Associate editor: I. Kimura

Therapeutic application of anti-arthritis, pain-releasing, and anti-cancer effects of bee venom and its constituent compounds

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Abstract

Bee venom (BV) therapy (BVT), the therapeutic application of BV, has been used in traditional medicine to treat diseases, such as arthritis, rheumatism, pain, cancerous tumors, and skin diseases. BV contains a variety of peptides, including melittin, apamin, adolapin, the mast-cell-degranulating (MCD) peptide, enzymes (i.e., phospholipase [PL] A_2), biologically active amines (i.e., histamine and epinephrine), and nonpeptide components which have a variety of pharmaceutical properties. BV has been reported to have anti-arthritis effects in several arthritis models. Melittin, a major peptide component of BV, has anti-inflammatory and anti-arthritis properties, and its inhibitory activity on nuclear factor kappaB (NF-κB) may be essential for the effects of BV. The anti-nociceptive effects of BV have also been demonstrated in thermal, visceral, and inflammatory pain models. Apcupoint stimulation (apipuncture) therapy into subcutaneous region may be important in the BV-induced anti-nociceptive effects. Multiple mechanisms, such as activation of the central and spinal opiod receptor, and $α_2$-adrenergic activity, as well as activation of the descending serotonergic pathway have been suggested. The inhibition of c-Fos expression in the spinal cord by BV apipuncture in several nociceptive models is also reported to be a possible mechanism. BV also has anti-cancer activity. The cell cytotoxic effects through the activation of PLA_2 by melittin have been suggested to be the critical mechanism for the anti-cancer activity of BV. 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.
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Keywords: Bee venom; Melittin; Apamin; Mast-cell-degranulating peptide; Adolapin; Procamines; Phospholipase A_2; Hyaluronidase; Histamines; Anti-arthritis effect; Anti-inflammatory effect; Anti-nociceptive effect; Anti-cancer effect

Abbreviations: 5-HT, 5-hydroxytryptamine; AP5, 5-aminophosphonovaleric acid; BV, bee venom; BVT, bee venom therapy; c-AMP, cyclic adenosine monophosphate; c-GMP, cyclic guanosine monophosphate; CH, chelerythrine chloride; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; COX, cyclooxygenase; CSPA, capsaicin-sensitive primary afferent; DNQX, 6,7-dinitroquinoxaline-2,3-dione; ERK, extracellular signaling-regulated kinases; H89, *N*-(2-[P-bromocinna-mylamino]ethyl)-5-isoquinoline sulfonamide hydrochloride; HA, hyperalgesia; IL, interleukin; iNOS, inducible NO synthase; i.t., intrathecal; MCD, mast cell degranulating; MIH, mirror image heat; MMP, matrix metalloproteinase; NO, nitric oxide; OA, osteoarthritis; ORLI receptor, opioid receptor-like I receptor; PG, prostaglandins; PKC, protein kinase C; PL, phospholipase; PSN, persistent spontaneous nociception; RA, rheumatoid arthritis; SK, small conductance Ca^{2+}-dependent K^+ channels; SMU, single motor units; TNF, tumor necrosis factor; TPA, 12-*O*-tetradecanoyl phorbol-13-acetate.

Contents

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1. Introduction

Bee venom (BV) therapy (BVT) is the therapeutic application of honeybee venom (HBV) to the treatment of various diseases. BVT has been used as a traditional medicine to treat a variety of conditions, such as arthritis, rheumatism, back pain, cancerous tumors, and skin diseases (Hider, 1988). BV contains at least 18 active components, including enzymes, peptides, and biogenic amines, which have a wide variety of pharmaceutical properties. BV might modify the immune system functions in the body and contribute to the increased production of cortisol (Vick et al., 1972). The healing potency of BV in the treatment of arthritis and rheumatism is initiated after stimulating the production of cortisol in the adrenal glands, which in turn has anti-inflammatory activity (Vick & Shipman, 1972). Recent studies have reported a variety of mechanisms for the anti-arthritis and/ or anti-inflammatory effects of BV and its constituents. The decrease in cyclooxygenase (COX)-2 and phospholipase (PL) A$_2$ expression and the decrease in the levels of tumor necrosis factor alpha (TNF-α), interleukin (IL)-1, IL-6, nitric oxide (NO) and oxygen reactive species (ROS) are suggested to be associated with the anti-arthritis effect of melittin (Murakami et al., 1997; Pelletier et al., 1998; Yang et al., 1999; Amin et al., 1999; Cernanec et al., 2002). Adolapin also has anti-inflammatory activity in carrageenan-, prostaglandin (PG)-, and adjuvant-induced rat hind paw edema and adjuvant polyarthritis. The effects of adolapin are presumably due to its ability to inhibit the PG synthesis system through COX inhibitory properties (Shkenderov & Koburova, 1982; Koburova et al., 1985). Apamin, a small conductance Ca^{2+}-activated K^{+} channel blocker, significantly inhibited both ovalumine-induced tracheal contraction and histamine release from lung tissues, suggesting that this compound reduces allergic airway inflammation through a mast cell stabilizing effect (Ichinose et al., 1995). The mast-cell-degranulating (MCD) peptide has an anti-allergic activity by inhibiting the release of histamine from mast cells (Buku, 1999). The MCD peptide binds to the mast cell receptors in a dose–response manner and has been found to partially inhibit the binding of IgE to this receptor (Buku et al., 2001).

Recently, it was found that melittin inhibited the DNA-binding activity of NF-κB, a critical transcriptional factor regulating inflammatory gene expression, by inhibiting IκB phosphorylation (Park et al., 2004, 2007). This result may be critical to understanding the anti-inflammatory and anti-arthritis mechanisms of BV and melittin, its major constituent.

There is increasing evidence suggesting that BV has anti-nociceptive effects on the thermal, visceral, and inflammatory pain responses. In this regard, BV has been used traditionally to relieve pain and treat chronic pain diseases. BV contains several bioamines, such as apamin, histamine, procamine, serotonin, and norepinephrine, which facilitate nerve transmission and healing in a variety of nerve disorders. This gives BV the ability to travel along the neural pathways from the spine to various trigger points and injured areas to help repair nerve damage and restore mobility (Banks et al., 1979; Shuba & Vladimirova, 1980). Acupoint stimulation into the subcutaneous region (acupuncture) rather than other injection sites may be important for the anti-nociceptive effects of BV. Subcutaneous apipuncture therapy of BV (apipuncture) reduces the visceral nociceptive effects (Kwon et al., 2001a, 2005). This BV treatment also reduces mechanical and thermal hyperplasia (Kwon et al., 2001b; Lee et al., 2001), formalin-induced pain behavior (Kim et al., 2003, 2005; Roh et al., 2006) and collagen-induced arthritic pain (Baek et al., 2006) as well as knee osteoarthritis (OA)-related pain (Kwon et al., 2001c). Multiple mechanisms have been suggested, such as activation of the central and spinal opiod receptor and α_2-adrenergic receptor as well as activation of the descending serotonergic pathway (Kwon et al., 2001a, 2005; Baek et al., 2006). The inhibition of c-Fos expression in the spinal cord by BV apipuncture in several nociceptive models was also reported to be a possible mechanism (Kwon et al., 2001b; Kim et al., 2003).

BV also has anti-cancer activity. Venom from a variety of animals including bees (Liu et al., 2002; Hu et al., 2006c; Moon et al., 2006; Putz et al., 2006), snakes (Feofanov et al., 2005; Yang et al., 2005; Son et al., 2007), spiders (Van Den Berg et al., 2002; Gao et al., 2005; Nagaraju et al., 2006), scorpions (Soroceanu et al., 1998; Wang & Ji, 2005), sea urchins (Borkow et al., 1992),

