A Novel Peptide from Vespa ducalis Induces Apoptosis in Osteosarcoma Cells by Activating the p38 MAPK and JNK Signaling Pathways

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Osteosarcoma (OS) is one of the most frequently found aggressive tumors in bone, which accounts for 5% of all childhood cancers.1 In the past decade, great efforts have been made in developing more effective therapies for patients with OS. A number of clinical trials have examined different doses of conventional drugs as multimodal therapies. However, most of these have failed.2,3 With the development of new adjuvant chemotherapies, 50% of OS patients were alive five years later. Nevertheless, the high rate of drug resistance and side effects has limited their wide application in the clinic.4 In order to ameliorate the therapeutic outcome in patients with OS, developing novel treatment modules that have better tumor selectivity and fewer side effects has become an urgent need.

Venom is secreted by organisms and contains many pharmacologically active constituents that might make a potential contribution to cancer therapies. For example, venoms from snake and bee have been demonstrated to kill tumor cells directly or through inhibiting tumor angiogenesis.5–7 Wasp venom has been used as a traditional Chinese medicine for its multiple functions. Similar to venoms derived from many other venomous animals, the venom of wasps contains a mixture of proteinaceous and non-proteinaceous constituents. Previous studies have determined the profiles of ectoparasitoid and endoparasitoid venoms.8–11 However, the activity of peptides in venoms from different animals remains largely unclear.

A major peptide compound in bee venom, melittin, could reportedly suppress cell proliferation and promote the apoptosis of human leukemic U937 cells. Moreover, melittin has been revealed to downregulate cell proliferation and promote the apoptosis of OS cells, as well as stimulate the marrow to produce leukocytes.12–14 These facts suggest that peptides in wasp venom are promising for OS therapy. In summary, we identified a novel peptide derived from Vespa ducalis (V. ducalis) Smith, and assessed its antitumor effects in OS cells.

Key words wasp; peptide; apoptosis; osteosarcoma; c-Jun N-terminal kinase (JNK); p38 mitogen-activated protein kinase (MAPK)

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MATERIALS AND METHODS

Reagents The chemicals used in the present study were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.). The Sephadex G-50 gel filtration column was obtained from GE Healthcare (Chicago, IL, U.S.A.). The C18 column was obtained from Phenomenex (Torrance, CA, U.S.A.). The antibodies used in the present study were obtained from Santa Cruz Biotechnology (Dallas, TX, U.S.A.).

Ethics Statement Three OS cell lines (MG-63, U-2 OS, and Saos-2) were obtained from ATCC (U.S.A.). The study protocol was approved by the Ethics Committee of the Institutional Review Board of the Second Xiangya Hospital of Central South University. The wasps (V. ducalis Smith) collected in the present study were very common, and are not protected species in Guanxi province, China.

V. ducalis Smith Wasp Venom Collection Adult V. ducalis Smith wasps were collected, stimulated with 5V of electricity, and subjected to venom collection. After electronic stimulation, approximately 0.1 µL of wasp toxin was collected from a single adult worker wasp. A total of 10 mL of venom was obtained from approximately 10000 wasps. The crude venoms were stored at −80°C.
**Peptide Purification** Peptides were purified, based on a previous study.\(^{15}\) The crude venoms obtained from *V. ducalis* wasps were dissolved in 0.1 M of phosphate buffer solution (PBS) containing 5 mM of ethylenediaminetetraacetic acid (EDTA). The crude venoms were separated using a Sephadex G-50 gel filtration column (Superfine, 2.6 cm in diameter and 100 cm in length) and eluted with 0.1 M of PBS (pH 6.0) at 280 nm. Then, 3.0 mL of each fraction was subjected to further experiments. Fractions that might have anticancer activity were further purified using C\(_{18}\) reverse-phase HPLC (RP-HPLC), as previously described,\(^{16}\) with the help of buffer A (0.1% [v/v] trifluoroacetic acid/water) and buffer B (0.1% [v/v] trifluoroacetic acid/acetoni trile).

**Mass Spectrometric Assays** Lyophilized HPLC fractions were dissolved in buffer A. Then, 0.5 \(\mu\)L of each sample was spotted onto a matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) (Bruker Daltonics, Bremen, Germany) plate with 0.5 \(\mu\)L of a-cyano-4-hydroxycinnamic acid matrix (10 mg/mL in 60% acetonitrile), and analyzed using an UltraFlex I mass spectrometer.

**Peptide Sequencing** The peptide sequences were identified via Edman degradation using a pulsed liquid-phase Procise\(^{®}\) Sequencer, Model 491 (Applied Biosystems, Foster City, CA, U.S.A.).

