

# Melittin exerts an antitumor effect on non-small cell lung cancer cells

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**Abstract.** Lung cancer accounts for a significant percentage of all cancer-associated mortalities in men and women, with non-small cell lung cancer being the most frequently occurring type of lung cancer. Melittin is the principal active component of apitoxin (bee venom) that has been reported to exert anti-chronic inflammatory and anti-cancer effects. In the present study, the antitumor effect of melittin was evaluated using *in vivo* and *in vitro* analyses. The results demonstrated that melittin significantly inhibited the epidermal growth factor-induced invasion and migration of non-small cell lung cancer cells. Subcutaneous injection of melittin at doses of 1 and 10 mg/kg significantly suppressed non-small cell lung cancer tumor growth by 27 and 61%, respectively. In addition, melittin significantly inhibited the secretion of vascular endothelial growth factor (VEGF) in non-small cell lung cancer cells. Furthermore, melittin decreased the protein expression of VEGF and hypoxia-inducible factor 1- $\alpha$ . Therefore, the antitumor activity of melittin may be associated with the anti-angiogenic actions of inhibiting the VEGF and hypoxia-inducible factor signaling pathways.

## Introduction

Lung cancer, additionally termed lung carcinoma or pulmonary carcinoma, is the leading cause of cancer-associated mortality worldwide and is characterized by uncontrolled cell growth in tissues of the lung. Lung cancer is the most fatal type of cancer in the USA, responsible for more fatalities than colorectal, breast and prostate cancers combined. An estimated

158,040 Americans are expected to have succumbed to lung cancer in 2015, accounting for ~27% of all cancer mortality in America (1-3).

It has previously been demonstrated that ~80-90% of cases of lung cancer are associated with cigarette smoking (4). Symptoms of lung cancer include, however are not limited to, coughing, chest pain and weight loss. The prognosis for a lung cancer patient is frequently poor, however outcomes have improved, due to the identification of certain mutations that maybe targeted for therapy (5). Depending on the stage of the disease, lung cancer treatment may include surgery, chemotherapy, radiation therapy or a combination of these.

It is reported that numerous active substances produced by animals, plants and microorganisms have been employed in the development of novel drugs to treat cancer (6). Of these identified substances, the peptide melittin in bee venom has been demonstrated to exhibit antitumor activity (7). Melittin is reported to exhibit the antitumor potential, and is isolated from bee venom, acting via differing mechanisms on the physiology of cancer cells (8). It is suggested that the cytotoxicity of melittin in tumor cell lines, and its action on signaling pathways, lead to the inhibition of cellular proliferation (9).

Melittin is the principal toxic component in the venom of the European honey bee (*Apis mellifera*) and is a cationic, hemolytic peptide that is small and linear and composed of 26 amino acid residues. The amino-terminal region of the peptide is predominantly hydrophobic, whereas the carboxy-terminal region is hydrophilic. This is due to the presence of a stretch of positively charged amino acids (10). The sequence of melittin is as follows: Gly-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Trp-Ile-Lys-Arg-Lys-Arg-Gln-Gln (6). It is reported that melittin targets a range of cancer cells, including those in leukemia, lung, renal, liver, bladder and prostate cancers (11). Heinen and da Veiga (6) reported that lytic peptide conjugates were all highly effective in targeting and destroying disseminated breast cancer metastases in lymph nodes, bones, lungs and other organs). In addition, Jeong *et al* (12) demonstrated that melittin suppresses epidermal growth factor (EGF)-induced cell motility and invasion by inhibiting the phosphoinositide 3-kinase/protein kinase B/mechanistic target of rapamycin (mTOR) signaling pathway in breast cancer cells. Furthermore,

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Shin *et al* (13) reported that melittin may be able to suppress the expression of hypoxia-inducible factor (HIF)-1 $\alpha$  and vascular endothelial growth factor (VEGF) through inhibiting the extracellular signal-regulated kinase and mTOR/p70S6K pathways in cervical carcinoma cells.

Therefore, the aim of the present study was to determine if melittin has a direct action on processes of non-small cell lung cancer cells.

## Materials and methods

**Ethical approval.** All animal experiments in the present study were approved by the Ethics Committee of Tongji University School of Medicine (Shanghai, China).

