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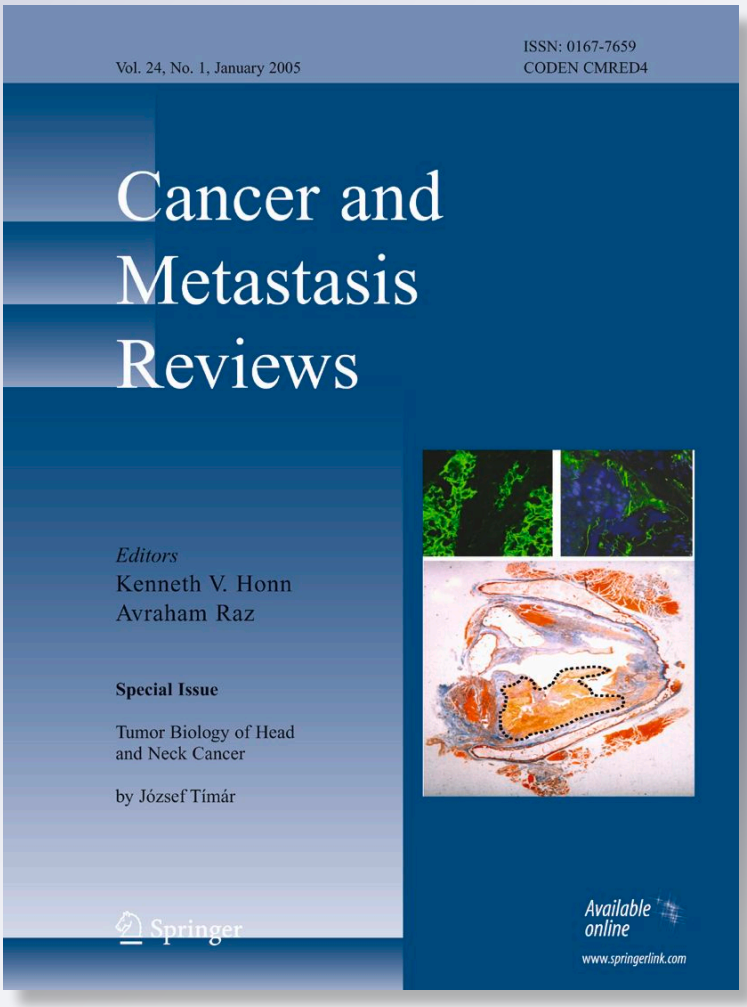

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NON-THEMATIC REVIEW

Bee venom in cancer therapy

Nada Oršolić

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Abstract Bee venom (BV) (api-toxin) has been widely used in the treatment of some immune-related diseases, as well as in recent times in treatment of tumors. Several cancer cells, including renal, lung, liver, prostate, bladder, and mammary cancer cells as well as leukemia cells, can be targets of bee venom peptides such as melittin and phospholipase A2. The cell cytotoxic effects through the activation of PLA2 by melittin have been suggested to be the critical mechanism for the anti-cancer activity of BV. The induction of apoptotic cell death through several cancer cell death mechanisms, including the activation of caspase and matrix metalloproteinases, is important for the melittin-induced anti-cancer effects. The conjugation of cell lytic peptide (melittin) with hormone receptors and gene therapy carrying melittin can be useful as a novel targeted therapy for some types of cancer, such as prostate and breast cancer. This review summarizes the current knowledge regarding potential of bee venom and its compounds such as melittin to induce cytotoxic, antitumor, immunomodulatory, and apoptotic effects in different tumor cells *in vivo* or *in vitro*. The recent applications of melittin in various cancers and a molecular explanation for the antiproliferative properties of bee venom are discussed.

Keywords Bee venom · Tumors · Cancer cells · Melittin · Phospholipase A2

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1 Introduction

Bee venom is a unique weapon in the animal kingdom. Bee venom apparatus has a prime role of defense to the bee colony. It is an efficient and complex mixture of substances designed to protect bees against a broad diversity of predators from other arthropods to vertebrates. Bee venom from the venom gland located in the abdominal cavity contains several biologically active peptides, including melittin (a major component of BV), apamin, adolapin, mast cell degranulating peptide, and enzymes (phospholipase A2, and hyaluronidase) as well as non-peptide components, such as histamine, dopamine, and norepinephrine [1, 2]. The composition of dry and fresh BV is given in the Table 1. The composition of fresh and dried BV differs mainly in regards to the volatile components; the overall biological activity is similar (Table 1). Bee venom as a non-steroidal anti-inflammatory drug has been used traditionally for the relief of pain and the treatment of chronic inflammatory diseases, such as rheumatoid arthritis and multiple sclerosis, in oriental traditional medicine [3, 4] as well as in recent times in treatment of tumors [5–9]. In the past few years, a number of studies regarding the beneficial role of bee venom were published stating that bee venom possesses radioprotective [10], antimutagenic [11], anti-inflammatory [12], antinociceptive [13, 14], and anticancer activities [5–9]. In addition, recent studies reported several effects of bee venom such as induction of apoptosis and necrosis and effects on proliferation, cytotoxicity, and growth inhibition of different types of cancer cells [5, 9, 15–18].

BV inhibits carcinoma cells proliferation and tumor growth *in vivo*. This inhibition of tumor growth was shown to involve stimulation of the local cellular immune responses in lymph nodes [5, 7]. Apoptosis, necrosis, and lysis of the tumor cells were found to be possible mechanisms by which bee venom inhibited tumor growth. Interestingly, it has been

Table 1 The major components of honeybee venom

Class of molecule	Component	Percent in dry venom	Concentration (nM) in a sting	Molecular weight	Biological effect
Proteins (enzymes)		15–17			
	Phospholipase A2; enzyme hydrolysing phospholipids	10–12	0.23	20,000	Cytotoxic effects against cancer cells; inflammatory effects; anti-tumor effects; destroys phospholipids and dissolves the cell membrane of blood bodies; lowers the blood coagulation and blood pressure; the strongest allergen and thus the most harmful BV component
	Phospholipase B; (cleavage of the toxic lysolecetin)	1			Detoxicating activity
	Acid phosphomonoesterase	1		55,000	
	Hyaluronidase catalyzes hydrolysis of hyoloronic acid, the tissue cement	1.5–2	0.03	41,000	Selectively attacks tissue hyaluronic acid polymers; increase the capillary permeability; immune response and tissue-spread properties; antigenic; catalyzes the hydrolysis of proteins, thus enabling the penetrating of BV into the tissue; dilates blood vessels and increases their permeability, causing an increase of blood circulation; allergenic
	Phosphatase	1			
	α-Glucosidase	0.6		170,000	
	Lysophospholipase	1	0.03		
Small proteins and peptides		48–58			
	Melittin; biologically active peptide	40–50	10–12	12,000(tetramer)	26 Amino acid; main biologically active component; enhance of PLA2 activity; cytotoxic effects against cancer cells; anti-inflammatory and anti-arthritic effects; membrane-active, diminishes surface tension of membranes; anti-inflammatory in very small doses; stimulates smooth muscles; increases capillary permeability increasing blood circulation and lowering the blood pressure, lowers blood coagulation, immunostimulatory and immunosuppressive; radiation protective influences the central nervous system; anticancer, antibacterial, antifungal, antiviral; higher doses are inflammatory and hemolytic
	Apamine; biologically active peptide	2–3	0.75	2,000	10 Amino acid; inhibition of Ca^{2+}-activated K^{+} channel; cytotoxic effect against cancer; nociceptive effect; anti-inflammatory properties; anti-inflammatory stimulating the release of cortisone, antiserotonine action; increases the defense capability; immuno-supressor, stimulates the central nervous system in very small doses; higher doses are neurotoxic

Table 1 (continued)

Class of molecule	Component	Percent in dry venom	Concentration (nM) in a sting	Molecular weight	Biological effect
	MCD peptide; mast cell degranulating peptide 401	2-3	0.6	2,500	22 Amino acid; anti-inflammatory and analgenic effect; histamine release (low dose); histamine release inhibition (high dose); anti-allergic effect; lyses mast cells, releasing histamine, serotonine, and heparine; melittine-like effect increasing capillary permeability increasing; anti-inflammatory; simulates the central nervous system
	Adolapine; biologically active peptide	0.5–1	0.06	11,500	Inhibition of PLA2 and COX activity; anti-inflammatory activity; inhibits the specific brain enzymes cyclooxigenase and lipooxigenase; decreases inflammations by, anti-rheumatic, decreases pain; inhibits the aggregation of erythrocytes; relatively low toxicity
	Protease inhibitor; biologically active peptides	0.1-0.8	0.07	9000	Inhibits the activity of different proteases like trypsin., chymotprypsin, plasmin, thrombin, thus decreasing inflammation, anti-rheumatic; low toxicity
	Tertiapine	0.1	0.03	2,500	Peptides, with an uncertain role in the physiological action of BV; antiradiation effects; cardiopep has antiarhythmic effects
	Cardiopep	<0.7		2,500	
	Procamine A, B	1–2	2	600	
	Small peptides (less than 5 a.a.)	13–15		≤600	
	Secapine	0.5–2	0.13	3,000	
	Pamine	1–3			
	Minimine	2–3		6000	
Phospholipids		1–3		700	
Physiologically active amines		3			Dilates blood vessels, increasing the permeability of blood capillaries and increases blood circulation; stimulates smooth muscles; allergenic
	Histamine; dopamine; noradrenalin; neurotransmitters	0.5–2; 0.13–1; 0.1–0.7	5–10; 2.7–5.5; 0.9–4.5	307.14; 189.64; 169.18	
Amino acids		1			
	γ-aminobutyric acid, α-amino acids	1; 0.5		150; 700	
Sugars	Glucose, fructose	2–4		180	
Volatiles (pheromones)	Complex ethers	4–8		200	Complex ethers, causing alarm of the bee colony and its defensive behavior
	Iso-pentyl acetate; n-butyl acetate; iso-pentanol; n-hexyl acetate; n-octyl acetate; 2-nonanol; *n*-decyl acetate; benzyl acetate; benzyl alcohol; (2)-11-eicosen-1-ol				
Minerals	P, Ca, Mg	3–4			

demonstrated that BV induces apoptosis in human leukemic cells; however, normal murine bone marrow cells have no effect on cytotoxicity [9]. Recently, Moon et al. [9] reported that key regulators in BV-induced apoptosis are Bcl-2 and caspase-3 in human leukemic cells through downregulation of mitogen-activated signal pathways. It has also reported that BV induces apoptosis through caspase-3 activation in synovial fibroblasts [19] and inhibition of cyclooxygenase (COX)-2 expressions in human lung cancer cells [15]. Although some studies have clearly demonstrated that BV and its components such as melittin possess antiproliferative and pro-apoptotic effects, the precise mechanisms responsible for these effects are largely unknown in tumor cells.