Table 1
Components of bee venom and their major characteristics

Components	MW	Contents (% dry BV)	Major characteristics
Peptides			
Melittin	2840	40–50	26 amino acid Enhance of PLA$_2$ activity Cytotoxic effects against cancer cells Anti-inflammatory and anti-arthritic effects
Apamin	2036	2–3	10 amino acid Inhibition of Ca^{2+}-activated K$^+$ channel Cytotoxic effect against cancer Nociceptive effect Anti-inflammatory properties
MCD peptide	2588	2–3	22 amino acid Anti-inflammatory and analgenic effect Histamine release (low dose) Histamine release inhibition (high dose) Anti-allergic effect
Adolapin	11,500	1	Inhibition of PLA$_2$ and COX activity Anti-inflammatory activity Analgesic effect
Protease inhibitor	9000	<0.8	
Minimine	6000	2–3	
Procamine A, B		1.4	
Secarpin		0.5	
Tertiapin		0.1	
Melittin F		0.01	
Cardiopep		<0.7	
Enzymes			
PLA$_2$	19,000	10–12	Cytotoxic effects against cancer cells Inflammatory effects Anti-tumor effects
Hyaluronidase	38,000	1.5–2	Selectively attacks tissue hyaluronic acid polymers Increase the capillary permeability Immune response and tissue-spread properties Antigenic
Glucosidase	170,000	0.6	
Acid phosphomono-esterase	55,000	1	
Amines			
Histamines	307.14	1.5	
Dopamine	189.64	0.13–1	
Norepinephrine	169.18	0.1–0.7	
Others			
Carbohydrates	307.14	1.5	
r-Aminobutyric acid	189.64	0.13–1	
B-Aminoisobutyric acid	169.18	0.1–0.7	

and sea coral (Schweitz et al., 1985; Meunier et al., 2000) has the capacity to kill cancer cells. The promise of this remedy exists with live honeybees, which make tumors disappear by killing cancer cells (Liu et al., 2002). The cytotoxic effect through the activation of PLA$_2$ by melittin is believed to be an important mechanism of anti-cancer activity of BV. Several cancer cells, including renal, lung, liver, prostate, bladder, and mammary cancer cells as well as leukemia cells, can be targets of melittin. The induction of apoptotic cell death through several cancer cell death mechanisms, including the activation of caspase and matrix metalloproteinases (MMP), is important for the melittin-induced anti-cancer effects (Holle et al., 2003; Moon et al., 2006). The binding of the cell lytic peptide (melittin) to the hormone receptors as well as gene therapy carrying melittin can be useful as a novel targeted treatment for some types of cancer, such as prostate and breast cancers (Li et al., 2004; Russell et al., 2004; Li et al., 2006; Hu et al., 2006a, 2006b, 2006c). Pharmaceutical companies are currently funding extensive research into the potential of venom as the next generation of cancer fighting drugs. The aim of this study is to review the data from a variety of experimental and clinical reports and describe the effectiveness of BV and possible mechanisms for how BV and/or its constituents relieve or prevent arthritis, pain, and cancer.

2. Components of bee venom

BV contains a variety of peptides including melittin, apamin, adolapin, and the MCD peptide. It also contains enzymes (e.g., PLA$_2$), biologically active amines (e.g., histamine and epinephrine) and nonpeptide components (including lipids, carbohydrates and free amino acids; Lariviere & Melzack, 1996; Table 1). Melittin is a small protein containing 26 amino acid residues and is the principal toxin in BV. The sequence of melittin is Gly-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Trp-Ile-Lys-Arg-Lys-Arg-Gln-Gln (Gevod & Birdi, 1984). Although it is water-soluble as a monomer or as a tetramer (Brown et al., 1980), this polypeptide readily integrates into and disrupts both natural and synthetic phospholipid bilayers (Lauterwein et al., 1979; Lavialle et al., 1980). Melittin also enhances the activity of PLA$_2$ (Shier, 1979) and has a variety of effects on living cells possibly through the disruption of this membrane (Lad & Shier, 1979). Fig. 1 shows the 3-dimensional structure of tetrameric melittin, as determined by X-ray crystallographic analysis at a 2-Å resolution. Each melittin chain contains 2 a-helical segments and its overall shape is that of a bent rod. Melittin is tetrameric at the concentrations prevailing in the venom sac of the bee and monomeric at the minimum concentrations needed for cell lysis (Terwilliger & Eisenberg, 1982). These structural characteristics may play an important role in the cytotoxic effects of melittin on cancer cells, as well as its possible anti-inflammatory effects.

Apamin is the smallest neurotoxin in BV and consists of 10 amino acids containing 2 disulfide bridges (Strong, 1990). Apamin has long been known as a highly selective inhibitor of the Ca^{2+}-activated K$^+$ channels (Banks et al., 1979). Current-clamp and voltage-clamp experiments have shown that apamin,
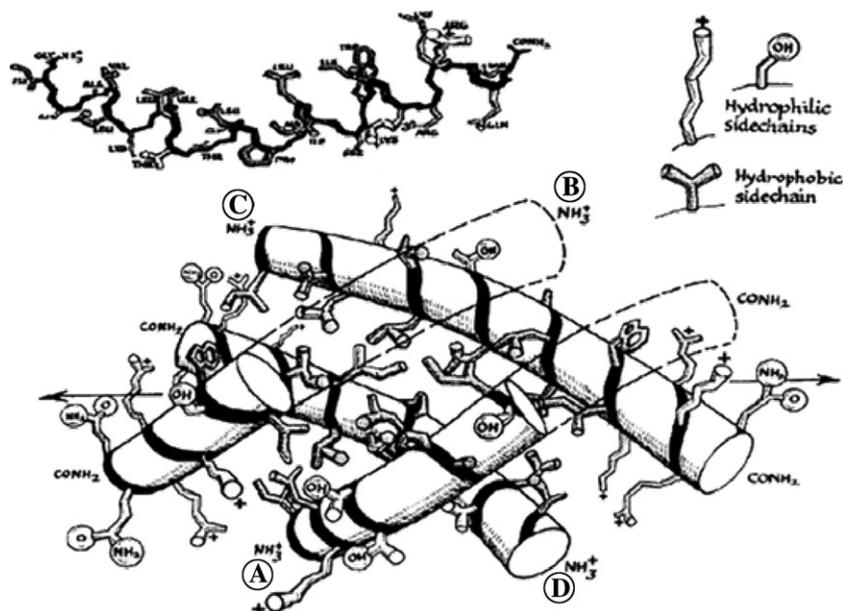
Fig. 1. The packing of melittin chains in the tetramer. Chain A is the chain built into the electron density map. Chain B is associated with chain A through an ∼2-fold axis approximately perpendicular to this page. Chains C and D are associated with chains A and B through a precise 2-fold axis almost in the plane of the page, as indicated (adopted with permission from Terwilliger & Eisenberg, 1982).

when applied externally, blocks the Ca^{2+}-dependent slow K^+ conductance specifically at low concentrations (0.1 μM) and mediates the long-lasting after-hyperpolarization in neuroblastoma cells and rat muscle cells in culture. There is an all-or-nothing control of the expression of the apamin-sensitive Ca^{2+}-dependent K^+ channel through innervation in the mammalian skeletal muscle. The rat brain contains an endogenous equivalent of apamin (Lazdunski et al., 1985). When administered, apamin crosses the blood–brain barrier and induces hyperexcitability (Habermann, 1972). The cloning of the small conductance Ca^{2+}-dependent K^+ channels (SK; Kohler et al., 1996; Shah & Haylett, 2000) has allowed molecular analysis of the apamin–receptor interaction and shown the importance of the pore region (Ishii et al., 1997). A recent study reported that a single amino acid located in the extracellular loop between the transmembrane segments S3 and S4 has a major impact on the sensitivity to apamin. This suggests that the human SK1 channel can be converted into a channel that is as sensitive to apamin as SK2, which is the SK channel with the highest sensitivity (Nolting et al., 2007). Apamin inhibits NO-induced relaxation of the spontaneous contractile activity of the myometrium in non-pregnant women (Modzelewska et al., 2003). These structural and pharmacological characteristics of apamin might play an important role in its cytotoxic effects on cancer cells and might also be related to its nociceptive activity.

Shkenderov and Koburova (1982) isolated another peptide in BV, known as adolapin, and reported it to have anti-inflammatory, analgesic, and COX inhibitory properties. The molecular masses of the polypeptide, as determined by SDS electrophoresis and amino acid composition, were reported to be with molecular weights of 11,500 and 11,092, respectively. It was found that adolapin also inhibits the activity of PLA_2 in BV. In addition, it inhibits the lipoxygenase from human platelets. Adolapin

increases the cyclic guanosine monophosphate (c-GMP) level in both the rat spleen and brain and decreases the cyclic adenosine monophosphate (c-AMP) level in the rat spleen. Similar to other non-steroid analgesics, adolapin has an antipyretic effect. In addition, adolapin has been shown to have an analgesic effect, which might be important for those who suffer pain as one of the symptoms. The analgesic action of adolapin has been tested using the "tail flick" method. The partial inhibition of the analgesic effect of adolapin induced by naloxone demonstrated the participation of a central mechanism of action (Koburova et al., 1985).

MCD peptide is a 22-amino acid bicyclic peptide that contains 2 disulfide bridges between $Cys^{3,15}$ and $Cys^{5,19}$. The sequence of the MCD peptide is as follows: lle-Lys-Cys-Asn-Cys-Lys-Arg-His-Val-lle-Lys-Pro-His-lle-Cys-Arg-Lys-lle-Cys-Gly-Lys-Asn (Buku et al., 2004). The MCD peptide has activities associated with allergies, which affects almost 20% of the population (Sutton & Gould, 1993). This peptide has intriguing biological properties. It releases histamine at very low concentrations (Habermann, 1972) and is one of the most potent natural histamine secretagogues (Mousli et al., 1990). On the other hand, this peptide has been found to inhibit mast cell degranulation at concentrations higher than those that would induce the release of histamine (Billingham et al., 1973; Hanson et al., 1974). This might be due to its interaction with the IgE molecule, which is associated with allergic reactions. It was suggested that disulfide exchange between IgE and the MCD peptide at high doses on the mast cell surface might inhibit the release of histamine, which would allow the MCD peptide to act as an anti-allergic agent (Buku, 1999). The MCD peptide binds to mast cell receptors in a dose–response manner and has been found to partially inhibit the binding of IgE to this receptor (Buku et al., 2001).