**Cell culture.** The human non-small cell lung cancer cell line, A549, was purchased from the Shanghai Institute for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% FBS at 37°C in an environment containing 5% CO<sub>2</sub>.

**Cell viability assays.** Cells at a density of 1x10<sup>3</sup> cells/well were plated in 96-well culture plates and cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS. Following a 24 h attachment period, media were replaced with 100  $\mu$ l of medium containing different concentrations (0, 0.5, 1, 2 or 4  $\mu$ g/ml) of melittin (cat no. M4171; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and cultured for 24 h. A total of 10  $\mu$ l Cell Counting Kit (CCK)-8 (cat no. CK04-3000T; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was added to each well for 4 h at 37°C in a 5% CO<sub>2</sub> incubator, then the absorption value was tested according to the manufacturer's protocol. The reaction product was quantified by measuring the absorbance value at 490 nm using a DU® 800 spectrophotometer (Beckman Coulter, Inc., Brea, CA, USA).

**Flow cytometric analysis of apoptosis.** A549 non-small cell lung cancer cells were seeded onto 6-well dishes at a density of 2x10<sup>6</sup> cells/ml. Following a 24 h period for attachment, the cells were treated with different concentrations of melittin (0, 0.5, 1 or 2  $\mu$ g/ml) for 24 h. Cells were treated with 1% para-formaldehyde for 15 min at room temperature, permeabilized with 10% Triton X-100 (cat no. 93443; Sigma-Aldrich; Merck KGaA) for 10 min at room temperature, and stained using the FITC-Annexin V Apoptosis Detection kit I (BD Biosciences, Franklin Lakes, NJ, USA), according to the manufacturer's protocol. The proportion of apoptotic cells was measured using a flow cytometer (BD FACS Aria™ III; BD Biosciences) and analyzed using FlowJo software (version 10; Tree Star, Inc., Ashland, OR, USA). All experiments were repeated three times (14).

**Transwell invasion assay.** Cell invasion serves a pivotal role in the progression of cancer. Non-small cell lung cancer cells (100  $\mu$ l cell suspension; 1x10<sup>6</sup> cells/ml) were plated on the Matrigel-coated upper chamber, and the serum-free media with or without drugs were added to the upper chamber of

the Transwell insert. The concentrations of melittin used were 0, 0.5, 1 and 2  $\mu$ g/ml, in combination with 20 ng/ml EGF. The lower chamber was filled with DMEM. Cells in the chamber were incubated for 24 h at 37°C and cells that invaded the lower membrane surface were removed using a cotton swab, fixed with methanol and stained with crystal violet for 30 min at room temperature. The cells that passed through the Matrigel and were located on the underside of the filter were counted. A total of 6 random fields were counted by light microscopy (13).

**Wound-healing assays.** The wound-healing assay is an effective tool for the investigation of the migration characteristics of cultured cells. Non-small cell lung cancer cells were seeded onto 6-well plates at a density of 5x10<sup>5</sup> cells/well. Following reaching 80% confluence, monolayers were scratched to create a wound, and then were washed twice with PBS to remove non-adherent cells. Media were then replaced with fresh serum-free medium containing different concentrations of melittin (0, 0.5, 1 or 2  $\mu$ g/ml), and 20 ng/ml of EGF for 24 h, prior to being photographed (12).

**Animal experiments.** A total of 18 BALB/C nude mice (six-weeks-old; male; weight, 20 $\pm$ 1 g) were purchased from the Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). Mice were kept under standard animal housing conditions (12-h light/dark cycle) with food and water *ad libitum* at a temperature of 20 $\pm$ 2°C and a relative humidity of 40-50%. BALB/C nude mice were subcutaneously injected with 0.1 ml Matrigel-containing non-small cell lung cancer cells (1.5x10<sup>6</sup> cells/ml) into the left and right flank. A total of 2 weeks following inoculation, tumors grew to 150-200 mm<sup>3</sup> and the mice were randomly divided into the following three groups (n=6 in each): Group A, administration with a subcutaneous injection of melittin (1 mg/kg) every day for 24 days; group B, administration with a subcutaneous injection of melittin (10 mg/kg) every day for 24 days; control, control group treated with vehicle control of PBS. Tumor growth was recorded daily until day 24. Following subcutaneous injections of melittin for 24 days, the mice were sacrificed, and the tumors were harvested and weighed using an electronic scale (CR5501; Care Electronic Scale Co., Ltd., Zhongshan, China). The tumor volume was calculated using the following formula: Tumor volume (V) = [length (L) x width (W)]<sup>2</sup>/2, in which the length is greater than the width. The average tumor volume in mm<sup>3</sup> was plotted for each third day, from day 9 until day 24. Data are presented as the mean  $\pm$  standard deviation (15).