**OS Cell Lines and Cell Culture** Human OS cell lines MG-63, U-2 OS and Saos-2 were purchased from ATCC, and cultured in a humidified atmosphere with 5% CO\(_2\) in RPMI 1640 medium (Gibco; Life Technologies, CA, U.S.A.) with 10% fetal bovine serum (FBS) and antibiotics, including 100 U/mL of penicillin and 100 U/mL of streptomycin at 37°C.

**Cell Survival Assay** Cell viability in response to the indicated peptides was examined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays, based on previous studies.\(^{17–19}\) The target cells were seeded in 96-well plates (0.8–1.2\(\times\)10\(^4\) cells/well) and exposed to a series of doses of the indicated peptide for different time-points. After co-incubation, 10 \(\mu\)L of MTT solution (5 mg/mL; Sigma) was added to each well. After 2–4 h of incubation at 37°C, the supernatant was discarded, and formazan crystals were dissolved using 100 \(\mu\)L of dimethyl sulfoxide (DMSO). Then, an OPTImax microplate reader (Molecular Devices, Sunnyvale, CA, U.S.A.) was used to analyze the absorbance at 570 nm. The percentage of cell survival was determined as follows: percentage of cell survival=mean optical density (OD) of peptide-containing wells/mean OD of DMSO-containing control wells. The IC\(_{50}\) values were calculated using the results obtained from three independent experiments.

**Cell Cycle and Apoptosis of OS Cells by Flow Cytometer Assays** Cells treated as described were fixed with 95% ethanol, washed with cold PBS and incubated with 150 mL of hypotonic fluorochrome solution, as previously described,\(^{20}\) in the dark at 4°C overnight. The apoptotic cells were stained using a CF488A-Annexin V and Propidium Iodide Apoptosis Assay Kit (Biotium), according to the protocols of the kit. Flow cytometry was employed on a FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, U.S.A.). DMSO-treated cells were used as controls.

**Immunoblotting Assays** MG-63 cells (1\(\times\)10\(^6\) cells/well) were seeded into a 6-well plate, transferred to serum-free Dulbecco’s Modified Eagle’s Medium (DMEM) and incubated for 16 h in a humidified 5% CO\(_2\) atmosphere at 37°C. After treatment with peptide for 12 h, the cells were collected and washed twice with ice-cold PBS. Then, cells were resuspended in 150 \(\mu\)L of extraction lysis buffer\(^{21}\) for 30 min at 4°C. Thereafter, the supernatant was collected and frozen after discarding cell debris. Total proteins were extracted, as determined by Bradford protein assay, subjected to 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and electro-blotted onto a polyvinylidene difluoride (PVDF) membrane. After blocking with 5% skim milk for three hours at 37°C, the blots were incubated with the primary antibody against \(\beta\)-actin, Bcl-2, c-Jun, poly ADP-ribose polymerase (PARP), phospho-stress-activated protein kinase/c-Jun N-terminal kinase (pSAPK/JNK), or p-p38 mitogen-activated protein kinase (MAPK) at 4°C overnight. Then, the blots were washed with TBST for three times, and incubated with a 1:1000 diluted horseradish peroxidase-conjugated secondary antibody for one hour. Finally, the blots were washed with Tris-Buffered Saline Tween-20 (TBST) for three times and analyzed using ECL (enhanced chemiluminescence; Tiangen Biotech, Beijing, China).

**Apoptosis Antibody Array** The PathScan\(^®\) Stress and Apoptosis Signaling Antibody Array Kit (Cell Signaling Technology, Danvers, MA, U.S.A.) was applied for the simultaneous detection of 19 signaling molecules related to stress response and apoptosis (the protein information is shown in Table 1).

**Statistical Analysis** All data were analyzed using Student’s t-test, followed by parametric one-way ANOVA, and were presented as mean±standard deviation (S.D.).

## RESULTS

**Purification and Identification of VACP1** Crude venom collected from *V. ducalis* was first separated using a Sephadex G-50 gel filtration column, and eluted with 0.1 M of PBS. The fractions were collected using an automatic collector at a rate of 3.0 mL/10 min. Three peaks were observed in the wasp toxin fractionations (Fig. 1A). The fractions with the highest peak were purified using RP-HPLC with 10–50%
buffer B for over 40 min (Fig. 1B), and these were further separated using the same column with 20% buffer B for over 30 min (Fig. 1C). The purity of the separated peptide was over 99%, as determined by mass spectrometry (Fig. 1D). This purified peptide was named as venom anti-cancer peptide 1 (VACP1).

Next, VACP1 was sequenced by Edman degradation, and it was found that the complete amino acid sequence of VACP1 was AQKWLKYWKADVKGFGRKIKKIWFG (Fig. S1). MALDI-TOF mass analysis revealed that its molecular mass was 3120.4 Da. VACP1 was identified as a novel peptide by a BLAST search based on the UniProt database.