1.1 Antitumor activity of bee venom and its components

Recently, many studies reported that some natural products inhibit tumor cell growth and metastasis and induce apoptosis suggesting a growing application of these natural compounds as an alternative medicine treatment of human tumors [20]. During the past two decades, peptides in bee venom containing melittin, as the BV major protein component, has attracted considerable attention for their potential use in cancer therapy [5–7, 21, 22]. Melittin, an amphiphilic peptide (26 amino acid residues) isolated from honeybee *Apis mellifera* [23], is known to exert a variety of membrane-perturbing effects such as hemolytic and antimicrobial activity [24]. Melittin also induces structural alterations of membranes including pore formation, fusion, and vesiculation [25–27]. These morphological changes of membranes brought about by melittin could be attributed to induction of hormone secretion [28], aggregation of membrane proteins [29], and change of membrane potential [30]. Furthermore, melittin stimulates various enzymes including G-protein [31], protein kinase C [32], adenylate cyclase [33], phospholipase C (PLC)2 [34, 35], and phospholipase D (PLD) [36–38]. Melittin has a role in the signal transduction; it directly stimulates nucleotide exchange by heterotrimeric GTP-binding proteins. In addition, melittin has also been shown to inhibit Gs activity by reducing the affinity of both GTP (or GTP-c-S) and GDP to Gs [39]. It has therefore been proposed that Gi stimulation and Gs inhibition are involved in melittin-induced inhibition of adenylyl cyclase. Interestingly, melittin represents the first metabostatic peptide that inhibits the intrinsic activity of G protein (Gs) activity [39].

These diverse effects implicate that melittin exerts multiple effects on cellular functions (Figs. 1 and 2). However, the mechanisms of the anti-cancer effects of melittin have not been fully elucidated.

In spite of diverse effects of melittin, this peptide has been widely accepted as an activator for phospholipase A2 (PLA2) in various intact cells. Bee venom secretory phospholipase A2, a member of the phospholipases A2 (PLA2) family of enzymes that catalyze the hydrolysis of the sn-2 fatty acyl ester bond of membrane glycero-3-phospholipids to generate free fatty acids and lysophospholipids [40–42]. In principle, sPLA2 enzymes can influence immunogenicity and proliferative capacities of tumor cells by various mechanisms. sPLA2-activity catalytically hydrolyzes and digests cell membrane components [43] and consequently disrupts the integrity of the lipid bilayers, thus making cells susceptible to further degradation. Direct protein interaction of PLA2 enzymes with cell surface receptors regulate a variety of biological activities including proliferation [44–46]. Immunostimulatory or cytotoxic action of sPLA2 reaction products like lysophosphatidylcholine and lipid mediators have been observed frequently [47–49]. Synthetic analoga of lysolipids, which structurally resemble reaction products of sPLA2 mediate anti-proliferative effects and cytotoxicity via blockades of both ERKs and PKB/Akt and thus has been suggested for cancer therapies [50–52].

Melittin, present in bee venom about 50–70%, is an antimicrobial peptid of bee venom. Some antimicrobial peptides display a wide range of biological activities and at

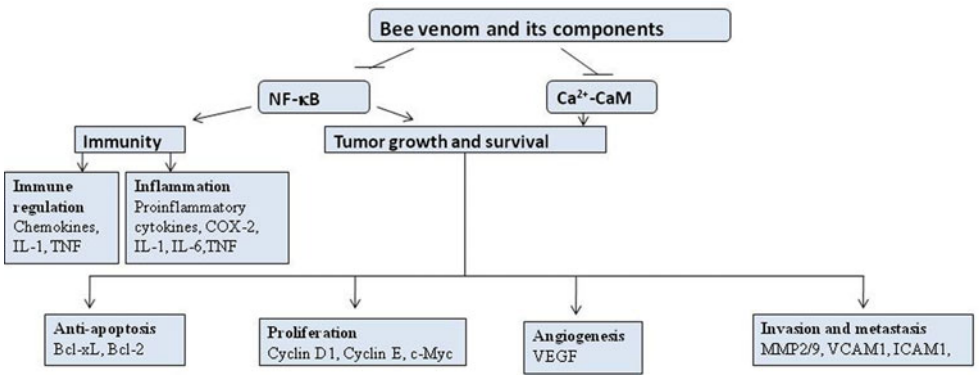

Fig. 1 Effect of bee venom components on calmodulin and NF-κB and their interection on immunity and tumor growth and survival. Abbreviations: *NF-kB* nuclear factor kappa B, *COX* cyclooxygenase, Ca^{2+}/*CaM* calcium-calmodulin complex, *VCAM1* vascular cell adhesion molecule, *ICAM1* inter-cellular adhesion molecule 1, *HIF-1* hypoxia-inducible factor 1, *MMP* metalloproteinase

