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ScienceDirect

Journal of the Chinese Medical Association 78 (2015) 574–583

www.jcma-online.com

Original Article

First report on the isolation of melittin from Iranian honey bee venom and evaluation of its toxicity on gastric cancer AGS cells

Amir Mahmoodzadeh [a], Hannaneh Zarrinnahad [b], Kamran Pooshang Bagheri [b], Ali Moradia [c], Delavar Shahbazzadeh [b,*]

[a] *Biochemistry Department, Shiraz Medical School, Shiraz University of Medical Sciences, Shiraz, Iran*
[b] *Biotechnology Research Center, Biotechnology Department, Venom and Toxin Laboratory, Pasteur Institute of Iran, Tehran, Iran*
[c] *Department of Biochemistry, Shahid Sadoughi University of Medical Sciences, Yazd, Iran*

Received July 12, 2014; accepted April 6, 2015

Abstract

Background: It has been previously reported that melittin, the main ingredient of honey bee venom, has anticancer properties. However, there appears to be no earlier study focusing on the isolation of melittin from Iranian honey bee venom (*Apis mellifera meda*), and evaluation of its effect on cancerous cells.
Methods: We isolated melittin using reversed-phase high performance liquid chromatography, and its potential toxicity on gastric cancer AGS cells was determined with an MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay. Furthermore, to ascertain whether melittin induces apoptosis or necrosis in these cells, morphological evaluation, DNA fragmentation assay, propidium podide and annexin-V-FITC dual staining, and flow cytometric analysis were also conducted.
Results: The results of our study suggested that melittin inhibited the proliferation of AGS cells in a dose and time-dependent trend. All of the above four distinct assays indicated that melittin induces necrosis in AGS cells at concentrations of $\geq$ 1 μg/mL.
Conclusion: The present study indicated that melittin has an anticancer effect on gastric cancer AGS cells and stimulates necrotic cell death in these cells.

Keywords: Apis mellifera meda; apoptosis; gastric cancer; melittin; necrosis

1. Introduction

Gastric cancer (GC) is one of the most common cancers in the world, in that ~1 million new cases of GC and > 700,000 deaths (12% of all cancer related deaths) are reported each year.[1] Unfortunately, current strategies in GC therapy are most of the time essentially ineffective.[2] Regarding this matter, there is an urgent need for the global medical community to conceive novel strategies to be used in the fight against this lethal disease. Various natural substances have been recognized as having anticancer properties.[3,4] One of these natural products is bee venom (BV) and its principal ingredient, melittin.[5]

Melittin ($C_{131}H_{229}N_{39}O_{31}$) is a cationic 26 amino acid residue long peptide and is reported to have strong hemolytic activity.[6] The first 20 residues (N-terminal) of the structure of melittin are predominantly hydrophobic amino acids, whereas the carboxyl-terminal of the peptide is mostly composed of hydrophilic residues.[7] This amphipathic entity allows the peptide to interact with phospholipid membranes.[8]

The effect of melittin on various cancer cell lines has been investigated broadly and its inhibitory effect on the proliferation of these cells has been demonstrated. In addition, it has been

Conflicts of interest: The authors declare that there are no conflicts of interest related to the subject matter or materials discussed in this article.

* Corresponding author. Dr. Delavar Shahbazzadeh, Biotechnology Research Center, Biotechnology Department, Venom and Toxin Laboratory, Pasteur Institute of Iran, P.O. Box: 1316943551, Tehran, Iran.
E-mail address: shahbazzadeh@pasteur.ac.ir (D. Shahbazzadeh).

http://dx.doi.org/10.1016/j.jcma.2015.06.008

evaluated whether melittin exhibits apoptotic or necrotic cell death in cancerous cells. Induction of apoptosis by melittin or BV has been reported in human lung cancer,[9] rheumatoid arthritis,[10] hepatocellular carcinoma,[11] human leukemic cells,[12] human breast cancer,[13] prostate,[14] and ovarian cancer cells.[15] It seems that melittin-induced apoptosis is mediated through the activation of the caspase-dependent pathway.[4] However, other studies have shown that melittin exhibits necrotic cytotoxicity in rat thymocytes,[16] murine skeletal muscles,[17] erythrocytes[18], lymphocytes,[19] lymphoblastoid cells,[20] and Caco-2 cells.[21] Identification of the precise mechanism of the effect of melittin on cancerous cells plays an important role in the future therapeutic application of this interesting peptide.