**VACP1 Suppresses OS Cell Proliferation** In order to test the functions of VACP1 on OS cell proliferation, three OS cell lines (MG-63, U-2 OS, and Saos-2) were subjected to treatment with 2 μM of VACP1 or methotrexate for 48 h. Then, cell viability was examined using MTT assays. Treatment with 2 μM of VACP1 significantly suppressed the proliferation of MG-63, U-2 OS and Saos-2 cells by approximately 72, 65 and 41%, respectively, while treatment with 2 μM of methotrexate only reduced cells growth by 15, 19 and 26%, respectively (Figs. 2A, B). Further assessment of the func-

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![Fig. 1. Purification of VACP1 Obtained from the Venom of *V. ducalis* Smith](image)

(A) The venom derived from *V. ducalis* Smith samples were filtrated using Sephadex G-50 gel and eluted using 0.1 M of phosphate buffer (pH 6.0) at a volume of 3.0 mL. (B) The asterisk-marked fraction was further purified by RP-HPLC. (C) The second purification of VACP1 via RP-HPLC. (D) The molecular mass was determined by mass spectrometry.

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![Fig. 2. Effect of VACP1 on the Growth of Osteosarcoma Cells](image)

(A) MG-63 cells were treated with DMSO (controls) and 2 μM of methotrexate or VACP1 for 48 h. The representative images of MG-63 cell morphology are marked as control, methotrexate, and VACP1. The right panel shows the HEK293 cells treated with 2 μM of VACP1 for 48 h. (B) Cell viability was examined by MTT assay. All data are presented as mean±standard error (S.E., n=5). **p<0.01, compared to the control group. (C) IC_{50} values for VACP1 in MG-63, Saos-2 and U-2 OS cells.
tions of different doses of VACP1 on OS cell proliferation revealed that the suppressive effect of VACP1 on OS cell proliferation was dose-dependent, with IC_{50} values of 0.52, 0.99 and 2.43 µM for MG-63, U-2 OS and Saos-2 cells, respectively (Fig. 2C). MG-63 cells were more sensitive to VACP1 treatment than the other two cell lines. The above findings revealed that VACP1 could suppress OS cell proliferation in a concentration-dependent manner. Meanwhile, no suppression was found on HEK293 cells after treatment with 2 µM of VACP1 for 48h.

**VACP1 Promotes MG-63 Cell Apoptosis** In order to examine whether VACP1 could increase OS cell apoptosis, MG-63 cells were treated with 0.2, 0.5 and 1.0 µM of VACP1 for 48h. Cell apoptosis was tested by Annexin V-propidium

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**Fig. 3. Apoptosis and Cell Cycle Progression of MG-63 Cells Exposed to VACP1**

MG-63 cells were cultured with DMSO (A) or VACP1 at concentrations of 0.2 (B), 0.5 (C) and 1.0 (D) µM for 48h. The sub-G1 increased by approximately 4.2% after treatment with 1.0 µM of VACP1 for 24h. After incubation with 1.0 µM of VACP1, 10 µM of SB203580 (E) or SP600125 (F) were added to cells for 48h. Cell apoptosis was examined using Annexin V-PI staining and flow cytometry. The representative flow cytometry analyses of cells using annexin V, and the cell cycle analysis results after 24h of treatment with control (G) and 1.0 µM of VACP1 (H) are shown.
iodide (PI) staining and flow cytometry assays. The apoptotic cell rate of MG-63 cells (Annexin V or PI positive cells) with DMSO was 9.6% (Fig. 3A), while the rate for those treated with 0.2, 0.5 and 1.0 µM of VACP1 were 29.0% (Fig. 3B), 37.0% (Fig. 3C) and 52.2% (Fig. 3D), respectively. However, in comparison to DMSO controls (Fig. 3G), there was no difference in the populations of cells in the G1, S and G2 phases after treatment with 1.0 µM of VACP1 (Fig. 3H). Thus, VACP1 concentration-dependently induced apoptosis, but this did not affect the cell cycle progression of MG-63 cells.