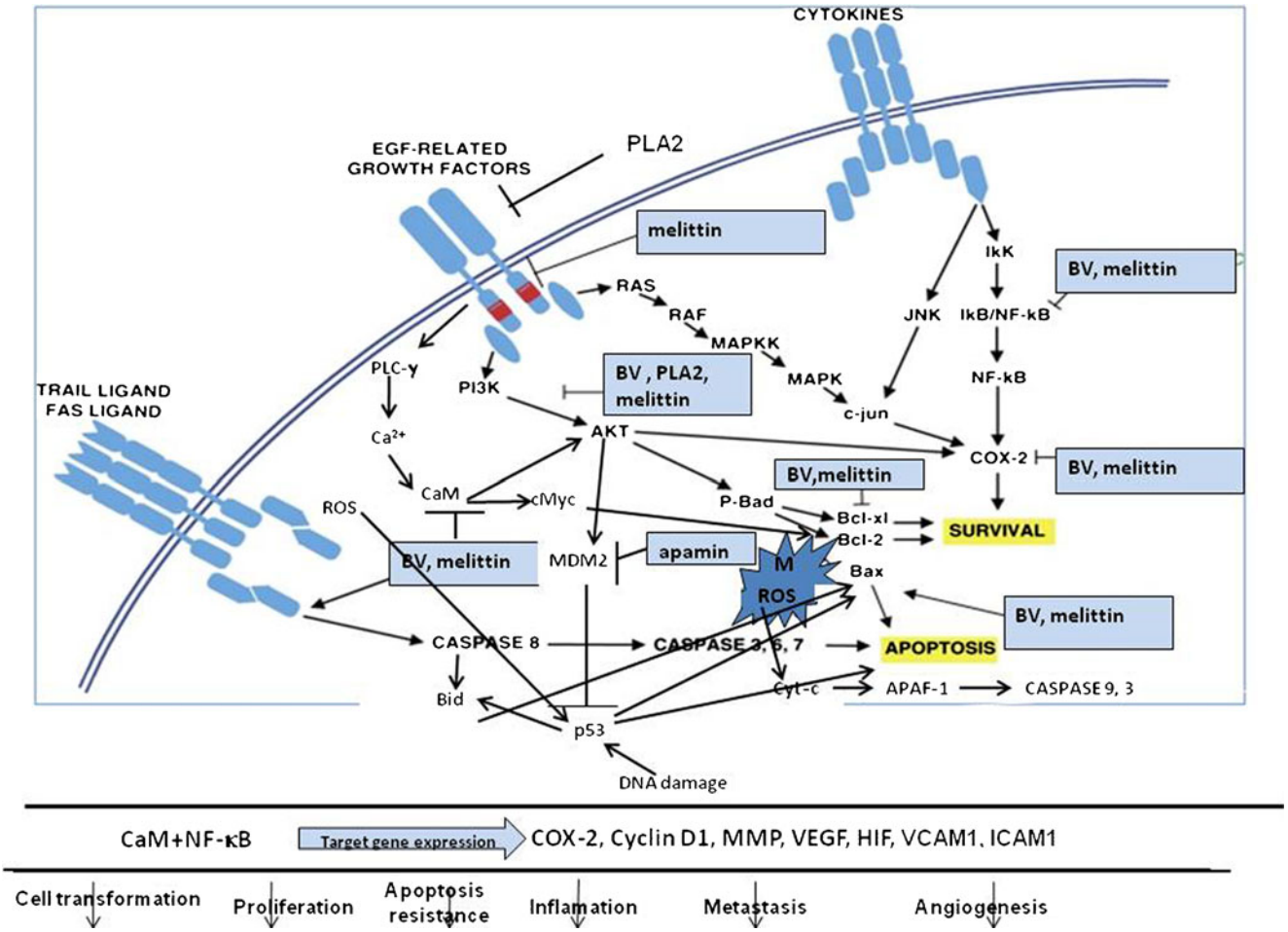

Fig. 2 The possible molecular target of bee venom and its components for inhibition of proliferation, metastasis, angiogenesis, and induction of apoptosis. The cell signaling pathways activated by bee venom components are numerous and different for different bee venom components. Multiple growth factor receptors (epidermal growth factor receptor, TNF receptor…..) are activated at the cell surface in tumorigenesis. Activation of these receptors activates several downstream signaling pathways. Among these pathways, the Ras-MAPK (such as ERK and JNK) pathways, the PI3K-AKT pathways, PLC-γ-CaM, and the NF-κB are important and are the targets of bee venom components. Some bee venom components inhibit the receptor at the surface either by dephosphoryating them or by inducing their degradation, which ultimately modulate the downstream signaling pathways important for proliferation, metastasis, angiogenesis, and apoptosis (for example, the synergistic effect of BV sPLA2 (bv-s PLA2) and phosphatidylinositol-(3,4)-bisphosphate (PtdIns(3,4)P2); the cytotoxic activity mediated by PtdIns(3,4)P2 and bv-sPLA2 is due to cell death resulting from a disruption of the membrane integrity, the abrogation of signal transduction and the generation of cytotoxic lyso-PtdIns(3,4)P2). Inhibition of AKT and ERK signaling by bee venom components is quite common, although in many cases, this inhibition is the resultat of growth factor inhibition. Inhibition of NF-κB signaling pathway by interfering with multiple targets of signaling is another common target of bee venom components. Some components of bee venom generate reactive oxigen species (ROS), which activate p53 family members and induce cell cycle arest and apoptosis. A probable model of calcium/calmodulin-induced AKT activation and cell survival is presented. Ca^{2+}-bound activated calmodulin (CaM) associates with AKT and transport AKT to the plasma membrane, where AKT binds to PI-3 kinase product such as phosphatidyl inositol-3,4,5-triphosphate (PIP3), and is subsequenntly activated byphosphorilation. Inhibition of PI-3 kinase, chelation of intracellular calcium, and inactivation of calmodulin, all result in AKT inhibition and apoptosis. In addition, Ca^{2+}/CaM plays a crucial role in angiogenesis. Ca^{2+}/CaM activates HIF-1 and consequently induces the expression of pro-angiogenic factors such as VEGF. Hypoxia-induced increase in intracellular Ca^{2+} concentration promotes both expression and transcriptional activity of HIF-1α by modulating the activity of Ca^{2+}/CaM-depedent enzymes, including CaM kinase and calcineurin. Furthemore, inhibition of Ca^{2+}/CaM by a CaM-dominant negative mutant, Ca^{2+}/CaM antagonist, or Ca^{2+} chelator down-regulates HIF-1 transcriptional activity and thus suppress angiogenesis. Therefore, Ca^{2+}/CaM might be a potent therapeutic targets for the treatment of angiogenesis-related diseases, including cancer. Stimulatory and inhibitory effects are indicated by *arrows* and *bars*, respectively. Abbreviations: *MAPK* mitogen-activated protein kinase, *MAPKK* mitogen-activated protein kinase kinase, *JNK* jun kinase, *IkB* inhibitor kinase B, *NF-kB* nuclear factor kappa B, *COX* cyclooxygenase, *PI3K* phosphatidylinositol 3 kinase, *Ca^{2+}/CaM* calcium–calmodulin complex, *VCAM1* vascular cell adhesion molecule, *ICAM1* inter-cellular adhesion molecule 1, *HIF-1* hypoxia-inducible factor 1, *MMP* metalloproteinase

least some of antimicrobial peptides isolated from insects, including melittin, cecropin-related peptides, and the magainins have exhibited antitumor activity on cells derived from mammalian tumors [6, 7, 53, 54]. Melittin can induce cell

cycle arrest, growth inhibition, and apoptosis in various tumor cells [55–59]. In addition, melittin has been shown to revert the transformed phenotype of H-ras transformed cells [60]. It has been demonstrated that melittin specifically selects against cells in culture that express high levels of the ras oncogene [61]. Melittin therefore exerts its anti-transformation effect(s) by specifically eliminating cells that express the oncoprotein. Further, acquisition of resistance to melittin is accompanied by a decrease in the number of copies of the ras genes, decrease in expression of the ras oncoprotein, and a concomitant reversion of transformed cells to a normal morphology in a strict dose-dependent manner [61]. Interestingly, it has been shown that the biochemical basis for melittin-mediated counterselection of ras-transformed cells is due to the ability of melittin to hyperactivate cellular phospholipase A2 in ras-transformed cells by the mediation of enhanced influx of calcium ions [60].

It was demonstrated that melittin is one of the most potent inhibitors of calmodulin activity and a potent inhibitor of cell growth and clonogenicity [7, 62]. There is also some evidence suggesting that calmodulin inhibitors are cytotoxic to malignant cells both *in vitro* [7] and *in vivo* [2, 6, 7, 54]. Drugs that inhibit the activity of calmodulin have been shown to inhibit DNA synthesis in a glioblastoma cell line to block the movement of chromosomes during metaphase to inhibit the growth of Chinese hamster ovary cells and to enhance the cytotoxicity of vincristine, doxorubicin, and bleomycin [62–64]. Lee and Hait [65] examined the inhibitory effect of melittin on the growth of C6 astrocytoma cells. They reported a good correlation between the activity of drugs as calmodulin inhibitors and their activity as inhibitors of cell growth. Lazo et al. [66] reported a similar mechanism for the cytotoxic effect of melittin in leukemic L1210 cells. Killion and Dunn [67] showed that 1210 leukemia cells are 2–4 times more sensitive to the cytolytic effects of melittin than normal DBA/2 mouse spleen and bone-marrow cells. Chueng [68] showed that melittin was capable of binding to calmodulin, which played a key role in cellular proliferation. Gest and Salomon [69] found that melittin inhibited the melanotropin receptor in M2R melanoma cell membranes. Studies demonstrated that melittin could induce an apoptotic cell death [19, 70, 71] and possess anti-tumor effects [53]. However, other results suggested that the pore-forming agents killed malignant cells by necrosis [55, 72]. The direct cytolytic/necrotic action of melittin has been reported in different cells [1, 55, 72, 73] and in a human lymphoblastoid cell line [74].

1.2 Synergistic antitumor effect of bee venom melittin and phospholipase A2

Melittin's action was thought to involve membrane pore formation [75–77] or membrane perturbation, resulting in the disruption of the membrane [78, 79] or both [80, 81]. Cell death was correlated with phospholipase A2 (PLA2) activation [82], since melittin was reported to be an activator of PLA2 both endogenously in intact cells, as well as in *in vitro* assays [83]. Saini et al. [38] demonstrated that melittin caused cell lysis in human monocytic leukemia cells (U937) at 10±15 min. Cellular hypertrophy (5 min) and aggregation (1 min) preceded cytolysis. Hypertrophy was reported to be due to a change in membrane permeability to ions [75]. Melittin rather than BV has a cytotoxic effect against several cancer cell lines, and its activation effect of PLA2 might be a target of melittin, suggesting that PLD plays a role in melittin-mediated membrane disruption/cytolysis through an uncharacterized signal transduction mechanism. It could be possible that membrane disruption or cytolysis of U937 cells by melittin is mediated by a PLD±calpain±fodrin±actin pathway. Calpain is a cytoplasmic, neutral protease thought to be involved in apoptosis [84, 85] that leads to the cleavage of fodrin, a cytoskeleton protein [86]. Fodrin cleavage results in a reduced ability of cells to crosslink actin filaments [87–89].

The activation of PLA2 might have a cytotoxic effect on cancer cells through several subsequent cellular changes. Arora et al. [90] also reported that melittin, a PLA2 activator, increased the calpain activity and cell necrosis in the hepatocellular carcinoma cell lines (N1S1 and McA-RH7777 cells). Melittin-induced cell necrosis was ameliorated by a calpain protease inhibitor, which suggests that PL-mediated calpain activation might be a therapeutic strategy for inhibiting cancer cell growth by melittin. The TNF-α-induced activation of cytosolic PLA2 is an important component of the signaling pathway leading to cell death. Wu et al. [91] suggested that melittin can be effective against leukemic cells, KG1a, CEM, and CEM/VLB100, which are relatively resistant to TNF-α. This is because melittin can activate low levels of cPLA2 activity in the KG1a cell line.

Effect of melittin on membrane permeability of L1210 cells was investigated by measuring the influx of EtBr, a fluorescent dye; the EtBr fluorescence rapidly increased when 5 mM melittin was added to a L1210 cell suspension. The observation of melittin-induced EtBr influx and LDH release confirms that melittin perturbs the L1210 cellular membranes. The membrane-perturbing effect of melittin has been observed in various mammalian cells as hemolysis of human erythrocytes [25], EtBr influx in HL60 cells [92], and LDH release in primary cortical neuronal cultures [93]. According to Lee [94], melittin exerts multiple effects on the composition of cellular lipids, especially on the release of free fatty acids from L1210 cells. Authors also concluded the lack of selective activation of phospholipase A2 by melittin.

Mufson et al. [95] reported that melittin could enter the phospholipid bilayers and exhibit surfactant activity. The association between melittin and the cellular membranes results in (1) a disturbance of the acyl groups of phospholipids, (2) increased phospholipid susceptibility to hydrolysis by PL, and (3) increased synthesis of PG from the arachidonic acid released from the phospholipids.

The synergistic effect of BV sPLA2 (bv-s PLA2) and phosphatidylinositol-(3,4)-bisphosphate (PtdIns(3,4)P2) in inducing cell death has attracted considerable interest. Putz et al. [96] demonstrated that the cooperation of bv-sPLA2 PtdIns(3,4)P2 was more effective than any of single component in the blocking of tumor cell growth. The growth inhibition induced by the combined action of bv-sPLA2 with either PtdIns(3,4)bisphosphate or PtdIns(3,4,5) trisphosphate was synergistic and accompanied by potent cell lysis. They suggested that the cytotoxic activity mediated by PtdIns(3,4)P2 and bv-sPLA2 is due to cell death resulting from a disruption of the membrane integrity, the abrogation of signal transduction, and the generation of cytotoxic lyso-PtdIns(3,4)P2.