**Measurement of VEGF levels using an enzyme-linked immunosorbent assay (ELISA).** Non-small cell lung cancer cells (1,000 cells/well) were seeded onto 96-well plates 6-well dishes. Following a 24 h attachment period, the cells were treated using different concentrations of melittin (0, 0.5, 1 or 2  $\mu$ g/ml) for 24 h. The culture supernatant of cells was collected individually, and the concentration of VEGF was measured using an ELISA kit (cat no. DVE00; R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's protocol.

**Western blot analysis.** Cultured cells were used in western blotting, as previously described (16), followed by homogenization

in lysis buffer (8 M urea, 0.2% SDS, 0.8% Triton X-100, 3% 2-mercaptoethanol), and the protein concentration was measured using a protein assay kit (cat no. 5000002) with bovine serum albumin as the standard (both from Bio-Rad Laboratories, Inc., Hercules, CA, USA). A total of 50  $\mu$ g protein/lane was separated by SDS-PAGE on a 10% gel and transferred onto nitrocellulose membranes. Membrane were blocked in 5% milk in TBS-Tween-20 for 1 h at room temperature. Western blot analysis was performed using the following diluted primary antibodies against: (HIF)-1 $\alpha$  (cat no. sc-10790; 1:200), VEGF (cat no. A-20; 1:250),  $\beta$ -actin (cat no. C-4; 1:500) (all from Santa Cruz Biotechnology, Dallas, TX, USA) and biotinylated anti-mouse IgG and biotinylated goat anti-rabbit IgG secondary antibodies (cat nos. 115-065-003 and 111-067-003; 1:100; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). The  $\beta$ -actin served as the internal control. Membranes were incubated with the primary antibodies for 1 h at room temperature, and subsequently with the secondary antibodies for 1 h at room temperature. The immunoreactivity was visualized using the Immobilon western blotting detection system (cat no. WBKLS0050; EMD Millipore, Billerica, MA, USA). Films were developed and scanned, and bands were quantified using Scion Image Analysis software, version 4.02 (Scion Corporation, Frederick, MD, USA).

**Statistical analysis.** One-way analysis of variance followed by the Tukey post-hoc test was performed to analyze the differences between different groups.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Cell viability.** Prior to evaluating the pharmacological potential of melittin on human non-small cell lung cancer cells, the cytotoxic effects of the drug were examined via a CCK cell viability assay. Each concentration of melittin was compared with the control group, and it was observed that melittin did not significantly inhibit cell viability at concentrations of up to 2  $\mu$ g/ml ( $P > 0.05$ ; Fig. 1A), therefore drugs at 2  $\mu$ g/ml were used in the following experiments.

**Flow cytometric analysis of apoptosis rate.** The authors detected the effects of melittin on apoptosis of non-small cell lung cancer cells. The results demonstrated that the apoptosis rate was significantly greater at 2  $\mu$ g/ml melittin, when compared with the control group (0  $\mu$ g/ml;  $P < 0.01$ ; Fig. 1B).

**Melittin inhibits the migration and invasion of non-small cell lung cancer cells.** The cytotoxic effects of melittin were examined using the CCK-8 kit prior to evaluating the pharmacological potential of melittin on non-small cell lung cancer cells.

Cell Transwell invasion assays were used to evaluate the inhibitory effects of melittin on the EGF-induced migration and invasion of non-small cell lung cancer cells. As demonstrated in Fig. 2, melittin inhibited EGF-induced (20 ng/ml) cell migration of non-small cell lung cancer cells. This inhibition was significant at melittin concentrations of 0.5, 1 and 2  $\mu$ g/ml, when compared with the control group ( $P < 0.05$ ; Fig. 2A).

