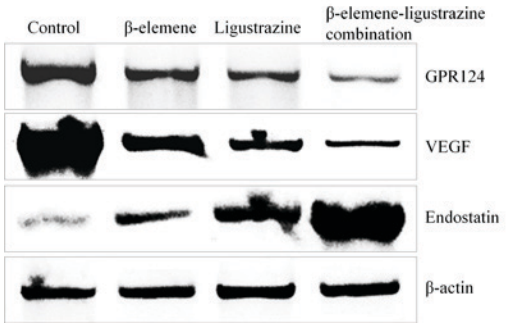

Figure 3. The protein expression of GPR124, VEGF and endostatin in mice. β-elemene, 1.2 mg/kg of β-elemene; ligustrazine, 1.2 mg/kg of ligustrazine; β-elemene-ligustrazine combination, 0.8 mg/kg of β-elemene and 0.4 mg/kg of ligustrazine.

mRNA expression and lowest endostatin expression like the OS-732 cells (Fig. 3, Table VII). -elemene-ligustrazine

Table VII. Quantitative analysis of GPR124, VEGF and endostatin mRNA expressions of tumor tissue in mice (Folds of control).

Treatment	GPR124	VEGF	Endostatin
Control	1.00±0.05	1.00±0.03	1.00±0.02
β-elemene	0.74±0.04[a]	0.69±0.02[a]	3.02±0.17[a]
P-value	(0.005)	(0.005)	(0.003)
Ligustrazine	0.46±0.06[a]	0.48±0.03[a]	6.71±0.29[a]
P-value	(0.003)	(0.004)	(0.005)
β-elemene-ligustrazine combination	0.16±0.03[a]	0.26±0.03[a]	7.33±0.26[a]
P-value	(0.005)	(0.002)	(0.004)

Results are presented as the mean ± SD of triplicate experiments. Differences between groups were statically analyzed using Duncan's new multiple-range test. [a]P<0.01 vs. the control group. β-elemene, 30 μg/ml of β-elemene; ligustrazine, 30 μg/ml of ligustrazine; β-elemene-ligustrazine combination, 20 μg/ml of β-elemene and 10 μg/ml of ligustrazine.

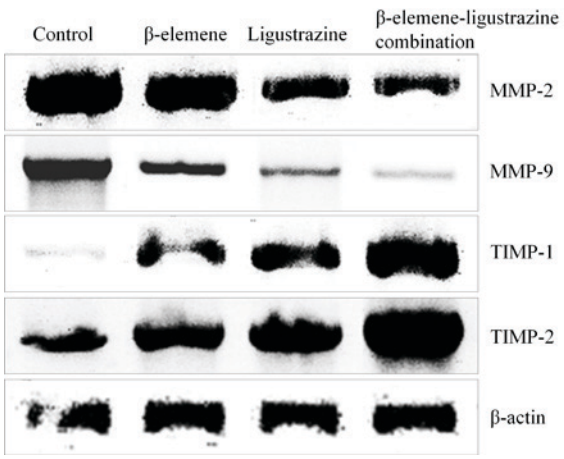

Figure 4. The proteinexpression of TIMP-1, TIMP-2, MMP-2 and MMP-9 in mice. β-elemene, 1.2 mg/kg of β-elemene; ligustrazine, 1.2 mg/kg of ligustrazine; β-elemene-ligustrazine combination, 0.8 mg/kg of β-elemene and 0.4 mg/kg of ligustrazine.

combination showed the weaker GPR124, VEGF expression and little raised endostatin expression compared than other group mice.

mRNA and protein expression of TIMP-1, TIMP-2, MMP-2 and MMP-9 in mice. The control mice had the lowest TIMP-1, TIMP-2 expression and highest MMP-2, MMP-9 expression (Fig. 4, Table VIII), and β-elemene-ligustrazine combination treated mice had the strongest TIMP-1, TIMP-2 expression and weaker MMP-2, MMP-9 expression than control mice.

mRNA expression of NF-κB, IL-8, CXCR4 and uPA in mice. β-elemene-ligustrazine combination decreased the NF-κB, IL-8, CXCR4 and uPA expression even less then the control mice (Table IX, and these expression in β-elemene and ligustrazine

Table VIII. Quantitative analysis of TIMP-1, TIMP-2, MMP-2 and MMP-9 mRNA expressions of tumor tissue in mice (Folds of control).