1.3 Antitumor and antimetastatic effect of bee venom components and its chemotherapeutical efficacy through the delivery system

Tumor metastasis is a complex process involving extensive interactions between tumor cell and host tissues. Tumor metastasis is the major cause of death in cancer patients. It can be roughly divided into the following steps: tumor cell dissociation, invasion, intravasation, distribution to distant organs, arrest in small vessels, adhesion to endothelial cells, extravasation, invasion of the target organ, and proliferation [97]. It has been shown that cell invasiveness and metastasis are regulated by multiple cues, including extracellular molecules such as cell adhesion molecules, proteases, angiogenesis factors, cytokines, and growth factors, and the underlying signaling transduction components such as Rac1. Rac1, a member of the Ras superfamily of small GTP (guanosine triphosphate)-binding protein is known to play important roles in the regulation of distinct microfilament-based structures, which is required for cell adhesion, migration, and invasion. It has been demonstrated that Rac1 can promote tumor cell migration and invasion for multiple types of cancer such as renal, breast, and liver carcinomas. In addition, it has been shown that tumor cells with high metastatic potential are more motile than non-metastatic cells [98] Recent paper Liu et al., [59] demonstrated that melittin inhibits tumor cell metastasis by reducing cell motility and migration via the suppression of Rac1-dependent pathway in nude mouse models, suggesting that melittin is a potential therapeutic agent for hepatocellular carcinoma (HCC). Melittin also inhibits the viability and motility of HCC cells, which correlates with its suppression of Rac1-dependent activity, cell motility, and microfilament depolymerization. It seems that the inhibition of MMP-9 expression and activity by bee venom through the suppression of p38/JNK and NF-κB expression is essential, signaling pathways (Fig. 1) for the bee venom and melittin-induced anti-metastatic and anti-invasive effects [99, 100]. Cho et al. [99] demonstrated that bee venom directly inhibits the invasive and migratory ability of MCF-7 cells via the suppression of MMP-9 expression without abolishing the expression of TIMP-1 and -2. In addition, the enzyme activity of MMP-9 was suppressed by bee venom and melittin but not by apamin and PLA_2. These results suggest that the specific inhibition of MMP-9 was regulated by a single component of the bee venom, which is melittin. These results indicate that bee venom is a potential anti-metastatic and anti-invasive agent, and this useful effect may expand future clinical research on the anti-cancer properties of bee venom.

Adhesion of circulating tumor cells to capillary endothelial cells is a crucial event in the retention of tumor cells in a specific organ [101]. Initial interactions between tumor cells and endothelium activates both tumor cells and endothelial cells through cytokines, free radicals, bioactive lipids, and growth factors, leading to the increased expression of adhesion molecules, which strengthens the initial adhesive bonds [102, 103]. In most cases, the lung is the first organ that tumor cells detached from primary tumors encounter, making it a major site for tumor metastasis. Oršolić et al. [7, 104] have shown that an experimental pulmonary metastasis of mammary carcinoma cells in mice can be effectively inhibited by BV at dose of 75 or 150 μg kg^{-1}. In addition, this study [7, 104] demonstrated that antitumor and antimetastatic effects of bee venom could be highly dependent on the route of injection and on close contact of bee venom and tumor cells. Bee venom significantly inhibited metastasis formation ($p<0.001$) only when administered by the same route as tumor cells at the time of tumor cell inoculation. In addition, we were particularly interested in antitumor activities of bee venom given intratumorally at different times after tumor cell inoculation and in clarification of the mechanisms by which bee venom produced these effects. When bee venom was injected intratumorally, tumors decreased in size; some sort of shrinkage of tumors occurred, and the delay of tumor growth was evident. Survival of bee venom treated mice was prolonged, as compared to control mice. Bee venom significantly inhibited tumor growth; tumor inhibition effect of bee venom was dose- and time dependent [54]. We have shown that inhibited tumor growth by bee venom was the consequence of apoptosis and/or necrosis of tumor cells or it was the result of activation of immune system by bee

venom [7, 105]. Anticancer effect of bee venom was confirmed by other authors [17, 59], demonstrating that in *in vivo* experiments, treatment with polypeptides in bee venom PBV (1.5 or 3 mg kg^{-1}) resulted in a significant retardation of SMMC-7721 cell growth in Balb/c nude mice ($p<0.05$), with the relative tumor inhibition of 31.4% and 48.2%, respectively. Huh et al. [106] demonstrated that BV played a remarkable role in inhibiting angiogenesis and metastasis via down regulation of VEGF and VEGFR-2 in lung cancer treatment. A similar effect of bee venom on tumor growth and metastasis was obtained in the experiment by Huh et al. [106] in Lewis lung carcinoma (LLC) models. Mice bearing subcutaneous LLC tumors treated with 1 or 10 μg mL^{-1} of BV showed reductions ranging between 49% and 62% in primary tumor volume and reduction of spontaneous pulmonary metastasis occurrences. Furthermore, BV delayed metastatic spread in mice which underwent primary tumor resection in the LLC tumor model; BV treatment in the spontaneous lung metastases model after primary tumor excision prolonged their median survival time from 27 to 58 days. It seems that BV could potentially offer long-term survival benefit when combined with surgery in the LLC tumor model.

Interestingly, research done by Ling et al. [107] reported inhibitory effect of recombinant adenovirus carrying melittin gene on hepatocellular carcinoma *in vitro* and *in vivo*. This suggests that animal toxin gene can be used as an antitumor gene. The induction of apoptosis in cancer cells by melittin gene therapy has also been demonstrated. The antitumor activity of melittin *in vivo* through gene therapy has been reported. Winder et al. [53] administered melittin repeatedly into a human bladder carcinoma-derived cell line to maintain the therapeutic levels and analyzed the resulting cell clones for their tumorigenicity in nude mice. The antimicrobial peptides such as cecropin magainins and melittin peptides rapidly associate with phospholipid cell membranes, move laterally in the membranes, and oligomerize, thereby leading to structural defects (e.g., pores) in the cell membrane. Alternatively, after intracellular transport, they act in a similar way on the membranes of internal organelles, inducing biochemical changes that cause cell death. Many groups have explored the unique potential of naturally occurring and synthetic peptides for cancer chemotherapy. By covalently fusing the membrane-active peptides with receptor-targeted peptide motifs, it has been possible to achieve very specific targeting to particular tumors for highly cytotoxic peptides [21, 108–110]. Most cell lytic peptides have an amphipathic structure, which preferentially bind and insert into negatively charged cell membranes. In contrast to normal eukaryotic cells with a low membrane potential, the cell membranes of prokaryotic and cancer cells have a large membrane potential. Therefore, many lytic peptides selectively disrupt the cancer cell membranes rather than those of normal cells.One such peptide, melittin, present in bee venom, has been shown to revert the transformed phenotype of H-ras transformed cells [60, 61]. Two analogs of the cecropin antimicrobial peptide, SB-7 and Shiva-1, have been shown to preferentially lyse a number of lymphoma- and leukemia-derived cell lines [111], and they are cytotoxic for a number of tumor-derived cell lines [112]. To achieve a similar effect *in vivo*, these peptides would have to be given repeatedly to maintain therapeutic levels, which may be pharmacologically unfavorable. The expression of the genes encoding such antimicrobial peptides in the desired cell type may circumvent these problems. Expression constructs in which the melittin or cecropin genes are placed under the transcriptional control of the murine leukaemia virus (MLV) promoter are reported. Introduction and expression of these peptides in tumor-derived human bladder carcinoma cells is associated with either reduced or no tumor growth when these cells are injected into nude mice. Studies by Winder et al. [53] suggest that such antimicrobial peptides such as melitin may be useful in gene therapy strategies for the treatment cancer.

Cytotoxic properties of immunoconjugates containing melittin-like peptide 101 against prostate cancer in *in vitro* and *in vivo* studies was investigated by Russel et al. [8]. Authors have shown beneficial effects against LNCaP-LN3 (LN3) or DU-145 human CaP cell xenografts when CaP-targeting Mabs–peptide 101 conjugates were injected i.p. or directly into the tumor. In both tumor models, specific antibody–peptide 101 conjugates significantly inhibited tumor growth compared with buffer, unconjugated antibody, or peptide alone; partial responses or development of stable disease occurred in some mice 3 weeks after initiation of treatment. Control antibody–peptide 101 conjugates showed more limited efficacy. Test-Mab–peptide 101 conjugates induced necrosis and hemorrhage in tumors, but sometimes with new growth around the dead areas, suggesting that the effects were limited. In addition, authors did not see toxicity in organs examined histologically (heart, kidney, liver), and while the peptide 101 has hemolytic properties *in vitro*, selective targeting through the use of MAbs prevented any long-term toxic effects, except in the tumors. Injection of peptide 101 alone caused necrosis/hemorrhage around the site of injection, but tumor volume was not affected; it would be anticipated that peptide 101 would be rapidly cleared as it is small. Intratumoral delivery of J591 Mab–peptide 101 immunoconjugates also provided tantalizing benefits and prolonged survival. This suggest that melittin and its analogs have properties advantageous for their use as toxins in immunoconjugates. These are (1) low toxicity of the peptides, limiting undesirable side effects; (2) their mode of action via interaction of the peptides with the plasma membrane

that is independent of internalization [75, 83, 113]; (3) the fact that they are cytolytic, not cytostatic, and their half-lives are limited *in vivo* by proteolysis, limiting toxicity of peptide released from its carrier. Tumor-specific antibody-melittin conjugates have been shown to selectively kill tumor cells *in vitro* [8] while in *in vivo* studies have shown a reduction in the average tumor volume and tumor burden in lytic peptide-treated animals. Ideally, antibody immunoconjugates should be used to target tumors of low burden (e.g., micrometastases, cancer cells shed during surgery) or after the tumor volume has been decreased by radiotherapy, brachytherapy, or hormonal treatment.

The phospholipase A(2) (PLA(2)) crotoxin, an antitumor protein that appears to act by interaction with epidermal growth factor receptors (EGFR), has recently shown activity in breast cancer in phase I clinical trials, it also displayed nonspecific neurotoxicity [114]. Ferguson and Duncan [114] investigated a novel dextrin-PLA(2) conjugate in aiming to reduce PLA(2) systemic toxicity but retain antitumor activity following alpha-amylase triggered degradation of dextrin in the tumor interstitium. Although the conjugate showed only approximately 36% enzyme activity compared to native PLA(2), it caused cytotoxic effects in MCF-7, HT29, and B16F10 cells at a level that was comparable to, or greater than, that seen for free PLA(2). Cytotoxic effects correlates with the level of EGFR expression.