3. Anti-arthrits effect of bee venom

3.1. Anti-inflammatory effect of bee venom

Rheumatoid arthritis (RA) is a common and severe disease with an ~0.5% prevalence in adults. It not only causes joint pain and severe disability but can also predispose an individual to an early death. RA is an inflammatory autoimmune disease whose the inciting stimulus is unknown. However, the cascade of immunological and inflammatory reactions has been determined. These reactions produce inflammatory synovitis that are promptly followed by irreversible joint and bone destruction (Bessis et al., 2002). OA is also the most common form of arthritis, which is known as "degenerative arthritis" or "degenerative arthritic disease." OA mainly occurs in middle-aged or elderly people. It attacks the weight-bearing joints, mainly the knee joint, and causes local degenerative changes in the cartilage, hypertrophy of the subchondral bones, excessive bone formation around the osteochondral regions, and a deformity of the joints accompanied by an inflammatory reaction. In RA, fibroblast-like synoviocytes, macrophages, T cells, B cells, plasma cells, neutrophils, mast cells, dendritic cells, and natural killer cells infiltrate the synovial tissue (Tak & Bresnihan, 2000). It is unknown how the inflammatory process is initiated because the inflamed synovium already has the histopathological features of chronic disease when symptoms are first noticed (Tak, 2001). However, the recruitment of inflammatory cells, local retention and cell proliferation contribute to the increased cellularity of the rheumatoid synovium in the clinically manifest stage of the disease. Therefore, the increased cellularity is partially dependent on a variety of immunological factors that enhance the influx of inflammatory cells into the synovium as well as the retention of cells (Buckley, 2003). Among the complex mechanisms that lead to inflammatory reactions, inflammatory mediators, such as NO and PG, which act on specific receptors located on the cell surface, might be involved in the inflammatory reactions (Cuzzocrea et al., 2002). Pro-inflammatory cytokines, such as IL-1 and TNF, activate the inducible NO synthase (iNOS) pathway in bone cells, and NO derived from this pathway potentiates the cytokine- and inflammation-induced bone loss. Elevated levels of PGs, which are involved in the complex interactions leading to the erosion of the articular cartilage and juxta-articular bone, have been also found in synovial cells treated with inflammatory mediators or in patients with RA (Williams & Shacter, 1997; Trebino et al., 2003). PLA_2 also plays an important role in different pathophysiological states of severe inflammatory diseases including RA (Green et al., 1991; Bomalaski et al., 1989, 1995). Therefore, useful agents that inhibit COX-2 as well as iNOS have potential as anti-inflammatory drugs and possibly as anti-arthritic drugs.

3.2. Bee venom therapy for arthritis

Western medical treatment of knee arthritis employs conservative methods such as medication, rest and exercise, and physical therapy, as well as surgical methods, such as arthroscopic debridement, osteomy, arthroscopic surgery, and total knee arthroplasty. Patients with mild symptoms are generally treated with physical therapy and non-narcotic medication. If the symptoms do not improve, a prescription of non-steroidal drugs can be considered if the patients have no contraindications for the drug. Steroids are administered to patients with exudative knee arthritis. These treatments have certain adverse effects, which highlights the need for safer and more effective treatments (Baumrucker, 2002; Zochling et al., 2004). BVT might be an alternative method. The anti-arthritis effects of BVT have been demonstrated using various animal arthritic models, such as adjuvant, carrageenan, or lipopolysaccharide (LPS)-induced arthritis. Chang and Bliven (1979) first reported that BV, when administered subcutaneously, suppressed the development of carrageenan-induced paw edema and adjuvant arthritis in rats in a dose-related manner. A single dose of BV administered subcutaneously on the day before or on the day of the injection of complete Freund's adjuvant (CFA) effectively suppressed the development of polyarthritis. This suppressive effect progressively decreased with increasing delay in administration. BV was reported to be most effective when mixed and injected (sub-plantar) with CFA, which is the disease-inducing agent. Similarly, antigens, such as egg albumin, prevented the development of arthritis when mixed with CFA and injected into the hind paw. This suggests that the mechanisms in the anti-arthritic action of BV involve a change in the immune response, possibly through antigen competition and anti-inflammatory activity through corticosteroids or by a yet undetermined mechanism.

Adolapin was isolated from BV (Shkenderov & Koburova, 1982), and shown to have potent analgesic activity, as demonstrated by the "writhing" test and by the Randall-Sellito's test. It was reported that adolapin has anti-inflammatory activity in a carrageenan-, PG-, and adjuvant-induced rat hind paw edema and adjuvant polyarthritis. The arthritis effect of adolapin is presumably due to its ability to inhibit the PG synthesis system. In the same year (1982), BV was also reported to suppress *Mycobacterium butyricum*-induced arthritis in Lewis rats. BV, administered at 2 mg/kg/day for 24 days, suppressed the primary and secondary inflammatory responses to the adjuvant but did not abolish them, as determined by the decrease in the swelling of the left and right hind paws and adjuvant-induced arthritis (Eiseman et al., 1982). This study found that although BV appears to suppress adjuvant-induced arthritis to a greater extent in female rats than in male rats, the alterations in the heme metabolism were similar in both genders. The observed changes in the heme metabolism elicited by BV or the adjuvant strongly suggest that perturbations of the immune system cause changes in the hepatic microsomal enzymes. Somerfield et al. (1986) reported that BV potently inhibits the human neutrophil production of superoxide (O_2^-) and hydrogen peroxide in a nontoxic and dose-dependent manner. This group also found that melittin, the major fraction of BV (50–70%) shows high-affinity calmodulin binding (Kd = 3 nM). Because binding to calmodulin inhibits the production of O_2^- in human neutrophils, Somerfield et al. (1986) also examined the effect of melittin and other BV peptides on the production of O_2^- in human peripheral blood leukocytes. They

reported that melittin inhibits the production of O_2^- both pre- and poststimulation in contrast to other BV fractions, which had no effect. Oxygen radicals and their derivatives from inflammatory cells have been implicated in the tissue damage that occurs during inflammation. They further demonstrated that this inhibition is the result of a direct effect on cells and suggested that melittin may serve as a prototype small (mol. wt., 1280), cationic, amphipathic, calmodulin-binding, membrane-active, superoxide-production-inhibiting peptide to provide a model peptide that might play a role in the in vivo regulation of radical production in the process of diseases including arthritis.

Ten years after the report of 2 in vivo studies by Shkenderov and Koburova (1982) and Eiseman et al. (1982), the effectiveness of BV in an adjuvant arthritic animal model was also shown. A BV (0.5 mg/kg, s.c.) injection in rats resulted in the significant suppression of adjuvant arthritis and the suppression of the hepatic acute-phase induction of the alpha$_1$-acid glycoprotein (AGP) gene at the early stages of disease development (Yiangou et al., 1993). The administration of the AGP to adjuvant arthritis rats accelerated the development of arthritis and increased the severity and duration of the disease. IL-1, IL-6, TNF-α, and glucocorticoids alone are not responsible for the HBV-mediated down-regulation of the AGP gene. This indicates that several factors are involved in AGP gene expression in adjuvant arthritis and HBV-treated adjuvant arthritis rats, and that the suppression of AGP by BV might help suppress the development of adjuvant arthritis. The BV pretreatment on carrageenan- and adjuvant-induced acute paw edema was analyzed quantitatively in normal animals (Lee et al., 2001, 2005). A pretreatment with BV before a carrageenan injection into the hindlimb suppressed both paw edema and thermal hyperalgesia. There is a positive correlation between the percentage change in paw volume and the expression of Fos-positive neurons in the spinal cord. This result suggests that the BV pretreatment has both anti-nociceptive and anti-inflammatory effects in carrageenan-induced inflammatory pain. A similar effect was observed in an adjuvant-induced arthritic rat (Kang et al., 2002). The administration of BV every other day for another 14 days suppressed the development of inflammatory edema and polyarthritis. The erosion of articular cartilage and inflammatory cell infiltration into the interphalangeal joint were also effectively suppressed in the treated groups. The same research group also reported that the subcutaneous administration of BV 1 day before injecting CFA effectively inhibits the inflammatory edema, polyarthritis, and bone changes in the right hind paw. BV inhibits the arthritic inflammation and bone changes in rats. BV suppresses the erosion of articular cartilage and inflammatory cell infiltrations into the interphalangeal joint. These inhibitory and suppressive effects are comparable to those induced by prednisolone (10 mg/kg, s.c.). Luo et al. (2006) reported that a BV-treatment injected hypodermically into rats for 14 days results in less swelling in the joints, a reduced circumference of the joints and lower joint scores. At the same time, the level of bone erosion and the infiltration of inflammatory cells in the synovium are also significantly reduced by the BV treatment. These results suggest that BV is effective in treating adjuvant-induced arthritis by reducing the level of synovitis, down-regulating the serum concentrations of cytokine TNF-α and IL-1β, and alleviating the bone erosion.