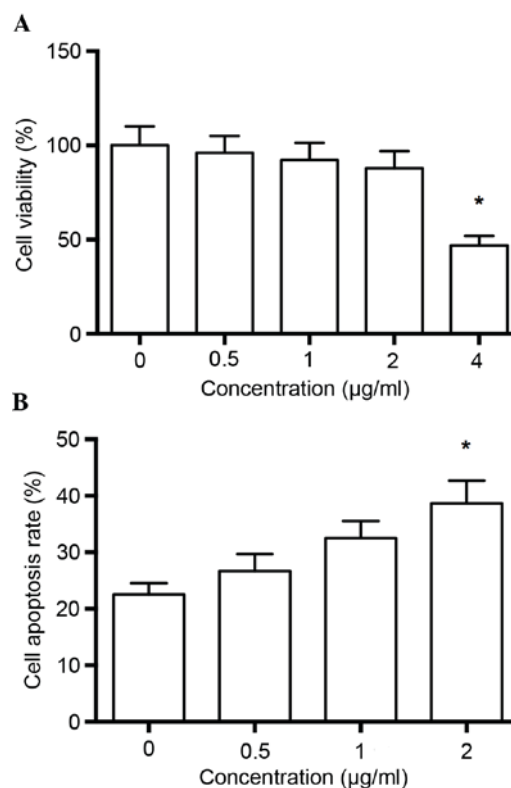


Figure 1. Melittin exerts various inhibitory effects on non-small cell lung cancer cells. (A) Non-small cell lung cancer cells were treated with different concentrations of melittin for 24 h. Cell viability was determined with a cell-counting kit assay, and demonstrated that 4  $\mu$ g/ml melittin resulted in a significant decrease to cell viability, when compared with control group. (B) Flow cytometric analysis indicated that melittin increased the apoptosis rate within non-small cell lung cancer cells, however only a concentration of 2  $\mu$ g/ml increased apoptosis rate significantly, when compared with the control group. Data are presented as the mean  $\pm$  standard deviation. \* $P < 0.05$  vs. 0  $\mu$ g/ml.

Furthermore, the inhibitory effect of melittin on cell invasion was similar to that on migration, with melittin significantly inhibiting the EGF-induced invasion in the Transwell invasion assays at melittin concentrations of 0.5, 1 and 2  $\mu$ g/ml ( $P < 0.05$ ; Fig. 2B).

**Melittin decreases levels of VEGF in non-small cell lung cancer cells.** Tumor growth and metastasis depend on angiogenesis triggered by chemical signals from tumor cells in the rapid growth phase. VEGF performs a critical role in the development of angiogenesis. To determine whether the inhibitory effect of melittin affects VEGF expression, the secretion of VEGF protein was examined via ELISA analysis under EGF-conditions in non-small cell lung cancer cells. As presented in Fig. 2C, the effects of melittin on the secretion of the VEGF protein increased significantly under the EGF-induced conditions, whereas melittin at concentrations of 0.5, 1 and 2  $\mu$ g/ml significantly decreased the secreted VEGF levels, as induced by EGF ( $P < 0.05$ ). VEGF expression is primarily regulated by HIF-1 $\alpha$ , so the authors further evaluated HIF-1 $\alpha$  and VEGF protein expression using western blot analysis. The results suggested that melittin decreased HIF-1 $\alpha$  and VEGF protein expression, which indicated that melittin may regulate VEGF levels by inhibiting HIF-1 $\alpha$  (Fig. 2D).