Apis mellifera meda (A. m. meda) is smaller than the European honey bee, and produces less honey and is more aggressive.[22] European honey BV has been studied extensively.[23,24] However, there are no published reports regarding the antiproliferative activity of *A. m. meda* venom components, especially melittin, on human gastric adenocarcinoma cell line (AGS) cells. The present study aimed to isolate melittin from Iranian honey BV and to evaluate its inhibitory effect on the proliferation of the gastric cancer AGS cell line.

2. Methods

2.1. Chemicals

We purchased 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT), dimethyl sulfoxide, triflouroacetic acid (TFA), acetonitrile, and melittin from Sigma–Aldrich (St. Louis, MO, USA). Annexin V-FITC Apoptosis Detection Kit (K-101-100) was obtained from Bio Vision (Mountain View, CA, USA). The materials that were used in the cell culture included RPMI-1640, fetal bovine serum (FBS), and antibiotics, and were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Bees

A hive of Iranian honey bees were selected and checked for disease and race. This hive was located in a bee keeping farm in the Koohrang region, Chaharmahal and Bakhtiari, a South-West province of Iran.

2.3. Bee venom collection

Honey BV was collected according to the electrical stimulation method[25] with a slight modification. Briefly, the wired glass plate of the venom collector was placed inside (top) of the hive. When bees covered the surface of the wired glass plate, the wires were electrified and a mild shock was applied to the bees for 10 seconds and then the current stopped for 20 seconds. In this method, the bees sting the surface of the glass plate in response to the electrical stimulation. Secreted venom from the bees' sting dried rapidly exposed to the air. Dried venom was scraped off with a sharp scalpel and transferred to the laboratory.

2.4. Venom profiling and isolation of melittin

Collected venom was dissolved in 1 mL ultra-pure water and then centrifuged at 16,000*g* for 10 minutes at room temperature. Soluble supernatant was filtered through a 0.2 μm membrane filter and stored at −20°C. In order to facilitate venom profiling and melittin isolation a high performance liquid chromatography (HPLC) system (Dr. Ing. Herbert Knauer GmbH, Berlin, Germany) was used. The HPLC system consisted of the following instruments: pump K-1000, UV detector 2550, manual injector with 20 μL loop, a computer system with ChromGate software, and a C18 column (Knauer, Eurosfer-100 C18, 250 mm × 4.6 mm). TFA/Water (1:1000) and TFA/ACN (1:1000) were used as solvent A and B, respectively. Flow rate was established as 1 mL/min in all of the separation processes. A linear gradient of 0–60% solvent B was applied for 55 minutes and the column elute monitored at 214 nm wavelength. The retention time of the isolated melittin was compared with an external standard (Sigma-Aldrich). The purity of the isolated melittin was evaluated with the same HPLC method. The melittin fraction was manually collected and lyophilized in a freeze dryer (Christ 2 alpha, Osterode am Harz, Germany).

2.5. Hemolytic activity

A hemolytic activity assay was performed according to the previously described method with slight modification.[26] Briefly, 2% red blood cells (RBC) suspension were transferred to a 96-well plate (100 μL per well). The RBCs were treated with melittin at final concentrations of 8 μg/well, 4 μg/well, 2 μg/well, 1 μg/well, 0.5 μg/well, 0.25 μg/well, 0.125 μg/well, and 0.0625 μg/well. The amount of 100 μL of supernatant from each well were transferred gently to a new 96-well plate and the optical density of released hemoglobin was measured at 540 nm with a microplate spectrophotometer (Epoch, BioTek, Luzern, Switzerland). The data were compared of a positive control (100 μL RBC and 100 μL Triton X-100 1%) and a negative control (100 μL RBC and 100 μL phosphate buffered saline), and all experiments were performed in triplicate. The percent of hemolysis was calculated as follows:

$$\text{Hemolysis percent} = [\text{OD}(\text{optical density})_{540(\text{Sample})} - \text{OD}_{540(\text{Neg.control})}]/(\text{OD}_{540(\text{Pos.control})} - \text{OD}_{540(\text{Neg·control})}) \times 100$$

2.6. Cell culture

AGS cell line was purchased from the Pasteur Institute of Iran Cell Bank (Tehran, Iran). AGS cells were cultured in RPMI-1640 medium supplemented with 10% FBS, penicillin 100 unit/mL, and streptomycin 100 μg/mL. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.