Barrajón-Catalán et al. [115] investigated lytic immunoliposomes in human breast cancer cells with different HER2 expression levels. Pegylated anti-HER2 immunoliposomes using the complete antibody (trastuzumab) were loaded with melittin decreased cancer cells viability in a dose–response manner and in correlation to their level of HER2 expression. The drastic morphological changes observed through bright-field microscopy suggest a very fast mechanism of cell death starting in less than 1 h after starting the cells' treatment, which might takes place primarily through membrane pore formation. Authors suggested that trastuzumab-resistant breast cancer cells (JIMT-1) can be also targeted using this approach.

Nanoparticles (size in nanometer range) provide a new mode of cancer drug delivery functioning as a carrier for entry through fenestrations in tumor vasculature allowing direct cell access. Importantly, tumor vessels are abnormal and have aberrant branching blind loops and tortuosity. Tumor vessels are leaky due to basement membrane abnormalities and to decreased numbers of pericytes lining rapidly proliferating endothelial cells [116]. This results in enhanced permeability for molecule passage through the vessel wall into the interstitium surrounding tumor cells. The size of the gaps between the leaky endothelial cells ranges from 100 to 780 nm depending on the tumor type [117–119]. This is in contradistinction to the tight endothelial junctions of normal vessels typically of 5 to 10 nm size. These particles allow exquisite modification for binding to cancer cell membranes, the microenvironment, or to cytoplasmic or nuclear receptor sites. This results in delivery of high drug concentrations to the targeted cancer cell, with reduced toxicity of normal tissue. Soman et al. [120] developed a specific strategy to synthesize a nanoscale delivery vehicle for cytolytic peptides by incorporating the nonspecific amphipathic cytolytic peptide melittin into the outer lipid monolayer of a perfluorocarbon nanoparticle. The favorable pharmacokinetics of this nanocarrier allows accumulation of melittin in murine tumors *in vivo* and a dramatic reduction in tumor growth without any apparent signs of toxicity. Incorporation of melittin onto the nanoparticles prolongs the melittin circulation time, thereby offering an increased stochastic probability of accumulation in the tumor and targeted binding to sites of angiogenesis. Thus, the antiangiogenic effect of melittin-loaded nanoparticles that triggers apoptosis in the target cell could be advantageous for treatment of early cancerous lesions, as demonstrated from the regression of papillae within epidermal dysplastic lesions in transgenic K14-HPV16 mice. The broad applicability of the melittin nanoparticle construct is evident in three distinct tumor lines employed for *in vivo* work: the B16F10 syngeneic in immunocompetent C57BL/6 mice, the human MDA-MB-435 xenograft in athymic nude (NCr-nu/nu) mice, and the K14-HPV16 transgenic in FVB/N mice. The accumulation of melittin-loaded nanoparticle in solid tumor and tumor growth suppression *in vivo* by endothelial permeability and retention effect (EPR) was documented as the dominant mechanism of action. Nanoparticles were targeted by incorporating a peptidomimetic αvβ3 integrin-binding ligand. The demonstrated efficacy of both nontargeted and αvβ3 integrin-targeted melittin-loaded nanoparticles indicates that the melittin-loaded nanoparticles could exert their antitumor effect either by nonspecific trapping in the abnormal tumor vasculature or binding to overexpressed integrins on angiogenic endothelial cells and that the contact-mediated transfer of melittin by nanoparticles produces cytochrome c release and apoptosis. Perfluorocarbon nanoparticles thus represent the first in a class of unique lipid-based delivery vehicles for melittin and other such cytolytic peptides with broad spectrum and multimodal antivascular and antitumor actions that could be exploited for anticancer therapy.

Holle et al. [121] showed that the melittin/avidin conjugate had strong cytolytic activity against cancer cells with a high MMP-2 activity; DU 145 prostate cancer cells and SK-OV-3 ovarian cancer cells. However, the conjugate exhibited very little cytolytic activity against normal L cells that displayed a low MMP-2 activity *in vitro*. *In vivo*, the size of the tumors injected with the melittin/avidin

conjugate was significantly smaller than the untreated tumors. Therefore, the melittin/avidin conjugate has potential use in cancer therapy. Therefore, many lytic peptides selectively disrupt the cancer cell membranes such as a 23 amino acid analog of melittin hecate-1. A novel approach for the treatment of endocrine tumors possessing luteinizing hormone receptors (LHR) was developed. Melittin and a fragment of a melittin-conjugated hormone receptor (e.g., hecate) were shown to have an anti-tumor effect in ovarian and testicular tumors. Gawronska et al. [122] reported that the melittin fragment (hecate) and conjugated to a 15-amino acid beta-chain of human chorionic gonadotropin (βhCG) destroyed human ovarian carcinoma cell line (NIH: OVCAR-3), established from the malignant ascites of a patient with progressive adenocarcinoma of the ovary, in a dose-dependent manner. Previous studies from Leuschner et al. [123] and Hansel et al. [124] have shown that the lytic peptide conjugate hecate–βhCG specifically targets prostate cancer cells expressing LH receptors to stop the growth of PC-3 prostatecancer xenografts in nude mice. *In vivo* study demonstrated that hecate–βhCG and hecate alone administered once per week during a period of 3 weeks inhibit the growth of OVCAR-3 human epithelial ovarian cancer xenografts in nude mice. However, the effect of lytic peptides on the OVCAR-3 xenografts in nude mice is not as pronounced as on prostate xenografts [123, 124]. A possible explanation for this phenomen may be based on differences in the number of LH receptors. The ability of the hecate–βhCG to destroy Chinese hamster ovary cells was significantly greater in cells transfected with the LH receptor than in receptor-negative wild-type cells. Furthermore, the cytotoxicity increased further when transfected cells were treated with $ZnCl_2$ to increase the number of LH receptors. Melittin fragment might be a candidate for treating cancer cells, and LHR may be involved in the anti-tumor activity of melittin and/or its conjugates as described Son et al. [14]. The hecate–CGbeta conjugate induces the rapid and cell-specific membrane permeabilization of LHR-expressing cells *in vitro*, suggesting a necrotic mode of cell death without the activation of apoptosis. This demonstrats the principle that the hecate–CGbeta conjugate provides a novel specific lead into gonadal somatic cell cancer therapy by targeting the destruction of LHR expressing tumor cells.

It was also shown that less differentiated cells (leukemic cells, tumor cells) were two- to fourfold more sensitive to the lytic effects of bee venom than normal splenocytes or bone marrow cells [67]; probably the reason for this is the loss of amino/carbohydrate binding structures which led to destruction of tumor cells that were in close contact with bee venom. It is likely that higher doses of bee venom exhibited toxic effect on tumor tissues as was shown for blood, muscle, and heart tissues in human and animals treated with bee venom or other components of it such as melittin and phospholipase A2 [7, 67].

1.4 Potentiation of chemotherapeutic lethality to cancer cells by bee venom components

DNA damage can arrest cell cycle progression to allow for repair and the prevention of the replication of the defect or to activate apoptosis (programmed cell death) to eliminate the cells with catastrophic mutations [125]. Numbered data indicates that bee venom inhibits DNA repair and that this may be the mechanism by which it increases chemotherapeutic lethality and inhibits recovery from chemotherapeutic-induced potentially lethal damage. Hait et al. [126] demonstrated that melittin was one of the most potent inhibitors of calmodulin activity and a more potent inhibitor of cell growth and clonogenicity than phenothiazines [126–128]. Drugs that inhibit calmodulin activity have been shown to inhibit DNA synthesis in the glioblastoma cell line [129], block the movement of chromosomes during metaphase [130], inhibit the growth of Chinese hamster ovary cells [64], and enhance the cytotoxicity of vincristine, doxorubicin, and bleomycin [62, 63, 131, 132].

It is known that some calcium antagonists and calmodulin inhibitors enhance the intracellular levels of antitumor agents by inhibiting their outward transport. The high intracellular melittin accumulation was directly related to the enhancement of the cytotoxicity of the antitumor agents. Treatment of exponentially growing cells with chemotherapeutic causes dose-dependent decrease in cell survival due to DNA damage. This lethal effect can be potentiated by the addition of a nonlethal dose of the bee venom. By preventing the repair of damaged DNA, bee venom also inhibits recovery from potentially lethal damage induced by chemotherapeutic in different cancer cells.

There are several possible mechanisms to explain the synergistic effect of chemotherapeutics and bee venom. First, the efflux of antitumor agents from cells may be controlled, possibly by calcium and calmodulin (calcium–calmodulin complex, CaM); for example, calmodulin inhibitors could directly inhibit calcium–calmodulin complex. Secondly, it might be possible that the energy-dependent drug exodus system in the membrane might be perturbed by the modifiers or by the change in calcium environment. Thirdly, the affinity of chemotherapeutics for intracellular targets may be altered by calcium antagonist-induced changes in the calcium environment or by calmodulin inhibitors. In addition, the differential toxic effect of bee venom on cancer cells might result with apoptosis or necrosis [62]. It is likely that the necrosis was potentiated with some components of bee venom (hyaluronidase, phospholipase A_2, and melittin).

1.5 Mode of action of bee venom and melittin at the cellular and molecular levels

1.5.1 Direct cytotoxicity of bee venom and its components on tumor cells

Requirements for close contact between MCa and bee venom for *in vivo* effect was also shown in *in vitro* studies [7]. The degree of growth inhibition of MCa cells in the presence of bee venom was dose dependent up to 24 h. It is likely that bee venom induce cytotoxic effect by inhibition of calmodulin or by induction of apoptosis and necrosis of tumor cells described in our studies [6, 78] and others [2, 5]. The mechanism by which agents exerting anticalmodulin activity inhibit the growth of cells is unknown. One possible mechanism might be the inhibition of a calmodulin sensitive enzyme such as cyclic nucleotide phosphodiesterase. The mechanism of inhibition appears to be mediated through the formation of a calcium-dependent high-affinity complex between calmodulin and melittin [2, 5, 60]. Although a phosphodiesterase was the first enzyme demonstrated to be activated by calmodulin, it is now known that numerous enzymes and structural proteins are dependent on this calcium-binding protein [2]. Calmodulin is essential for many processes that are necessary for normal cellular function, including the assembly and disassembly of microtubules, calcium extrusion from cells by a calcium–magnesium ATPase, and the activation of numerous intracellular enzymes, such as protein kinases, phosphatases, and cyclic nucleotide phosphodiesterase [2, 5, 60]. Interfering with any of these known functions, calmodulin would be potentially toxic to cells. Other cellular functions inhibited by calmodulin inhibitors may or may not be mediated exclusively through the inhibition of calmodulin. These effects include the depletion of intracellular ATP, destabilization of membranes and inhibition of protein kinase C [2, 5, 6, 60]. It has also been reported that direct contact of bee venom components with different tissues (cells) caused instability of cell membrane receptors and damaged of enzyme systems bound to cell membrane such as cation activated adenosine triphosphatases, damaged acetylcholine esterases, or inhibited thromboplastic potency of tissue [2, 5, 60].