Treatment	MMP-2	MMP-9	TIMP-1	TIMP-2
Control	1.00±0.04	1.00±0.04	1.00±0.15	1.00±0.15
β-elemene	0.84±0.02[a]	0.66±0.03[a]	4.02±0.30[a]	2.88±0.18[a]
P-value	(0.005)	(0.005)	(0.002)	(0.003)
Ligustrazine	0.65±0.03[a]	0.32±0.03[a]	6.12±0.25[a]	5.12±0.21[a]
P-value	(0.004)	(0.004)	(0.003)	(0.005)
β-elemene-ligustrazine combination	0.42±0.02[a]	0.11±0.03[a]	7.89±0.36[a]	7.39±0.35[a]
P-value	(0.005)	(0.003)	(0.007)	(0.004)

Results are presented as the mean ± SD of triplicate experiments. Differences between groups were statically analyzed using Duncan's new multiple-range test. [a]P<0.01 vs. the control group. β-elemene, 30 μg/ml of β-elemene; ligustrazine, 30 μg/ml of ligustrazine; β-elemene-ligustrazine combination, 20 μg/ml of β-elemene and 10 μg/ml of ligustrazine.

Table IX. Quantitative analysis of NF-κB, IL-8, CXCR4 and uPA mRNA expressions of tumor tissue in mice (Folds of control).

Treatment	NF-κB	IL-8	CXCR4	uPA
Control	1.00±0.03	1.00±0.03	1.00±0.03	1.00±0.03
β-elemene	0.74±0.03[a]	0.75±0.03[a]	0.68±0.06[a]	0.65±0.03[a]
P-value	(0.004)	(0.004)	(0.005)	(0.008)
Ligustrazine	0.36±0.04[a]	0.65±0.04[a]	0.55±0.02[a]	0.35±0.03[a]
P-value	(0.005)	(0.003)	(0.007)	(0.007)
β-elemene-ligustrazine combination	0.12±0.03[a]	0.41±0.02[a]	0.18±0.02[a]	0.22±0.02[a]
P-value	(0.003)	(0.005)	(0.005)	(0.004)

Results are presented as the mean ± SD of triplicate experiments. Differences between groups were statically analyzed using Duncan's new multiple-range test. [a]P<0.01 vs. the control group. β-elemene, 30 μg/ml of β-elemene; ligustrazine, 30 μg/ml of ligustrazine; β-elemene-ligustrazine combination, 20 μg/ml of β-elemene and 10 μg/ml of ligustrazine.

Table X. Quantitative analysis of caspase-3, caspase-8 and caspase-9 mRNA expressions of tumor tissue in mice (Folds of control).

Treatment	Caspase-3	Caspase-8	Caspase-9
Control	1.00±0.12	1.00±0.12	1.00±0.18
β-elemene	3.87±0.20[a]	4.52±0.16[a]	2.84±0.15[a]
P-value	(0.004)	(0.004)	(0.007)
Ligustrazine	5.26±0.26[a]	5.87±0.26[a]	5.26±0.18[a]
P-value	(0.004)	(0.005)	(0.009)
β-elemene-ligustrazine combination	7.54±0.36[a]	8.26±0.26[a]	8.03±0.31[a]
P-value	(0.005)	(0.008)	(0.005)

Results are presented as the mean ± SD of triplicate experiments. Differences between groups were statically analyzed using Duncan's new multiple-range test. [a]P<0.01 vs. the control group. β-elemene, 30 μg/ml of β-elemene; ligustrazine, 30 μg/ml of ligustrazine; β-elemene-ligustrazine combination, 20 μg/ml of β-elemene and 10 μg/ml of ligustrazine.

treated mice were higher than β-elemene-ligustrazine combination treated mice, but lower than control mice.

mRNA expression of caspases in mice. β-elemene-ligustrazine combination could increase the caspase-3, caspase-8 and caspase-9 expression in tissues which is greater as compared to the control group mice (Table X). β-elemene and ligustrazine treated mice also had stronger caspase-3, caspase-8 and caspase-9 expression than control group mice, and these effects of ligustrazine treated mice were stronger than β-elemene treated mice.

Discussion

A great deal of experimental researches showed that the tumor cell with the volume, less than 2 mm^3 mainly depends on diffusion for supply of energy and oxygen, waste excretion as well as to maintain its own survival till the growth of any blood vessel in tumor (20-22). At this stage, the apoptosis and the hyperplasia of tumor cells are in the balance. The volume of tumor can remain unchanged for several months or years, no metastasis will happen in this time period (23).