3.3. Possible mechanisms of anti-arthritis effect of bee venom

Nam et al. (2003) reported that the water fraction of BV, less than 20 kDa, might contain a major effective component. They compared the anti-inflammatory activity of the *n*-hexane, ethyl acetate, and aqueous partitions of BV (*Apis mellifera*) by measuring the in vitro COX activity and level of pro-inflammatory cytokine production (TNF-α and IL-1β). The aqueous partition of BV had strong inhibitory effects on COX-2 activity but did not inhibit the COX-1 activity. The aqueous partition was further subfractionated into 3 parts according to the molecular weight, namely BV fraction 1 (above 20 kDa), BV fraction 2 (between 10 and 20 kDa) and BV fraction 3 (below 10 kDa). BV fractions 2 and 3 strongly inhibited the COX-2 activity and COX-2 mRNA expression in a dose-dependent manner, without having any cytotoxic effects. All 3 subfractions inhibited the production of TNF-α and IL-1β. This study first suggested the pharmacological activities of BV on the anti-inflammatory process include the inhibition of COX-2 expression and the blocking of pro-inflammatory cytokine (TNF-α, and IL-1β) production, and particular fraction may contain an active constituent that inhibits the inflammatory reaction.

The decrease in COX-2 and PLA$_2$ expression, and the decrease in the levels of TNF-α, IL-1, IL-6, NO and ROS were reported be associated with the anti-arthritis effect of BV, suggesting that the anti-arthritis effect of BV might be related to its anti-inflammatory effects. In order to gain better insight into the action mechanisms of BV, our group reevaluated the anti-arthritis effect of BV using arthritis animal models, and examined the anti-arthritis mechanisms for the effect of BV and melittin in a murine macrophage cell line, Raw 264.7 cells, as well as in synoviocytes obtained from RA patients. Similar to other findings (Murakami et al., 1997; Pelletier et al., 1998; Amin et al., 1999; Yang et al., 1999; Cernanec et al., 2002), we also found that BV had an anti-arthritis effect (Park et al., 2004). A BV treatment decreased the level of tissue swelling and osteophyte formation in a chronic arthritis model as well as edema formation in an acute model (Park et al., 2004). However, the dose (μg/kg) used in their study was much lower than the dose (0.1–0.5 mg/kg) used in other studies (Pelletier et al., 1998). The different source, extraction or purification methods of BV and composition, as well as the different animal models or other unknown factors might be responsible for this discrepancy. These results confirmed that the anti-arthritis effects of BV are associated with its anti-inflammatory effect. In a further mechanism study, our group identified the anti-inflammatory properties of BV and its molecular action mechanism. BV at 0.5–5 μg/mL, which is not cytotoxic, inhibited the LPS-induced production of PGE$_2$ and NO in Raw 264.7 cells. BV was also effective in inhibiting the generation of inflammatory mediators in the synoviocytes obtained from RA patients. The inhibitory effect of BV was comparable to indomethacin, a well-known COX-2 inhibitor. Therefore, the inhibitory effect of BV on the

inflammatory reaction in adjuvant-induced arthritis might be partly due to the inhibition of COX-2 expression. In inflammatory arthritis, there is evidence suggesting that the affected tissues produce a large amount of NO that can behave as a pro-inflammatory to cause tissue injury (Dennis et al., 1992; Tischfield, 1997). In this regard, our group also showed that BV decreases the level of NO and TNF-α generation, which is involved in the influx of inflammatory cells, the erosion of joint cartilage and bone destruction in the inflammatory arthritis (Green et al., 1991; Hoffmann et al., 1992). It was also found that BV has an inhibitory effect on the LPS-induced generation of ROS and the release of calcium. Therefore, the anti-arthritic effect of BV may be related to its multiple inhibitory effects on the generation of inflammatory mediators, such as PGE_2, NO, and TNF-α as well as on the release of ROS and intracellular calcium (Kortesuo et al., 1992). In more precise mechanistic studies, our group showed that BV inhibits the DNA binding and transcriptional activity of NF-κB in Raw 264.7 cells, synoviocytes, and THP-1 cells, which are a monocyte cell line, in a dose-dependent manner (Park et al., 2004). The promoter region of the murine gene encoding iNOS and COX-2 contains NF-κB binding sites (Spiecker et al., 2000). This inhibitory effect is consistent with the decrease in the release of IκB detected in the cytosol through the suppression of IκB phosphorylation and the decrease in the translocation of the p50 subunit of NF-κB. This result suggests that either BV or melittin inhibits the DNA-binding activity of NF-κB by inhibiting IκB phosphorylation, thereby preventing p50 translocation, resulting in a decrease in the expression of the inflammatory gene. The direct interaction of BV (by measurement with melittin) with the p50 and IκB kinase (IKKα and IKKβ), which are upstream molecules regulating NF-κB, were Kd = 4.6×10^{-6} M, 1.34×10^{-9} M, and 1.01×10^{-9} M, respectively, as evidenced by surface plasmon resonance analysis. Therefore, this strong protein–protein interaction is likely to modify the activities of IKKα and IKKβ and inhibit the release of IκBα and IκBβ (or p50 translocation) and decrease the DNA binding activity of NF-κB. Fig. 2 shows the possible preventive mechanism of BV (melittin) in the inflammatory reaction. It was reported that synthetic melittin inhibits the enzymatic activity of secretory PLA_2 ($sPLA_2$) from the synovial fluid obtained from RA patients by binding to PLA_2. This indicates that the disruption of the key inflammatory enzymes might be a potential therapeutic target of BV or the components of BV and that the down regulation of the anti-inflammatory genes is important (Saini et al., 1997). In this regard, the study reported by Yin et al. (2005) is indicative. Yin et al. (2005) performed microarray analysis to determine the global gene expression profiles in a human chondrocyte-like cell line treated with BV. Human chondrosarcoma cells, HTB-94, were treated with BV, LPS, or both. Of the 344 genes profiled in their study, with a cut-off of a 4-fold change in expression: (1) 35 were down-regulated after the BV treatment; (2) 16 were up-regulated and 7 were down-regulated after the LPS treatment; and (3) 32 were down-regulated after co-stimulation with BV and LPS. This study showed that a BV treatment reversed the LPS-induced up-regulation of genes such as IL-6 receptor, MMP-15, TNF-α (ligand) superfamily-10,

caspase-6, and tissue inhibitor of metalloproteinase-1 (TIMP-1), which are mostly involved in inflammation. This suggests that the altered inflammation regulatory genes will play an increasingly important role in advancing our understanding of the pharmacologic actions of BV in the treatment of arthritis.

Although the anti-inflammatory effect of BV is the most implicated mechanism in the effect of BV on arthritis, other mechanisms are also noteworthy. Hong et al. (2005) examined the inhibition of cell growth and the induction of apoptosis in human rheumatoid synovial fibroblasts because RA is an autoimmune disease that is characterized by synovial proliferation, infiltration of the synovial lining by lymphocytes and macrophages, and a paucity of apoptosis (Veis et al., 1993; Firestein, 1996). In RA, the fibroblasts that reside in the synovial lining significantly increase in number, display a transformed phenotype, and destroy the adjacent cartilage and bone. It was demonstrated that rheumatoid synovial cells treated with BV for 24 hr exhibited apoptotic features. In addition, BV-induced apoptosis in rheumatoid synovial cells by decreasing the expression of B-cell leukaemia/lymphoma-2 (BCL_2) and increasing the expression of BCL_2-associated X protein (BAX) and caspase-3. This suggests that BV inhibits the proliferation of rheumatoid synovial cells by inducing apoptosis through the activation of caspase-3. Moreover, the induction of synovial fibroblast apoptosis might contribute to the synovial hyperplasia that is associated with RA.