Acquisition of resistance to bee venom could be accompanied by a decrease in the number of copies of ras genes, decrease in the expression of the ras oncoprotein, and concomitant reversion of transformed cells to a normal morphology, as suggested by Sharma [60]. Our results on other cells (HeLa and V79) showed that glutathione levels might be involved in their resistance to bee venom.

The growth inhibitory effect of bee venom measured by cell numbers after exposing cells for periods of 72 h demonstrated that the inhibitory effect on V79 cells was more effective when compared to HeLa cells. Growth inhibition with bee venom on HeLa and V79 cells was dose-dependent; IC_{50} for HeLa cells was 3 μg mL^{-1}. The same dose of bee venom killed about 90% V79 cells.

The polypeptides in bee venom (PBV) produced a significant growth inhibition against SMMC-7721 human hepatoma cell line [17]. A significant inhibition was observed over the concentration of PBV ranging from 2 to128 μg mL^{-1} ($p<0.05$). The IC50 value of PBV in SMMC-7721 cells was 6.68 μg mL^{-1} while in the same concentration of PBV, relative viability of Hela cells was 20.3%. In addition, the cell proliferation was evaluated by immunohistochemistry assay by Ki67 (a nuclear protein expressed in proliferating cells) labeling index as an easy and reproducible method to determine the growth fraction of malignant and normal tissue [133, 134]. The results showed that the expression of Ki67 was inhibited significantly by PBV and that the proliferation index was decreased from 97.0% to 10.2%. Flow cytometric analysis revealed a prominent cell arrest in G0/G1, which is a hallmark of cells undergoing apoptosis [135]. Accumulation of PBV-treated cells in G0/G1 phase seemed to be responsible for cytostatic effects. Other studies suggested that PBV induced the apoptotic cell death [5, 15, 70].

The possible explanations for different sensitivity of cancer cells may be the results on differences in the average doubling time in log phase (for example,V79 cells was about 12 h, while for HeLa cells was about 20 h), alternations of membrane topography, the heterogeneous nature of tumor cells which contain subpopulations varying in their sensitivity to the cytotoxic effects of calmodulin, and calcium and magnesium levels as well as in the presence amino-group/carbohydrate binding sites on cell membranes for BV component binding as suggested by Killon and Dunn [67]. The amino groups appear to be essential for blocking the melittin-mediated lysis because glucose, galactose, and the N-acetyl derivatives produced no inhibition. This suggests that bone marrow cells are rich in membrane binding sites for carbohydrates, which decreases in mature spleen cells and are virtually absent after a neoplastic transformation.

Since the number of cells present in culture is a reflection of both proliferation and death of cells, the inhibition of growth by calmodulin inhibitors could be due to the inhibition of proliferation rather than to cytotoxicity. However the work of Balk et al. [136] strongly suggests that both calcium and magnesium play significant roles in the initiation of cell replication. Disturbance in the homeostasis, these divalent cations leads to a defect in the regulation of cell growth or development of a cation-indepedent initiation mechanism. Other evidence indicates that extracellular calcium is necessary for DNA synthesis and cell proliferation in normal cells but not in cancer cells [137].

Putz et al. [96] demonstrated that bee venom secretory phospholipase A2 and phosphatidylinositol-homologs cooperatively disrupt membrane integrity, abrogate signal transduction, and inhibit proliferation of renal cancer cells. Jang et al. [15] demonstrated that BV induces apoptosis and causes selective inhibition of COX-2 in NCI-H1299 human lung carcinoma cells. BV treatment resulted in an increase in bax expression and a decrease in bcl-2 expression as well as in an up-regulation of caspase-3 activity. It was demonstrated that BV exerts two profound effects on cells of the human lung carcinoma cell line NCI-H1299; BV induces apoptotic cell death and selective inhibition of COX-2 mRNA expression.

BV caused growth arrest or cytotoxic effects in hepatocellular carcinoma, leukemia, osteosarcoma and mammary carcinoma cells [6, 7, 9, 56, 57]. Recent studies reported that BV inhibits cyclooxygenase-2 and induces caspase-3 activation in human lung cancer cells and synovial fibroblasts, respectively [15, 19]. Liu et al. reported that BV induces apoptotic death in mouse melanoma cells [5]. According Tu et al. [20], BV-triggered apoptosis was accompanied by ROS generation, translocation of Endo G (endonuclease G) and AIF (apoptosis-inducing factor), JNK activation (cJun N-terminal kinases), and cytosolic calcium elevation in human melanoma A2058 cells. Growing of evidence indicates that apoptosis-induced factors (AIF) and endonuclease G (Endo G), which are mitochondrial proteins, are major apoptosis executors [138–140] involved in the caspase-independent cell death pathway [141]. Immunostaining results with polyclonal antibodies against AIF or Endo G showed that the translocation of AIF and Endo G from mitochondria to the cytosol and nucleus had occurred within 10–30 min after BV administration, indicating that both molecules might be involved in BV-induced apoptosis. This finding also provides a significant cue to cancer treatment, since many cancer cells carry mutations in apoptotic-regulated genes such as p53, Bcl-2, or those affecting caspase-signaling pathways. These defects render treatment with traditional chemotherapeutic agents ineffective. BV may be a potent and novel therapy for treatment of patients with melanoma or other cancer.

1.5.2 Bee venom and mellitin are promising agents in induction tumor cell apoptosis

Previous studies have reported that bee venom caused growth arrest or cytotoxic effects in hepatocellular carcinoma, leukemia, osteosarcoma, and mammary carcinoma cells [7, 9, 56, 57]. Also, proliferation of melanoma cells, activity of apoptotic enzyme (bcl-2 and caspase-3) in leukemic cells, and ERK and Akt signaling pathway of renal cancer cells were all regulated and/or suppressed by bee venom [5, 9, 14, 15]. Recent studies have reported that bee venom induced apoptosis in breast cancer cell line and lung cancer cell line [14]. Some recent studies reported that bee venom inhibits cyclooxygenase-2 and induces caspase-3 activation in human lung cancer cells and synovial fibroblasts, respectively [15, 19]. Liu et al. [5] reported that BV induces apoptotic death in mouse melanoma cells. However, the underlying mechanisms are largely unknown. A recent study by Tu et al. [20] demonstrated anticancer effects of bee venom in human melanoma A2058 cells and its mode of action at the cellular and molecular levels. The authors found that honeybee venom induced calcium-dependent but caspase-independent apoptotic cell death in human melanoma A2058 cells but not in normal skin fibroblast Detroit 551 cells. Finally, Tu et al. [20] suggested that treatment of A2058 cells with melittin, the major component of bee venom, resulted in similar elevation of calcium levels and cell killing effects, suggesting that melittin is the major determinant in bee venom-triggered cell death [57]. Observations by Ip et al. [142] indicated that bee venom induced cell cycle arrest and apoptosis in human cervical epidermoid carcinoma Ca Ski cells via a Fas receptor pathway involving mitochondrial-dependent pathways and is closely related to the level of cytoplasmic Ca^{2+} in Ca Ski cells. Flow cytometric analysis demonstrated that bee venom induced the production of reactive oxygen species, increased the level of cytoplasmic Ca^{2+}, reduced mitochondrial membrane potential which led to cytochrome c release, and promoted the activation of caspase-3 which then led to apoptosis. Bee venom also induced an increase in the levels of Fas, p53, p21, and Bax, but a decrease in the level of Bcl-2. The activities of both caspase-8 and caspase-9 were enhanced by bee venom, promoting caspase-3 activation, leading to DNA fragmentation (Fig. 2).

Apart from the caspase-dependent pathway, authors also showed caspase-independent pathway through expression of apoptosis-inducing factor (AIF) and endonuclease G (Endo G) in the Ca Ski cells. Similar mechanism of bee venom was confirmed in human breast cancer MCF7 cells [143], U937 cells [144], and in human MG63 osteosarcoma cells [57]. Data by Wang et al. [145] suggest that melittin can synergize with tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) in the induction of human hepatocellular carcinoma cells (HCC) apoptosis and that the combination of melittin with TRAIL may be a promising therapeutic approach in the treatment of TRAIL-resistant human cancer.

Moreover, there might be other components in BV that can trigger cell death at high dosage via a Ca^{2+}-independent manner. It was reported that a secretory phospholipase A2 (PLA2), a minor component of BV, synergistically acted with phosphatidylinositol-(3,4)bisphosphate to disrupt membrane integrity and caused subsequent cell death in renal cancer cells [146, 147]. Putz et al. [96] demonstrated that bv-sPLA2 and phosphatidylinositol-(3,4)-bisphosphate

synergistically generate tumor lysates which enhance the maturation of immunostimulatory human monocyte-derived dendritic cells. Such tumor lysates which represent complex mixtures of tumor antigens and simultaneously display potent adjuvantproperties meet all requirements of a tumor vaccine. This data suggests that bv-sPLA2 action may exert inhibitory effects on tumor cells, while comitantly promoting immunostimulatory capacities of moDCs. Increasing evidence also suggests a modulatory role of phospholipids in the differentiation process of moDCs, thus affecting the immunogenic potential of antigen-presenting cells [148–150]. Phospholipids like phosphatidylinositols (PtdIns) and phosphorylated forms thereof exert their role either as precursors of second messengers, or directly by interacting with proteins to orchestrate the spatio-temporal organization of key intracellular signal transduction pathways [151, 152]. It is possible that antitumor action and immune activation through cooperation of bee venom secretory phospholipase A2 and phosphatidylinositol-(3,4)-bisphosphate may be implicated in cell survival, control of proliferation, and regulation of the cell cycle [151, 153, 154]. In agreement with these reports, Putz et al. [96] assumed that PLA2 might also be responsible for the BV-induced apoptotic process. It seems that bee venom and its compounds have multiple functions in various cell types [2]. Thus, the intracellular targets for melittin have been reported by several groups, such as calcium channel, calmodulin, phospholipase A2 (PLA2) and D (PLD), mitochondrial F1-ATPase, sphingomyelinase, and Rac 1 [2, 57, 145].