In addition, the infiltration and metastasis occur easily after generation and growth process of blood vessel, GPR124 plays a role in migration and differentiation in endothelial cells. The growth and the metastasis of tumor need the generation of new vessels. In addition, the expression of GPR124 has been found in some tumors. Tumor recurrence and metastasis are the main reason in the failure of treatment (24). In the research for liver cancer tissue and para-cancerous tissue which adopts immunohistochemical, western blot and RT-PCR technologies, the expression of GPR124 in liver cancer tissue is positive. But the one in para-cancerous tissue mainly is negative. Based on the analysis for clinical data, it is found that the expression of GPR124 is positively related to tumor size, node quantity, TNM staging and microvascular invasion. Meanwhile, it is also found that the total survival rate of GPR124 high expression group is somehow lower than the one of low expression group. This shows that the expression of GPR124 can promote the development of cancer as well as affect the prognosis (25).

The expressions of MMPs, TIMP-1 and TIMP-2 all occur in various tumors. MMPs also have the important function in promoting the tumor invasion and metastasis. As a natural inhibitor, it does have the higher expression but also will result in the poor prognosis. The over expression of TIMP-1 and TIMP-2 is the reactive over expression after MMPs rise (26). When the balance between them is broken, MMPs will pay a dominant role. However, recently, there are many evidences by many different studies shows that TIMP-1 is a kind of multifunctional protein. In addition of inhibiting MMP activity, it also has the unique function to stimulate tumor, participating in the process of cell apoptosis, cell proliferation and tumor angiogenesis etc., which has explained the mechanism to hint the poor prognosis in the breast cancer with its increased content (27). In addition of inhibiting MMP hydrolytic activity, it also has the unique cytokine-like function, capable of participating in the proliferation, apoptosis of tumor cell and tumor angiogenesis through signal transduction pathway (28).

VEGF is the key regulator of tumor metastasis. The research proves that VEGF can promote the permeation, activation, survival, proliferation, infiltration and migration of endothelial cells; it is closely related to tumor metastasis and lymphoangiogenesis (29). Meanwhile, it also can block the signal pathway of VEGF to inhibit the growth and metastases of tumor. Endostatin can inhibit the endothelial cell proliferation of tumor vessel, and in a dose-dependent manner. Moreover, it also can inhibit the endothelial cell proliferation through competing with fibroblast growth factor, stopping phase G0/G1 transforming to phase S of cell cycle and in many other ways, as a result there'll be change in cell signaling pathway and can effectively inhibit the growth of multiple primary solid tumors as well as tumors which were metastasis. The expression and the function of multiple cytokines required by the growth of tumor cell also depend on NF-κB (30). The growth factor, EGF can activate NF-κB to cause the growth of solid tumor. VEGF is the principal member of angiogenic factor family, whose transcription is regulated and controlled by NF-κB (31).

When the activity of NF-κB is inhibited, it can reduce the angiogenic factor, VEGF and IL-8 in both *in vitro* and *in vivo* remarkably to reduce the tumor angiogenesis. In promoting the metastasis of tumor, the activity of NF-κB can make the factor expression of cell line of high-metastatic breast cancer, MMPs and uPA as well as some cells high. The further research finds that NF-κB can regulate the motility of breast cancer cell line through directly increasing the expression of CXCR4. Moreover, the expression of cell line, CXCR4 which has higher pulmonary metastasis (32).

In the molecules of signaling pathways and pathways of apoptosis, caspase family has an important role, caspase family do the major work in cutting off cell signaling, recombination cytoskeleton, closing dna replication as well as repair, destroying DNA and nuclear structure throughout the downstream effect (33). Caspase family also induces apoptotic bodies in order to perform apoptosis by inducing apoptosis in tumor cells, especially in chemotherapy resistant tumor cells, the action of drugs can be enhanced by mediating the activity of caspase-3 and caspase-8 (34). Caspase-9 can regulate chemically induced dipolymer infiltration, other caspase activation and apoptosis in mitotic as well as in non-dividing cells (35).

Through various parts of experiments, this research finds that the combined effect of β-elemene and ligustrazine can promote the apoptosis in tumor more precisely. Especially, it is proved by the help of many molecular experiments that the combined effect of β-elemene and ligustrazine can regulate and control the expression of GPR124, and significantly affect the metastasis expression of relevant tumor so as to realize the purpose to control the tumor metastasis. The tumor can be effectively inhibited by regulating the expression of GPR124 in bone cancer cell and we can effectively control its migration.

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