3.4. Effectiveness of bee venom in acupuncture

In BVT, the method of injecting BV might be important for improving its effectiveness. In this point, it is important to understand the effectiveness of BV acupuncture (BVA or apipuncture) (Lee et al., 2005a). BVA is growing in practice as a type of herbal acupuncture and is used primarily to relieve the pain of inflammatory diseases. Many animal studies have shown that BVA can induce anti-inflammatory activity (Doh et al., 1995; Lee & Kim, 1999; Yim et al., 2000; Do et al., 2001; Kwon et al., 2001d; Lee et al., 2001; Choi et al., 2002; Kim et al., 2002, 2003; Seo et al., 2003). Several studies suggest that the effects of BV are intensified by acupuncture stimulation, which may help achieve the therapeutic goals. Research and a review by Dr. Lee's group have provided evidence for BVA relieving the symptoms of RA and OA. Comparative studies of the acupoint versus non-acupoint stimulation on an adjuvant induced RA animal model with BVA were carried out to examine the potential site specificity (Kwon et al., 2001b, 2002; Seo et al., 2003; Kim et al., 2003). A direct injection of BV into acupoint ST36 (known as Zusanli) in an animal model of chronic arthritis produced a potent anti-nociceptive effect compared with an injection in a non-acupoint, which suggests that this alternative form of acupoint stimulation using BV can be applied to pain relief. The effectiveness of BVA at acupoints ST36 and BL23 was demonstrated in a type II collagen-induced rat arthritis model, which also showed that a decrease in the proteolytic enzyme activities and level of ROS-induced oxidative damage to the synovial fluid. In this model, BVA at acupoint BL23 more significantly decreased the proteolytic enzyme activities and the
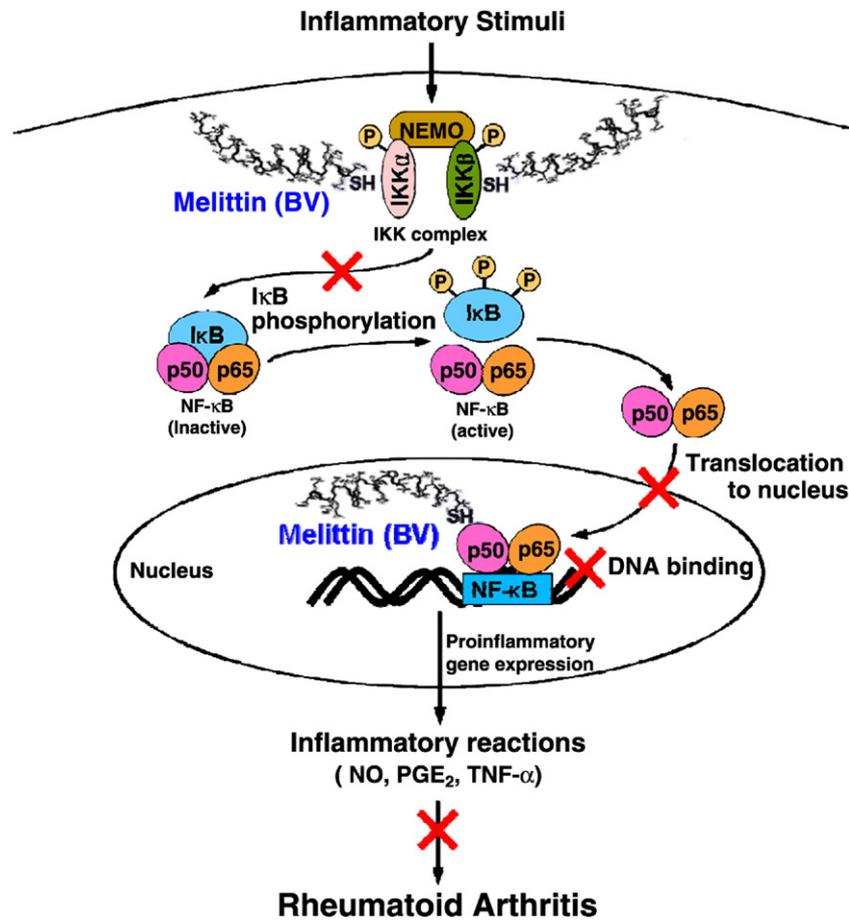
Fig. 2. Proposed anti-arthritic effect of BV (melittin). NF-κB activity is stimulated by many inflammatory stimuli. The IKK complex, which consists of the kinases IKKα and IKKβ and the regulatory subunit NEMO (also known as IKKγ), is a point of convergence for all 3 signaling pathways. The IKK complexes phosphorylate IκBα, which leads to its degradation and allows the NF-κB dimers to enter the nucleus, where they bind to the cognate DNA binding sites and activate the expression of the proinflammatory gene. Proposed anti-arthritic mechanism of BV (melittin) through its anti-inflammatory effects. BV (melittin) inhibits the release of IκB through the inhibition of IKKs. This inhibition might be due to an interaction between the sulfhydryl (SH) group of IKKα and IKKβ with BV (melittin) molecule, which results in NF-κB inactivation, and thus reduces the generation of inflammatory mediators. BV (melittin) may also interact directly with p50 of NF-κB and thereby inhibit the translocation of p50 into the nucleus. P, phosphorus; Ub, ubiquitin; NF-κB, nuclear factor-κB; IκB, inhibitor of NF-κB; IKK, IκB kinase; NEMO, NF-κB essential modulator.

level of ROS-induced oxidative damage to the synovial fluid proteins (Kim et al., 2002) compared with that from other treatment methods (Doh et al., 1995; Kim et al., 2002; Choi et al., 2002). In addition, BVA at acupoint BL23 showed more significant anti-inflammatory effectiveness on knee arthritis induced by carrageenan in rats. Lee et al. (2001) reported that BV administered at acupoint ST36 prior to injecting carrageenan inhibited the carrageenan-induced edema and paw size, demonstrating a correlation between the change in the rates of edema and the expression of Fos-positive neurons in the spinal cord. BVA at acupoint GB34 in LPS-induced arthritis significantly decreased the number of white blood cells, the infiltration of leukocytes and fibroblasts into synovial joints and the levels of CD56, IL-1, IL-2R, CD54 and CD106 in the synovial membrane when compared with the control (Lee & Kim, 1999; Do et al., 2001). Compared with an arbitrary non-acupoint located on the back, BVA at acupoint ST36 in adults Sprague-Dawley rats significantly decreased the paw-licking time in the late phase of the formalin test and markedly inhibited the

expression of Fos in the spinal cord induced by a formalin injection (Yim et al., 2000; Kim et al., 2003). This suggests that the effects of BVA depend on the locations of the injection in that an injection in the acupoints has much stronger effects than an injection in the non-acupoints. Lee et al. (2004) also reported that BVA at acupuncture point (Zusanli) near both knees twice a week for a total of 5 times on a murine type-II collagen-induced arthritis (CIA) model significantly decreased the incidence of arthritis, the mean arthritis index and the number of arthritic limbs compared with a control group. In this study, among the serum proinflammatory cytokines, the production of TNF-α in the BV group was suppressed compared with the control group. An examination of the histopathology of the joints of murine type II CIA showed decreased inflammatory signs and less lymphocyte infiltration after the BVA therapy. Using the rat CIA model, Baek et al. (2006) demonstrated that BVA injected into the Zusanli acupoint (ST36) had an anti-nociceptive effect. Furthermore, the anti-nociceptive effect of BVA was blocked by a pretreatment with yohimbine (an α_2-adrenergic receptor

antagonist, 2 mg/kg, i.p.), but not by naloxone (a mu-opioid receptor antagonist). This suggests that BVA can relieve the inflammatory pain associated with CIA and the anti-nociceptive effect of BVA might be mediated by the α_2-adrenergic receptor.

Suh et al. (2006) also reported the effectiveness of BVA and the possible mechanisms of action on the development of CIA in rats. The BVA treatment significantly decreased the activity of a comprehensive range of cytoplasmic, lysosomal and matrix protease types, along with the levels of free radical-induced protein damage (determined a protein carbonyl derivative) in the synovial fluid obtained from collagen-induced rats compared with the normal rats. The level of free radical-induced damage to the synovial fluid proteins was ∼3 times higher in CIA than in normal rats. However, BVA decreased the level of oxidative damage to the synovial fluid proteins caused by ROS. They concluded that the activation of proteolytic enzymes and free radicals are likely to be of equal importance as protein damaging agents in the pathogenesis of RA. Moreover, the development of novel therapeutic strategies for the latter disorder should include both protease inhibitory and free radical scavenging elements. In addition, they further demonstrated that a protease inhibitory element is needed to inhibit the action of a broad range of enzymatic mechanistic types (cysteine, serine, metallo proteases and peptidases), and BVA might be an effective RA modulator, inhibiting the protease activities and removing ROS.

3.5. Effect of bee venom on arthritis patients in clinical trials

One randomized controlled and two uncontrolled clinical trials examined the effect of BV on RA. Ten patients who were diagnosed with RA and met the ACR (American College of Rheumatology) 1987 revised criteria for a diagnosis of RA, received BVA therapy twice a week for 3 months. The study showed remarkable improvement in 2 patients, good improvement in 5 cases, and effective improvement in 2 cases (Kwon, 1998). The tender joint counts, swollen joint counts and the duration of morning stiffness was significantly lower in the patients after BVA therapy than before. Lee et al. (2003) also performed a randomized controlled trial to evaluate the effects of BVA in RA patients. Each group was treated with either BVA or a normal saline injection on the acupoints twice a week for 8 weeks. The tender joint count, swollen joint count, morning stiffness, pain, health assessment questionnaire, erythrocyte sedimentation rate, and C-reactive protein level were significantly lower in those given BVA therapy over a 1- or 2-month period compared with the control group. One randomized controlled trial and one uncontrolled trial examined the effect of BVA on OA. Of 70 knee arthritis patients treated with BVA, excellent improvement was observed in 11 cases (15.7%), good in 31 cases (44.3%), and improved in 16 cases (22.9%) (Wang et al., 2001; Kwon et al., 2001c). They also examined whether or not the direct administration of BV into an acupoint is a clinically effective and safe method for relieving the pain of patients with OA of the knee compared with traditional needle acupuncture. Four weeks of the BVA treatment produced substantial pain relief compared with the group receiving the

traditional needle acupuncture and the infrared themography score correlated with the level of pain relief. Wang et al. (2001) and Kwon et al. (2001c) also demonstrated that the majority (82.5%) of subjects receiving BVA reported substantial pain relief compared with those who received the traditional acupuncture therapy. The therapeutic efficacy was favorable irrespective of the disease duration (acute, subacute, or chronic stage), arthritic type (unilateral or bilateral knee OA and radiological severity). However, rigorous trials with a large sample size and adequate design, as well as an optimized dosage and BV concentration, will be needed to define the role of BV in the treatment of arthritis.