Phospholipase A2-independent Ca^{2+} entry and subsequent apoptosis induced by melittin in human MG63 osteosarcoma cells was demonstrated by Chu et al. [57]. Furthermore, there is a causal relationship between Ca^{2+} overload and apoptosis, namely, melittin induced $[Ca^{2+}]i$ increases in MG63 osteosarcoma cells via Ca^{2+} influx through L-type Ca^{2+} channels, without evoking Ca^{2+} release from stores. Melittin has been shown to cause death in several other cell lines, via either apoptosis or necrosis. In thymocytes, it is thought that the activation of phospholipase A2 by melittin induces necrosis but not apoptosis [72]. Zhao et al. [138] show that melittin causes apoptosis in several cell lines via activation of phospholipase A2. In four murine tumor cell lines, melittin was thought to cause Ca^{2+} influx without inducing apoptosis [55]. Since promoting apoptosis is a strategy for cancer drug discovery, it is likely that bee venom, as well as mellitin, are promising agents in induction tumor cell apoptosis. However, Park et al. [155] recently demonstrated that low concentration BV (1, 10, and 100 ng mL^{-1}) possess a potent suppressive effect on anti-apoptotic responses of tumor necrosis factor (TNF)-alpha with actinomycin (Act) D-treated hepatocytes and suggest that these compounds may contribute substantial therapeutic potential for the treatment of liver diseases.

1.5.3 Reactivation the p53 pathway by apamin

The tumor suppressor p53 has been implicated in a growing number of biological processes, including cell cycle arrest, senescence, apoptosis, autophagy, metabolism, and aging. Activation of p53 in response to oncogenic stress eliminates nascent tumor cells by apoptosis or senescence. The p53 tumor suppressor pathway blocks tumor development by triggering apoptosis or cellular senescence in response to oncogenic stress. A large fraction of human tumors carry p53 mutations that disrupt DNA binding of p53 and transcriptional regulation of target genes. Reconstitution of wild-type p53 *in vivo* triggers rapid elimination of tumors. Therefore, pharmacological reactivation of mutant p53 is a promising strategy for novel cancer therapy [156, 157].

The activity and stability of the tumor suppressor protein p53 is negatively regulated by the oncogenic proteins MDM2 and MDMX; the cellular process is initiated by MDM2/MDMX binding to the N-terminal transactivation domain of p53 [158]. It has been demonstrated that antagonists that block the p53-binding pocket of MDM2/MDMX kill tumor cells both *in vitro* and *in vivo* by reactivating the p53 pathway, resulting in cell cycle arrest, senescence, or apoptosis [159]. Since MDM2 andMDMX act synergistically in tumor cells, they may be highly attractive molecular targets for anticancer drug development. Thus, recent papar Li et al. [160] demonstrated that a bee venom-derived peptide apamin could provide a scaffold for p53 inhibitors to treat cancer. Apamin, a highly specific blocker of Ca^{2+}-activated K^+ channels of small conductance, [161] converted the 18 amino acid residue bee-venom neurotoxin into several potent peptide inhibitors of the p53–MDM2/MDMX interactions with different specificities. The rational design of these apamin-derived p53 activators are termed stingins. Stingin 5, a peptide that combines the amino acids of a p53-MDM2 inhibitor and a bee venom neurotoxin, had 15-fold higher binding affinity for MDM2 than did a small molecule p53 inhibitor (Fig. 2). Inhibiting interactions between p53 and MDM2 is known to have an antiproliferative effect in some cancer cells. Since the tumor suppressor p53 inhibits tumor growth primarily through its ability to induce apoptosis, reactivation of p53 by apamin in such tumors should trigger massive apoptosis and eliminate the tumor cells [162].

1.5.4 Inhibition of angiogenesis and MMP as a target for anticancer and antimetastatic effect of bee venom

Angiogenesis plays a vital role in growth, intravasation, and metastatic spread of cancer [163, 164]. Inhibition of angiogenesis provides a good chance of preventing cancer from becoming malignant [106, 165]. It is widely accepted that tumor-induced angiogenesis is initiated by angiogenic

cytokines such as bFGF and VEGF that are expressed in the tumor itself [106, 165]. This process depends on vascular cell proliferation, migration, and tube formation of endothelial cells.

BV played a remarkable role in inhibiting angiogenesis and metastasis via downregulation of vascular endothelial growth factor (VEGF), VEGFR-2 and blockaded the VEGFR-2 signaling pathways by interfering with the activation of Akt and p42/44 MAPK. BV also inhibited VEGF-induced proliferation, migration, and capillary-like tube formation of human umbilical vein endothelial cells (HUVECs). Results Huh et al. [106] suggest that the tumor-specific anti-angiogenic activity of BV takes effect during different stages of tumor progression by blocking the tyrosine phosphorylation of VEGFR-2 and validate the application of BV in lung cancer treatment.

The invasion and metastasis of cancer cells are known to be the primary causes of cancer progression [166]. These are complicated processes involving a group of proteolytic enzymes such as matrix metalloproteinases, which participate in the degradation of tissue barriers such as the extracellular matrix (ECM) and basement membrane [167, 168]. Matrix metalloproteinases (MMPs) play an important role in tissue repair, angiogenesis, apoptosis, tumor invasion, and metastasis [169]. One of the members of MMP family, the type IV collagenase (MMP-9) is critical for cell migration and can lead to invasion and metastasis of cancers [170].

BV has anti-cancer activities on several types of cancer cells including lung, liver, breast, prostate, and mammary cancer cells as well as leukemia cells [7, 9, 56, 57]. Previous studies reported that the cytotoxic effects on cancer cells work through the activation of PLA2, caspase and MMP, which destroy cancer cells and is suggested as an important basic mechanism for the anti-cancer activity of BV [9, 99, 100, 121, 169–171]. BV has also been reported to induce apoptosis in cancer cells both *in vitro* and *in vivo*. It is likely that the promotion of apoptotic cell death through several cancer cell death mechanisms, including the activation of caspases and MMP such as MMP-2 and MMP-9, is essential for the bee venom and melittin-induced anti-cancer effects [9, 121, 169, 171]. Recently, studies [99, 100] have shown that suppression of MMP-9 expression contributes to the anti-tumor properties of BV and melittin in Caki-1 cells and MCF-7 cells, while melittin/avidin conjugate had strong cytolytic activity against cancer cells with a high MMP-2 activity such as DU 145 prostate cancer cells and SK-OV-3 ovarian cancer cells.

Controversial properties of bee venom were confirmed by Hamedani et al. [172, 173] who demonstrated the stimulatory effect of bee venom on matrix MMP-2 and MMP9 activities at doses 0–0.05 μg mL^{-1} and the inhibitory effect at dose higher than >0.05 μg mL^{-1}.

1.5.5 Immunomodulatory effect of bee venom

The immune system represents a primary defense against invading pathogens, non-self components, and cancer cells. Inflammation is a basic process by which the body reacts to infection, irritations, or other injuries and is recognized as a type of non-specific immune response. Bee venom consists of several biologically active agents such as phospholipase A2, apamin, adolapin, melittin, and mast cell degranulating peptide [1, 2]. Apart from antitumor action, melittin and phospholipase A2 are the two major ingredients thought to be responsible for inducing irritation and the well-known allergic reaction associated with bee stings. Nam et al. [174] elucidated the effects of BV on Th1/Th2 lineage development under *in vivo* conditions by intraperitonial injection BV to BALB/c mice. Thus, Nam et al. [174] demonstrated that BV enhances the Th1 cell-dominated immune response by increasing the expression of IFN-γ mRNA, without altering the Th2 cell response in both *in vitro* and *in vivo* conditions. Interestingly, the *in vivo* effect of BV on T cells was significantly increased compared with the *in vitro* data, suggesting that antigen presenting cells (APCs) also play a role in BV-mediated T cell responses. However, the study of Nam et al. [174] demonstrates that BV directly influences $CD4^+$ T cell immune activity without APCs.

Recently, it has been demonstrated that bee venom secretory phosphatase A2 (bv-sPLA2) acts as an adjuvant by triggering the maturation of monocyte-derived dendritic cells (moDCs). Putz et al. [96] demonstrated that bv-sPLA2 and phosphatidylinositol-(3,4)-bisphosphate synergistically generate tumor lysates which enhance the maturation of immunostimulatory human monocyte-derived dendritic cells; this combination displayed antitumor effect with concomitant stimulation of the immune system. Such tumor lysates which represent complex mixtures of tumor antigens and simultaneously display potent adjuvant properties meet all requirements of a tumor vaccine. This observation by Putz et al. [96] strongly suggests the further exploration of the therapeutic potential of these compounds in cancer immunotherapy. In addition, Putz et al. [96] showed that induced maturation of human moDCs by BV can enhance expression of CD83 and improves stimulation in allogeneic mixed leukocyte reactions due to inflammatory reaction. Influence on inflammation and immune responses has also been attributed to phospholipids and related compounds [175]. Today, it is known that inflammation serves to recruit immune cells to the site of vaccine administration and to promote DC maturation which favors the induction of the desired immune responses [176, 177], which could be important in cancer immunotherapy.

Some study confirmed that injection of mellitin induced paw edema in mice [178]. Furthermore, injection of whole

BV into the hind paw of mice has been shown to produce local inflammation [7, 179].