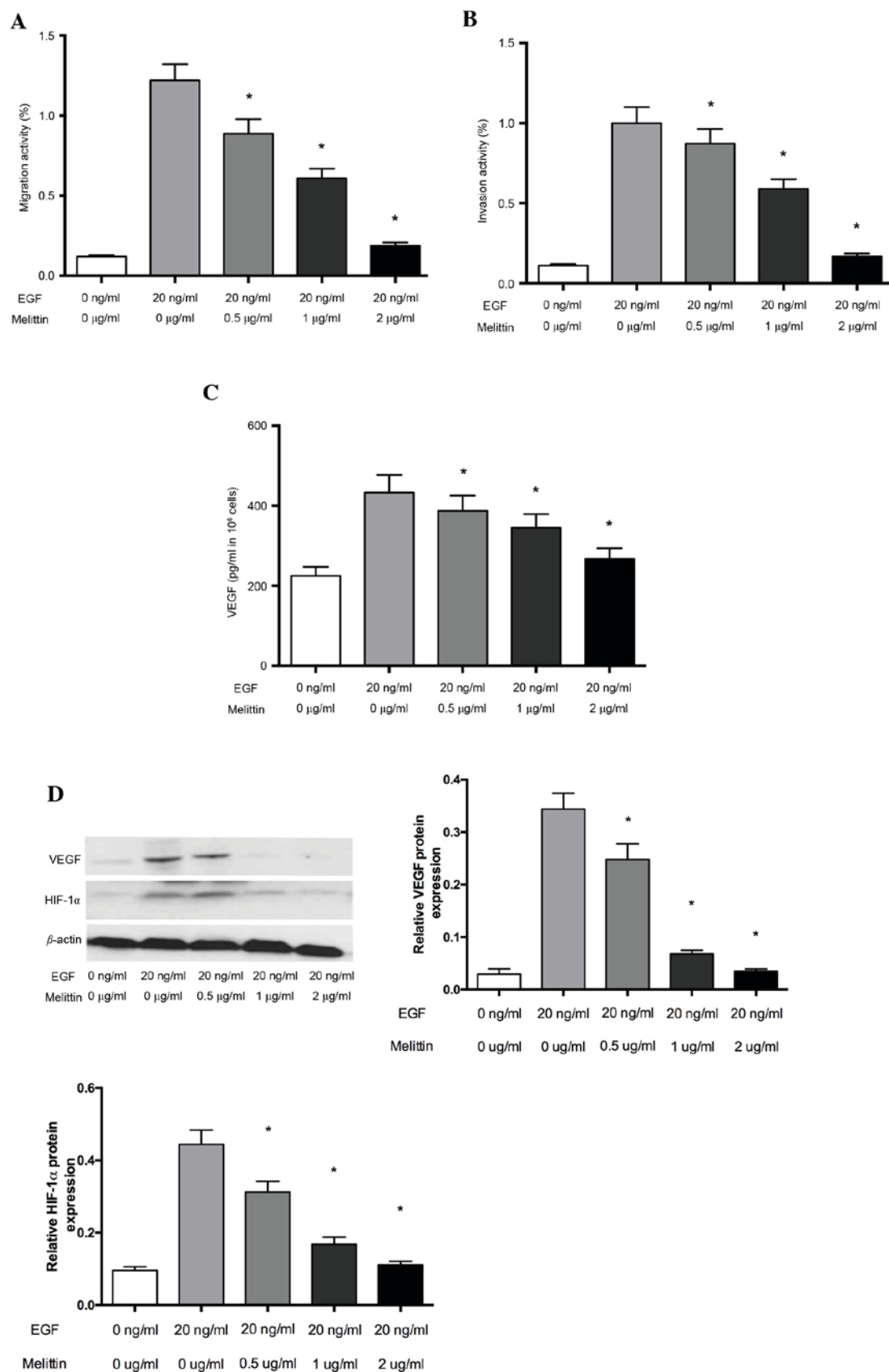


Figure 2. Melittin exerts various inhibitory effects on non-small cell lung cancer cells detected with differing assays. (A) A wound-healing migration assay was conducted and demonstrated that melittin inhibited the EGF-induced migration of non-small cell lung cancer cells, when compared with the control group (0  $\mu$ g/ml EGF, 0  $\mu$ g/ml melittin). (B) An invasion assay identified that melittin inhibited EGF-induced invasion, when compared with the control group (0  $\mu$ g/ml EGF, 0  $\mu$ g/ml melittin). (C) An enzyme-linked immunosorbent assay demonstrated that melittin inhibited the secretion of VEGF in non-small cell lung cancer cells, when compared with the control group (0  $\mu$ g/ml EGF, 0  $\mu$ g/ml). (D) Western blot analysis demonstrated that VEGF and HIF-1 $\alpha$  expression decreased by increasing the concentration of melittin. Data are presented as the mean  $\pm$  standard deviation. \*P<0.05 vs. control. VEGF, vascular endothelial growth factor; HIF-1 $\alpha$ , hypoxia-inducible factor-1 $\alpha$ ; EGF, epithelial growth factor.