4. Pain release effect of bee venom

4.1. Nociceptive effect of bee venom

Cutaneous tissue injury often causes persistent spontaneous pain, hyperalgesia and allodynia (pathological pain). Hyperalgesia is characterized by a lower pain threshold and increased pain sensitivity to mechanical and heat stimuli that are normally painful. On the other hand, allodynia is characterized by an abnormal pain sensitivity to mechanical stimuli that does not normally provoke pain. Reorganization of the spinal neuronal systems is known to occur as a result of a peripheral injury. Several mechanisms or the involvement of receptors have been reported. Neuropathic and inflammatory pain induces neuronal plasticity through the N-methyl-D-aspartate (NMDA) receptors. Therefore, antagonists of the glutamate receptors subclass $NMDA_1$ receptor prevent plastic alterations in the nervous system. Tissue injury and inflammation induce the prolonged activation of the excitatory amino acid systems followed by the excessive activation of an intracellular cascade of secondary messengers, protein phosphorylation, and the activation of transcription factors and gene expression. Excitatory amino acids increase the level of Ca^{2+} influx, protein kinase C (PKC) translocation and enhance the production of NO. Increased spinal c-GMP levels occur in parallel to the thermal and mechanical hyperalgesia and tactile allodynia caused by a chronic constriction injury of the sciatic nerve. The mechanisms responsible for the hyperalgesia in chronic pain might involve not only NO itself but also peroxynitrite, which is the product of a reaction between NO and the superoxide radical O_2^-, that can lead to the formation of the free radicals OH and NO_2^- (Tal, 1996). The recognition of persistent pain as a disease entity should encourage changes in the overall approach to its management. Although it is important to treat the underlying event that initiates the pain, it is also necessary to identify and treat the physiological changes that are initiated within the nervous system and perpetuate the pain process. The neurochemistry of painful conditions is relevant to the pharmacotherapy of pain. Serotonin reuptake inhibitors and noradrenergic tricyclic drugs (e.g., antidepressants) can alter the pain threshold. Drugs that modulate the γ-aminobutyric acid activity (e.g., clonazepam) and NMDA-associated calcium channels (e.g., gabapentin) can be effective in treating the burning sensation that is associated with neuropathic pain caused by

peripheral neuropathy, radiculopathy, or spinal cord injury. Opioids produce analgesia by interfering with the transmission of the pain signal. They bind to the pain-sensing receptors (μ, δ, and κ) within the central nervous system, block the release of substance P, and have postsynaptic effects on the dorsal horn (Kondo et al., 2005).

A BV injection can produce an initial nociceptive effect as well as a prolonged anti-nociceptive effect. BV contains many potential pain-producing substances including melittin, histamine, and PLA$_2$. Therefore, it is not surprising that several reports described a nociceptive effect after an intraplantar injection (Table 2). There are several papers on the nociceptive effects and possible mechanisms of BV (Luo et al., 1998). Following an intraplantar BV injection, there is an increase in the number of Fos-like immunoreactive neurons in the superficial and deep layers of the dorsal horn of anesthetized rats. In contrast, systemic morphine suppressed c-Fos expression in both superficial and deep layers of dorsal horn in a dose-dependent manner, and the latter region was much more sensitive to morphine than the former. These findings show that prolonged neuronal activities in the superficial and deep layers of the dorsal horn were essential for mediating the BV-induced tonic pain. Wu et al. (2002) further confirmed the effect on the spinal cord Fos expression and relevant nociceptive behaviors by BV in the rat hind paw. The nociceptive behavioral responses (spontaneous pain and hyperalgesia) after a BV injection were assessed after the intrathecal administration of c-fos antisense oligodeoxynucleotide, sense oligodeoxynucleotide and saline before injecting BV into adult male Sprague-Dawley rats. Pretreatment of the rats with the c-fos antisense oligodeoxynucleotide decreased the flinching response and primary thermal hyperalgesia but had no significant effects on mechanical hyperalgesia and secondary thermal hyperalgesia. At the same time, the antisense oligodeoxynucleotide treatment also decreased the expression of the Fos protein within the lumbar region of the spinal cord ipsilateral to the injection. This shows that the Fos protein contributes to the activation of the spinal dorsal horn neurons and the generation and/or maintenance of spontaneous pain and primary thermal hyperalgesia induced by a subcutaneous injection of BV.

Table 2
Nociceptive and anti-nociceptive effect of BV and its components administered subcutaneously

Administration	Results	Mechanism (receptor involvement)	References
Nociceptive effect			
BV	Induction of persistent pain, flinching response and primary thermal hyperalgesia	Prolonged neuronal activities of c-fos protein expression in the superficial and deep layers of the dorsal horn	Luo et al., 1998; Wu et al., 2002
	Firing of the wide-dynamic-range neurons in the spinal dorsal horn, mechanical allodynia and hyperplasia	Activation of NMDA receptor, activation of 5-HT1A receptor, spinal neurokinin 1/2 receptors or ORLI receptor	Chen et al., 1999, 2000; Zheng & Chen, 2001; You et al., 2002; Chen et al., 2003; Wang et al., 2003; Sun et al., 2004
	Contralateral hypersensitized wind-up and after-discharge of the spinal withdrawal reflex	Activation of the spinal non-NMDA receptor	You & Arendt-Nielsen, 2005
	Long-term spontaneous nociception and inflammation, and contralateral heat hyperalgesia	Activation of the peripheral capsaicin-sensitive afferents, increase of dorsal root and axon reflex, and activation of spinal PKC and PKA	Li et al., 2000; Chen & Chen, 2001; Chen et al., 2006a, 2006b
	Persistent nociceptive response	Activation of the ATP P2x-purinoceptor, inactivation of NO–c-GMP–K(+) channels	Zheng & Chen, 2000; Mixcoatl-Zecuatl et al., 2006
BV and melittin	Pain and hypersensitivity	Production of the spinal neuronal changes	Li & Chen, 2004
	Nociceptive responses	Activation of the capsaicin sensitive afferent fiber	Shin & Kim, 2004
Melittin	Heat hyperalgesia and biphasic vasomotor reflex responses	Increase of the sympathetic vasomotor tone	Koyama et al., 2002; Sumikura et al., 2006
	PSN, primary heat and mechanical hypersensitivity, MIH hypersensitivity	Activation of the spinal PKC and PKA	Li & Chen, 2003
	PSN, mechanical and heat hypersensitivity	Activation of the spinal ERK pathway	Yu & Chen, 2005
Anti-nociceptive effect			
BV	Visceral anti-nociception	Activation of the alpha 2-adrenoceptors	Kwon et al., 2001a, 2005
	Decrease in mechanical and thermal hyplerplasia	Reduction of c-Fos expression in the spinal cord	Kwon et al., 2001b
	Pain relief of knee OA		Kwon et al., 2001c
	Anti-nociceptive effect on the formalin-induced pain behavior	Decrease in Fos expression in the spinal cord	Kim et al., 2003; Roh et al., 2006
	Anti-nociceptive effect on the formalin-induced pain behavior, Antihyperalgesic and antiallodynic effects	Activation of the α$_2$-adrenergic and serotonergic components of the DPIS	Roh et al., 2004; Kim et al., 2005
	Decrease in acute paw edema and thermal hyperalgesia	Parallel reduction in neuronal Fos expression	Lee et al., 2001
	Anti-nociceptive effect on type II CIA	Activation of the α$_2$-adrenergic receptor	Baek et al., 2006

DPIS, descending pain inhibitory system.