In our data, the responses of regional lymph node cells were increased in animals treated with bee venom [7], as was similarly shown by Schneider and Urbanek [180]. The results related to the lytic activity of popliteal lymph node cells on MCa cells [7], indicating that bee venom is a strong activator of antitumor lytic activity of lymphoid cells deriving from the regional lymph node. Inactivity of spleen cells in this respect indicates that concentration of bee venom is an important factor for activation of antitumor lytic activity in mice. The possible mechanism(s) of antitumor lytic activity may include factors related to activation of cytotoxic T lymphocytes. Local treatment with bee venom increased the $CD8^+$-T cell subset and led to a progressive reduction of the immune index ($CD4^+/CD8^+$ ratio) in favor of $CD8^+$ cells. A low $CD4^+/CD8^+$ ratio in lymph node, of 0.75, is observed in the treated group compared with the control group (1.43). There was a significant difference between the two groups ($p<0.01$). The $CD4^+/CD8^+$ ratio in spleen between control and treated group was 1.45 and 1.35, respectively. This implies that the phenotype of $CD8^+$ cells increased by local stimulation of regional lymph nodes by bee venom may have an important role in tumor cytotoxicity. Moreover, according to Magnan et al. [181] and Ribardo et al. [182], the release of precursors of pro-inflammatory mediators caused by bee venom components such as phospholipase A2 and melittin might increase synthesis of IL-1 and TNF-α in monocytes and the cellular response of T lymphocytes. These findings contrast to those indicating that bee venom has an inhibitory effect on immunocompetent cells [183]. Moreover, iv treatment with bee venom increased the response of spleen cells to polyclonal mitogens [7]. Consistent with this observation is a report of Schneider and Urbanek [180], who also found an increased stimulation index among lymphocytes from lymph nodes when cultured with bee venom *in vitro*. Thus, bee venom might have direct and indirect action on tumor cells by stimulating the host cells, mainly macrophages and cytotoxic T lymphocytes [181–183]. In addition, adding the bee venom to K562 cell line could significantly increase the production level of IFN-β only on day 8 post-treatment. It seems that time- and dose-dependent response as well as the type of treated cell line could determine the immunosuppressive and/or immunostimulant property of bee venom that could be effective in future therapeutic strategies.

1.6 Possible application of bee venom components, perspective, and conclusions

BV and its constituents has anti-cancer activity on numbered tumor cells including renal, lung, liver, prostate, bladder, melanoma, osteosarcoma, and mammary cancer cells, as well as leukemia cells. Taken together, the data presented here support and extend our findings and the findings of the others that bee venom has direct antitumor effect *in vivo* and *in vitro* [5–9, 17, 96, 120]. BV components such as melittin, phospholipase A2, and apamin may be an important alternative medicine to treat cancer. Recent studies have demonstrated diverse mechanisms of anticancer effect of BV and its components. Different *in vitro* and *in vivo* studies have shown that bee venom component modulate different gene involved in the regulation of proliferation, apoptosis, metastasis, and angiogenesis (see Fig. 2). Thus, numbered studies have demonstrated that apoptosis, necrosis, and lysis of tumor cells could be the possible mechanisms by which bee venom inhibited tumor growth. Concomitant to these is the development of local cellular immune response in lymph node draining the region of bee venom introduction. The route of injection, time- and dose-dependent response, as well as the type of treated cell line could determine the immunosuppressive and/or immunostimulant property of bee venom. The inhibitory ability of BV and its constituent on calmodulin activity and induction apoptosis/necrosis in cancer cells through the different way may be a promising therapeutic approach in the treatment of human cancer. Therefore, Ca^{2+}/CaM might be a potent therapeutic targets for the treatment of angiogenesis-related diseases, including cancer. Ca^{2+}/CaM plays a crucial role in angiogenesis. Ca^{2+}/CaM activates HIF-1 and consequently induces the expression of pro-angiogenic factors such as VEGF. Hypoxia-induced increase in intracellular Ca^{2+} concentration promotes both expression and transcriptional activity of HIF-1α by modulating the activity of Ca^{2+}/CaM-depedent enzymes, including CaM kinase and calcineurin. Furthermore, inhibition of Ca^{2+}/CaM by Ca^{2+}/CaM antagonist such as melittin might suppress angiogenesis.

Chu et al. [57] demonstrated that melittin could induce apoptosis in a phospholipase A2-independent manner, while Zhao et al. [138] showed that melittin causes apoptosis in several cell lines via activation of phospholipase A2. In addition, Wang et al. [145] demonstrated that the combination of melittin with tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) may be a promising therapeutic approach in the treatment of TRAIL-resistant human cancer. Several studies suggested the cell cytotoxic effect through the activation of PLA2, caspase, and MMP, which destroy cancer cells as an important basic mechanism for the anti-cancer activity of BV [9, 121]. Selective effect of major component bee venom, melitin, might be a desirable therapeutic peptide, since melittin preferentially hyperactivates PLA2 in ras oncogene-transformed cells resulting in their selective destruction. This suggests that the hyperactivation of PLA2 by melittin might be a target for the cytotoxic effect of melittin against

cancer cells. Moreover, the difference between a low membrane potential in normal eukaryotic cells and a large membrane potential in cell membranes of prokaryotic and cancer cells provides that many lytic peptides selectively disrupt the cancer cell membranes rather than those of normal cells. Thus, Son et al. [14] reported that melittin did not inhibit the growth and cloning efficiency of normal cells at a concentration that prevents the proliferation of tumor cells such as lung tumor cell lines (human small-cell cancer-derived cell line IRSC-10 M and adenocarcinoma-derived cell line A459). This difference in responsiveness suggests that different growth-signaling pathways are triggered in histologically distinct lung tumor cell lines and normal cells. As a consequence, the susceptibility of tumor cells to phenotype modifiers needs to be considered in cancer therapy. Melittin exerted multiple effects on cellular lipids including various types of FFAs [94]. Production of various lipid metabolites could lead to a broad alteration of the lipid bilayer structure and consequently affect cellular functions in a variety of different ways. Moreover, Huh et al. [106] have provided positive data for the safety and efficacy of BV as a potential anti-angiogenesis strategy against lung cancer through down-regulation of VEGF and VEGFR-2. The effectiveness of BV in disrupting lung tumor growth and metastasis provides a promising basis toward further studies and clinical application of BV for lung cancer treatment. Liu et al. [59] showed that melittin can inhibit cell motility drastically and prevent hepatocellular carcinoma (HCC) metastasis *in vivo* through the suppression of the Rac1-dependent pathway. Furthermore, this study concludes that melittin is a potential novel drug for HCC.

Some studies also suggest the possible novel use of bee venom in the enhancement of the cytotoxicity of the antitumor agents by inhibitors of calmodulin. Bee venom and melittin as calmodulin inhibitors can enhance the intracellular levels of antitumor agents by inhibiting their outward transport, thus increasing directly their cytotoxicity to tumor cells [62, 63, 131, 132]. Reinforcement of the cytotoxic effect of cytostatics using bee venom or its components as well as preventing recovery or acquisition of resistance of tumor cells is an important modality in the treatment of tumors and could find its application in the future.

Bee venom components have a wide range of targets that lead the suppression of inflammation, which has been closely linked with cancer. NF-κB is a major mediator of inflammatory pathways (Fig. 1). Activation of NF-κB results from various stimuli that activate kinases and subsequently the IKK complex driving the proteasomal degradation of the NF-κB inhibitor (IκB). Activation of NF-κB, concomitant to its translocation in the nucleus, allows the onset of transcription of a wide spectrum of genes. Some of them are noted in Fig. 2. Disruption of the NF-κB activity can occur by the use of bee venom compounds able to target the kinase cascades that phosphorylate the IKK complex, compounds that inhibit the activity of IKK, prevent the proteasomal degradation of IκB or either the translocation of NF-κB in the nucleus. These molecules might be the subject of new therapeutic approaches. Thus, it is easy to understand the rationale of attempt to use the NF-κB/IL-6 pathway as a potential therapeutic target in hormone-refractory metastatic prostate cancer wherein growth, proliferation, or apoptosis resistance are supported and mediated by this signaling. It seems that the effect of BV components in inhibition of NF-κB and calmodulin, together with the induction of apoptosis and direct lysis of cells, could be crucial in inhibiting tumor growth.

A conjugation of the cell lytic peptide (melittin) with hormone receptors, and gene therapy carrying melittin can be useful as a novel targeted therapy for some types of cancers such as prostate and breast cancer because these delivery system can more effectively to kill cancer cells [8, 17, 22, 56]. Soman et al. [120] demonstrated that synthetic nanoscale vehicles such as perfluorocarbon nanoparticles can deliver a potent model cytolytic peptide (melittin) by flexible passive and active molecular targeting to kill both established solid tumors and precancerous lesions.

Next steps include developing a genetically encoded or nanoparticle-based system to deliver stingin into cancer cells which is an important peptid in inhibition of the interactions between p53 and MDM2. Stingins represent a novel class of p53 activators and are superior in many aspects to the existing miniprotein antagonists of MDM2/MDMX. Coupled with a therapeutically viable delivery modality, stingins may have the potential to be used as antitumor agents for clinical use. Since the loss of p53 function is a characteristic of almost all human tumors, reconstitution of p53 function by apamin is possible and practical as a promising antitumor strategy [156].

An indirect mechanism of bee venom, based on stimulation of the local cellular immune response in lymph node, to inhibit tumor growth and to promote its rejection was also observed. In addition, bee venom secretory phospholipase A2 in combination with phosphatidylinositol-(3,4)-bisphosphatecan can also display antitumor effect with concomitant stimulation of the immune system.

Numbered findings suggested that BV could be used as a chemotherapeutic agent against tumors. Furthermore, it is likely that more attention should be paid to natural inhibitor of tumor growth such as bee venom and other products of beehive in testing their antitumor activity for future possible use in clinical practice. It seems that nanoparticles as delivery vehicles for melittin, stignin, and other cytolytic peptides with a broad spectrum, multimodal antivascular, or

antitumor actions, could be exploited for anticancer therapy. *In vitro* and *in vivo* as well as clinical trials have demonstrated that BV therapy may be an important modality in traditional medicine to treat different types of cancer.

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