**VACP1 Could Activate the p38 MAPK and JNK Signaling Pathway**

The mechanism of apoptosis is complex and involves many pathways. In order to explore the potential apoptosis pathways altered by VACP1 treatment in OS cells, an apoptosis antibody array containing 19 proteins, including p-p38 MAPK, p-JNK, protein kinase B (Akt) and caspase-7, was used. Changes in these apoptosis-related proteins were detected in MG-63 cells in response to 0.2, 0.5 and 1 µM VACP1 treatment. It was found VACP1 treatment could increase the phosphorylation levels of p38 MAPK and JNK in MG-63 cells in a dose-dependent manner (Fig. 4). Next, the activation of the p38 MAPK and JNK signaling pathways were validated by Western blot. Consistently, VACP1 increased JNK and p38 phosphorylation in a dose-dependent manner, with no function on the total protein levels of JNK or p38 MAPK (Fig. 5A). Moreover, VACP1 concentration-dependently induced the cleavage of PARP and caspase 3, promoted the phosphorylation of c-Jun, and decreased the protein level of Bcl-2, which is a well-established anti-apoptotic protein. These results suggest that VACP1 stimulates the activation of p38 MAPK and JNK signaling, along with the cleavage of apoptosis markers, PARP and caspase 3. In order to confirm whether VACP1 induces apoptosis though the p38 MAPK and JNK signaling pathways, inhibitors of p38 (SB203580) and JNK (SP600125) were used to block these pathways. As shown in Fig. 3E, no apoptosis was found in MG-63 cells after incubation with 1 µM of VACP1 and 10 µM of SB203580 for 48 h. Furthermore, the addition of 10 µM of SP600125 to MG-63 cells was able to abrogate the apoptotic effect of VACP1 after 48 h (Fig. 3F). Moreover, the VACP1-induced phosphorylation of p38 MAPK was suppressed by 10 µM of SB203580 (Fig. 6A). Similar to SB203580, 10 µM of SP600125 also decreased p-p38 and p-JNK expression levels. The above findings suggest that VACP1-induced apoptosis may be mediated by activating the p38 MAPK and JNK signaling pathways.

**DISCUSSION**

In the present study, a new found peptide VACP1 derived from the wasp venom of V. ducalis Smith was identified. The amino acid sequence of VACP1 was AQKWLKYWKADKVKGFBCKKKIKIWF, and the molecular mass was 3120.4 Da. Functional studies have demonstrated that VACP1 inhibited the growth and induced apoptosis of OS cells, accompanied by cleavage of PARP and caspase 3. Further mechanistic studies have shown that VACP1 might exert its biological effects through the activation of p38 MAPK and JNK signaling.
Apoptosis has been identified as a key event in a large number of physiological processes, including fetal development, and in the pathogenesis of almost all kinds of diseases. The dysregulation of cell death and/or cell apoptosis contribute to many diseases, including cancers. The pro- and anti-apoptotic members of the Bcl-2 family interact with each other to maintain homeostatic balance within cells through complex mechanisms. This protein family is also involved in the stress signal transduction pathways of the endoplasmic reticulum (ER), and in maintaining ER homeostasis. The induction of apoptosis results in the activation of a series of caspases, including caspase-3. Cancer cell apoptosis escape is the main cause of the hyperproliferation of cancer cells. Hence, targeting apoptosis is one of the major strategies for blocking the development of cancer treatments. Venom represents a rich source of anticancer drugs. In the present study, we isolated VACP1 from wasp venom and found that it could suppress OS cell proliferation in a concentration-dependent manner. Furthermore, it was revealed VACP1 potently induced OS cell apoptosis, accompanied by caspase-3 activation and a decrease in Bcl-2. Moreover, PARP plays a pivotal role in DNA damage repair, and has been regarded as a potential target for anticancer therapy. These results show that VACP1 treatment led to PARP cleavage, which is a marker of cell apoptosis. The above findings indicate that VACP1 might suppress OS cell proliferation, at least partially, through the induction of apoptosis.

The p38 MAPK pathway participates in the modulation of inflammatory mediator production, as well as the modulation of cell proliferation, differentiation, survival, migration and invasion in many kinds of cancers. JNK signaling has dual physiological and pathological functions. It promotes cell survival and proliferation, and also promotes cell apoptosis. The functions of JNKs and p38 MAPK in the modulation of cell proliferation and apoptosis are presently being explored for the development of targeted therapies. Several mouse models have shown that p38 MAPK signaling suppresses the formation of breast, lung and liver tumors in vivo. Furthermore, mice deficient in Gadd45a, a JNK and p38 MAPK pathways activator, exhibited the promotion of Ras-induced breast tumorigenesis and decreased apoptosis. The present results revealed that VACP1 could activate the JNK and p38 MAPK signaling pathway in OS cells, suggesting that VACP1 may induce the apoptosis of OS cells, at least partially, through activating the p38 MAPK and JNK signaling pathways.

In summary, a novel peptide VACP1 derived from wasp venom was isolated and characterized. VACP1 potently inhibited cell proliferation and promoted the cell apoptosis of OS cells, and this was concomitant with the activation of the JNK and p38 MAPK signaling pathway. The present study demonstrates that VACP1 is a potential candidate for OS therapy.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

REFERENCES