4.2. Role of N-methyl-D-aspartate receptor

Chen et al. (1999) examined the roles of the peripheral excitatory amino acids receptor subtypes, NMDA and non-NMDA receptors, on the persistent nociception on the BV-induced increase in the firing of the wide-dynamic-range (WDR) neurons in the spinal dorsal horn of urethane-chloralose anesthetized cats. A BV injection into the cutaneous receptive field resulted in a single phase of increased firing of WDR neurons over the background activity. The local pre-administration of a NMDA receptor antagonist 5-aminophosphonovaleric acid (AP5), or non-NMDA receptor antagonists 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 6,7-dinitroquinoxaline-2,3-dione (DNQX) into the BV injection site suppressed the increased neuronal firing when compared to a pretreatment with a local injection of saline or dimethylsulfoxide. However, the same treatment with CNQX and even a higher dose of DNQX did not inhibit the neuronal firing induced by the BV injection, suggesting that the suppressive action of local AP5 or CNQX is not the result of a systemic effect. This indicates that the activation of the peripheral NMDA receptors is involved in both induction and maintenance of BV-induced persistent nociception. On the other hand, the activation of the non-NMDA receptors is only involved in the induction of the persistent firing of the dorsal horn of WDR neurons induced by the BV injection. The BV-induced heat hyperalgesia identified in the injection site might be different from that identified in the contralateral hindpaw because the former co-exists with the mechanical hyperalgesia while the latter does not. Chen's group reported that a BV injection into the contralateral pawpad of conscious rats caused primary heat and mechanical hyperalgesia as well as contralateral heat hyperalgesia. In addition, they demonstrated that both NMDA and non-NMDA receptors in the spinal cord are involved in processing by intrathecal with each antagonist pretreatment. Moreover, they also reported that an injection of BV could produce secondary heat hyperalgesia in a region distant from the injection site, which has similar characteristics to contralateral heat hyperalgesia (Chen & Chen, 2000). They also showed that the NMDA receptors are involved in either the development or maintenance of the secondary and the contralateral heat hyperalgesia but without demonstrating a role in the processes of primary heat and mechanical hyperalgesia. The secondary heat hyperalgesia observed in the injected hind limb is likely to share the same neural mechanisms with that identified in the non-injected site through co-activation of the NMDA receptors (Chen et al., 2000). You et al. (2002) also reported that a BV injection-induced mechanical allodynia and hyperalgesia, which was involved with the NMDA receptor. Furthermore, You and Arendt-Nielsen (2005) also showed that BV elicited monophasic long lasting (∼1 hr) single motor unit (SMU) electro myographic responses without any resting state. The mechanically and electrically evoked responsiveness of the SMU were enhanced significantly by an ipsilateral BV injection, whereas electrically but not mechanically enhanced evoked responses (including wind-up and after-discharge) were observed at the non-injection site of the contralateral hind paw. The maintenance and development of BV-induced contralateral hypersensitivity of the spinal withdrawal reflex to noxious electrical stimulation depends on the different central pharmacological receptors, suggesting that the NMDA receptors may also play an important role in the BV-induced contralateral central hyperexcitability and sensitization.

4.3. Other mechanisms of nociceptive effect of bee venom

The involvement of other pathways in the BV-induced nociceptive effects has been also reported. Zheng and Chen (2000) reported the involvement of the adenosine triphosphate (ATP) P2x-purinoceptor in the persistent nociceptive response induced by a BV injection into the plantar surface of a hindpaw in a conscious rat by evaluating the continuously flinching reflex of the injected paw. An intradermal injection of 5 μg melittin, which is the principal toxin of BV, into the volar aspect of the forearm of humans caused severe pain, followed by a sustained increase in skin temperature. The topical application of 10% lidocaine gel did not significantly suppress the melittin-induced pain, but markedly suppressed both the increase in peak temperature and the area of the temperature increase. This suggests that 5 μg of melittin is sufficient to produce pain in humans, and a 10% lidocaine gel differentially decreases the melittin-induced axon reflex without having any significant analgesic effect. A subcutaneous injection of melittin into the posterior surface of one of the hind paws of rats produced an immediate tonic nociceptive response, which was observed as a persistent spontaneous paw flinching reflex (Li & Chen, 2004). Both the intensity and time course of the melittin response was also monophasic and dose-dependent. As an accompanied consequence, the heat and mechanical hypersensitivity (hyperalgesia and allodynia) as well as the inflammatory responses (paw swelling and plasma extravasation) were induced by the melittin injections. In the electrophysiological recordings, an injection of the same 3 melittin doses into the cutaneous receptive field produced an immediate, dose-dependent increase in the spontaneous spike discharges of the WDR neurons in the spinal dorsal horn, which are believed to be responsible for the spinally organized nociceptive flexion reflex. The melittin-induced ongoing spike responses are similar to the behavioral flinching reflex in terms of both the duration and frequency. Furthermore, the responsiveness of the WDR neurons to both heat and mechanical stimuli was enhanced significantly by the melittin injection. They suggested that melittin is responsible for producing the long-term spinal neuronal changes as well as the persistent spontaneous nociception (PSN), heat/mechanical hypersensitivity, and inflammatory responses induced by the experimental bee sting.

The roles of the descending facilitatory pathway from the rostral medial medulla (RMM) in the development of PSN and hyperalgesia by the BV treatment were also reported (Chen et al., 2003). Bilateral lesions of the RMM inhibited the persistent spontaneous flinching reflexes in the BV test. Bilateral lesions of the RMM prevented the development of the BV-induced heat hyperalgesia that occurred in the non-injected paw. However, they had no effect on the primary heat and mechanical

hyperalgesia occurring in the injected paw, which provided behavioral evidence that the tonic activation of the descending facilitatory pathway contributes to the establishment of BV-induced PSN, which is referred to as mirror image heat (MIH) hyperalgesia and central sensitization, but not the primary heat and mechanical hyperalgesia. Shin and Kim (2004) reported the involvement of capsaicin-sensitive afferent fibers showing that an intraplantar injection of melittin increased the discharge rate of the dorsal horn neurons only with C fiber input from the peripheral receptive field, which was completely blocked by the topical application of capsaicin to the sciatic nerve. This suggests that both melittin and BV induce nociceptive responses through the selective activation of the capsaicin-sensitive afferent fibers. The roles of the primary afferent fibers in development of the BV-induced PSN and hyperalgesia was also reported in the sciatic nerve or both the sciatic and saphenous nerves of rats that had been topically treated with capsaicin under pentobarbital anesthesia, which had been administered to destroy the capsaicin-sensitive primary afferent (CSPA) fibers (Chen & Chen, 2001). The destruction of the CSPA fibers of the sciatic nerve or both the sciatic and saphenous nerves produced only 34% or 69% inhibition of the mean total number of BV-induced paw flinches. In the BV-treated rats, the destruction of the CSPA fiber in the sciatic nerve completely blocked the development of the heat and mechanical hyperplasia in the BV injection site. However, the destruction of the CSPA fibers in both the sciatic and saphenous nerves blocked the development of both heat and mechanical hyperplasia in the whole BV-treated hind paw and heat hyperalgesia in the non-injected hind paw. This suggests that the CSPA (C- and A delta-) fibers play an important role in mediating either the heat or the mechanical hyperalgesia induced by the BV injection, and play a partial role in the BV-induced nociceptive process. Furthermore, in addition to the sciatic nerve, the saphenous nerve is also involved in mediating the BV-induced PSN as well as heat and mechanical hyperalgesia. Chen et al. (2007) also demonstrated that the peripheral capsaicin-sensitive primary afferents pathway also modulated the long-term inflammatory pain induced by BV. Melittin-induced hyperalgesia in humans was also reported in those evoked by capsaicin. In 6 healthy volunteers, 10 μg of melittin was injected intradermally. Two-way ANOVA revealed a significant increase in the pain-rating index. These results show that a melittin injection induces slowly developing secondary heat hyperalgesia (Sumikura et al., 2006). Koyama et al. (2002) also clarified the interaction between the nociceptive inputs and the vascular changes through the noxious stimulation with intradermal melittin on the vasomotor control of the distal extremities in human volunteers. A biphasic response of the skin temperature was found: the skin temperature of both fingers and hands increased well above the control level before the melittin injection but decreased immediately after the injection. This suggests that the initial increase was due to the release of the sympathetic vasomotor tone and the later decrease was interpreted as a sympathetic vasoconstrictor reflex induced by the noxious stimulus. Chen et al. (2006a) also reported a neurogenic mechanism. They showed that an injection of BV-induced an increase in the volume of the injected paw, and

increased effect of the sciatic nerve transection (SCT), and the local capsaicin treatments compared with that of the L4–L6 dorsal rhizotomy (DRT) treatment. A local injection of capsaicin into the sciatic nerve produced the significant inhibition of the BV-induced decrease in the paw withdrawal mechanical threshold of the injected paw but had no effect on the paw withdrawal latency. This suggests that neurogenic components are probably involved in the maintenance and development of the BV-induced inflammatory pain response through dorsal root reflex and axon reflex mechanisms. In addition, the CSPA might play differential roles in the development of the BV-induced static and dynamic mechanical allodynia.