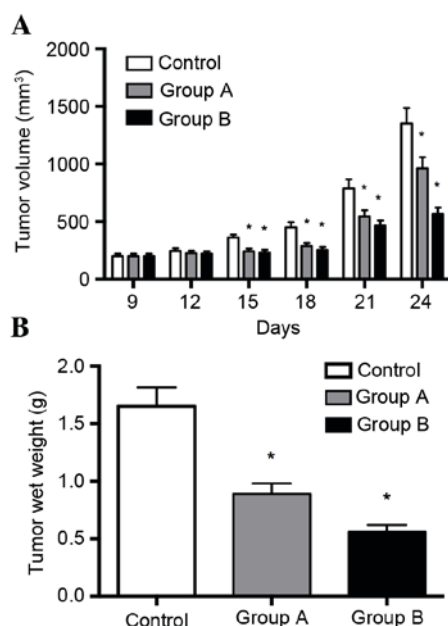


Figure 3. Melittin inhibits tumor growth of non-small cell lung cancer cells injected subcutaneously into mice. The (A) volume and (B) wet weight of the tumors were evaluated, and these assays demonstrated that melittin inhibited tumor growth of non-small cell lung cancer cells. The inhibition of tumor growth is greater with a dose of 10 mg/kg melittin (group B), than with 1 mg/kg (group A). Data are presented as the mean  $\pm$  standard deviation. \* $P < 0.05$  vs. control.

**Melittin inhibits tumor growth in xenograft models of lung cancer.** The *in vitro* data demonstrated that melittin exerts a tumor suppressor role on non-small cell lung cancer cells. To explore this role *in vivo*, tumor growth was assessed using tumor xenograft mouse models. As presented in Fig. 3A, melittin (at concentrations of 1 or 10 mg/kg) inhibited tumor growth of non-small cell lung cancer cells. Tumor growth was significantly decreased by the two concentrations of melittin when recordings were taken on days 15, 18, 21 and 24, when compared with the control group ( $P < 0.05$ ). Tumors in experimental groups were smaller compared with those in the control group, and tumors in the 10 mg/kg melittin group were smaller than those in the 1 mg/kg melittin group. Melittin significantly inhibited tumor growth by 27% (1 mg/kg/day) and 61% (10 mg/kg/day).

## Discussion

Lung cancer is a type of cancer that is initiated in lung tissue. There are three primary types of lung cancer, including non-small cell lung cancer, small cell lung cancer and lung carcinoid tumor (17). Of these, non-small cell lung cancer is the most frequently occurring form, responsible for ~85% of all lung cancer diagnoses (18). Squamous cell carcinoma, adenocarcinoma and large cell carcinoma are all subtypes of non-small cell lung cancer. Lung cancer claims more lives each year than colon, prostate, ovarian and breast cancers combined (19). As a result, it is crucial to develop a novel drug for the treatment of lung cancer (20).

Melittin is a major peptide constituent of bee venom that has been proposed as one of the potential drugs for anticancer

therapy (21). Certain studies have suggested that melittin inhibits the melanotropin receptor in M2R melanoma cell membranes (22), whereas other studies have demonstrated that melittin kills malignant cells by acting as a pore-forming agent (23).

Angiogenesis is known to serve a crucial role in tumor growth, tumor propagation and metastasis formation (24). Therefore, as anti-angiogenesis is an efficient method in anti-tumor treatment, the present study tested the anti-angiogenesis potential of melittin. The results indicated that it is effective, as the results of western blot analysis presented a decrease in VEGF when cells were treated with doses of melittin. It is reported in previous studies that melittin exerts an effect on proliferation and induces growth inhibition and apoptosis via several cancer cell death mechanisms. These mechanisms include the activation of caspases and matrix metalloproteinases in different types of cancer cells (25,26). Melittin has been suggested to suppress tumor growth by inhibiting VEGFR-2, thereby indicating that the antitumor activity of melittin may be associated with anti-angiogenic actions via inhibition of VEGFR-2 and inflammatory mediators involved in mitogen-activated protein kinase signaling (12).

The results of the *in vivo* tumorigenicity tests demonstrated that the melittin injection suppressed tumor wet weight and tumor size. Melittin significantly inhibited tumor growth by 27% (1 mg/kg/day) and 61% (10 mg/kg/day) over the study period.

In conclusion, the findings suggested that melittin exerts an antitumor effect on lung cancer cells *in vitro* and *in vivo*, indicating the potential of melittin for the treatment of lung cancer.

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