The involvement of other pathways in the BV-induced nociceptive effect has been reported. Li et al. (2000) examined the roles of spinal PKC in the induction and maintenance of both the PSN and the contralateral heat hyperalgesia induced by the BV injection and identified the effects of an intrathecal (i.t.) pretreatment with a PKC inhibitor, chelerythrine chloride (CH), in conscious rats. The intrathecal pre-treatment with CH at 3 doses, 0.01, 0.1 and 1 nM, had a dose-dependent suppressive effect on the flinching reflex when compared with the pre-saline control group. Post-treatment intrathecally with the drug at the highest dose used (1 nM) also reversed the effect on the established contralateral heat hyperalgesia. Li and Chen (2003) also reported that an injection of BV produces different types of pain and hypersensitivity, including PSN, primary heat, and mechanical hypersensitivity (hyperalgesia) and MIH hypersensitivity, and that the changes in the spinal neurons are likely to be responsible for the production of these pain-related behaviors. In addition, they suggested that the central processes of primary heat hypersensitivity involve the activation of the spinal PKC pathway, while spinal PKA is likely to be involved in the primary mechanical hypersensitivity induced by a subcutaneous BV injury. They showed that the BV-induced primary heat hypersensitivity could be blocked by an i.t. pre- or post-treatment with a PKC inhibitor, CH, while a PKA inhibitor, *N*-(2-[P-bromocinnamylamino]ethyl)-5-isoquinoline sulfonamide hydrochloride (H89), had no effect. However, the BV-induced primary mechanical hypersensitivity could be blocked by a pre- or post-treatment with H89, whereas CH had no effect. Both the pre- and post-treatment with H89 had suppressive effects on both the induction and maintenance of the BV-induced PSN and MIH hypersensitivity. Yu and Chen (2005) reported that the extracellular signaling-regulated kinases (ERK) pathway is involved in the induction and maintenance of the persistent ongoing nociception, pain hypersensitivity and inflammation.

Wang et al. (2003) suggested a facilitating role for the 5-hydroxytryptamine (5-HT) 1A receptor in BV-induced inflammatory pain. An injection of BV into the hind paw of a rat can causes acute inflammation along with spontaneous pain, heat hyperalgesia, and mechanical hyperalgesia/allodynia. The 5-HT1A receptor is the main receptor subtype in the spinal dorsal horn that mediates the function of 5-HT in nociception. However, 1 or 4 hr after the subcutaneous BV challenge, the expression of the 5-HT1A receptor mRNA in the ipsilateral lumbar spinal cord was increased significantly. Moreover, the

antisense oligodeoxynucleotide knockdown of the spinal 5-HT1A receptor attenuated the spontaneous pain and reversed the heat hyperalgesia in the rats injected with BV. Liu et al. (2005) also reported that different sets of 5-HT receptor subtypes work at different stages of the inflammatory pain induced by the BV injection into the plantar surface of the unilateral hindpaw. An injection of BV into the rat lumbar dorsal root ganglion (DRG) significantly increased the mRNA levels of the 5-HT(1A), 5-HT(1B), 5-HT(2A), and 5-HT(3) receptor subtypes at 1 and 4 hr after the injection, while the increase in the level of the 5-HT(2C), 5-HT(4), 5-HT(6) and 5-HT(7) receptor subtype mRNA was detected only after 4 hr. There were no such changes in the mRNA expression of the 5-HT(1D), 5-HT(1F), and 5-HT(5A) receptor subtype. The up-regulation of the 5-HT(1A), 5-HT(1B), and 5-HT(2A) receptor subtype mRNA was also observed in the contralateral DRG at 4 hr. 5-HT(1E), 5-HT(2B), and 5-HT(5B) receptor subtype mRNA was not detected in the rat DRG.

Zheng and Chen (2001) reported the presence of spinal neurokinin receptors during the development of persistent nociception and hyperalgesia in response to thermal and mechanical stimuli induced by a BV injection with the pre- or post-treatment with a non-selective antagonist of the (NK1/2) receptors, [D-Arg1, D-Trp7,9, Leu11] substance P (spantide), and a selective NK3 receptor antagonist, (S)-(N)-(1-(3-(1-benzoyl-3-(3,4-dichlorophenyl) piperidin-3-yl)propyl)-4-phenylpiperidin-4-yl)-N-methyl acetamide (SR142801) in a conscious rat. They suggested that both the induction and maintenance of the PSN involves the activation of the spinal NK1/2 receptors. In contrast, activation of the spinal NK1/2 receptors is only involved in inducing the primary and secondary thermal hyperalgesia stimulated by the BV injection but has no effect on primary mechanical hyperalgesia. The spinal NK3 receptor does not appear to be involved in the BV-induced behavioral responses that are characterized by spontaneous pain and thermal and mechanical hyperalgesia. Sun et al. (2004) examined the spinal activity of an endogenous ligand for the opioid receptor-like I (ORLI) receptor, nociceptin, on the spontaneous nociception, hyperalgesia and inflammation induced by BV injection. Pretreatment with an intrathecal injection of nociceptin at 3 doses (3, 10, and 30 nM) suppressed the spontaneous paw flinching reflex. A pre-treatment with 10 nM nociceptin before injecting the BV had no significant effect on the occurrence of the primary heat and mechanical hyperalgesia. Moreover, a post-treatment with the same dose again 3 hr after the BV injection had no effect. A pre-treatment with nociceptin had no effect on the BV-induced increase in paw thickness and volume and plasma protein extravasation. These results suggest that intrathecal nociceptin has no effect on primary heat and mechanical hyperalgesia and inflammation but has a dose-dependent anti-nociceptive effect on the BV-induced PSN by activating the spinal ORLI receptor.

The nociceptive effects of the other components have also been reported. Chen et al. (2006b) recently identified the active components of BV that cause inflammation and pain. Five major peptidergic subfractions were separated, purified, and identified from the BV. Four active peptidergic components were characterized as apamin, MCD peptide, PLA$_2$-related peptide, and melittin. All 5 sub-fractions were effective in producing local inflammatory responses (paw edema) in rats but had different efficacies. Among the 5 sub-fractions identified, only the MCD peptide, the PLA$_2$-related peptide and melittin could produce the ongoing pain-related behaviors observed as paw flinches, while only apamin and melittin produced thermal and mechanical hypersensitivity. They also found that among them, melittin was the most potent polypeptide in producing local inflammation, ongoing pain and hypersensitivity. They suggested that the peripheral transient receptor potential vanilloid receptor 1 is likely to be involved in the melittin-produced ongoing pain and heat hyperalgesia but is not involved in mechanical hyperalgesia after a treatment with a single dose of capsazepine, which blocks the thermal nociceptor transient receptor potential vanilloid receptor 1. Apamin has a significant effect on the peripheral nociceptive action by blocking the large- and small-conductance Ca^{2+}-activated K$^+$ channels (Ortiz et al., 2005). It was also reported that an injection of a K$^+$ channel blocker, apamin, inhibited the anti-nociception of MV8612 against both phases of formalin-induced nociception (Santos et al., 2003). Apamin is also known to be involved in the lumiracoxib-induced local and intrathecal anti-nociception as well as the gabapentin-induced spinal anti-allodynia by inactivating the NO–c-GMP–K$^+$ channels (Lozano-Cuenca et al., 2005; Mixcoatl-Zecuatl et al., 2006). Granados-Soto et al. (2002) also reported that apamin significantly decreases sildenafil-induced local peripheral anti-nociception in a dose-dependent manner by activating the NO–c-GMP–PKG–K$^+$ channel pathway.

4.4. Anti-nociceptive effect of bee venom

Although an injection of BV has been reported to cause tonic pain and hyperalgesia, there is also evidence suggesting that BV can also have anti-nociceptive effects on inflammation. In this regard, BV has been used traditionally to relieve pain and treat chronic inflammatory diseases. A subcutaneous injection of BV into an acupoint, which is known as acupuncture, has been used to produce a potent analgesic effect. Several experimental studies have also demonstrated this effect (Table 2). Dr. Lee's group is the one of the leading research groups that has demonstrated the anti-nociceptive effect of BV. Kwon et al. (2001b) reported an anti-nociceptive effect of BV injections into a specific acupoint (Zusanli) in an animal model of chronic arthritis compared with an injection into a non-acupoint. BVT significantly reduced the arthritis-induced nociceptive behavior (i.e., the nociceptive scores for mechanical hyperalgesia and thermal hyperalgesia). These anti-nociceptive/anti-inflammatory effects of BV were observed from 12 days through to 21 days after the BV treatment. In addition, the BV treatment significantly suppressed the adjuvant-induced Fos expression in the lumbar spinal cord at 3 weeks after the adjuvant injection. Finally, an injection of BV into the Zusanli acupoint had a significantly stronger analgesic effect against arthritic pain than a BV injection into a more distant non-acupoint. They also showed that a BV injection into the Zusanli acupoint has both anti-inflammatory and anti-nociceptive effects on Freund's adjuvant-induced arthritis in rats. This is in contrast to that

observed with a subcutaneous injection and intraplantar injection of BV and melittin, which produce robust nociceptive behavior. These findings suggest that BVA is a promising alternative long-term treatment of inflammatory pain.

The anti-inflammatory and anti-nociceptive effects of BV in a localized inflammatory reaction state have also been reported (Lee et al., 2001). In normal animals, a BV injection into the hindlimb was found to slightly increase the level of Fos expression in the spinal cord without producing any detectable nociceptive behavior or hyperalgesia. In contrast, a pretreatment with BV before the carrageenan injection suppressed both the paw edema and thermal hyperalgesia evoked by the carrageenan. In addition, there was a positive correlation between the percentage change in paw volume and the expression of Fos positive neurons in the spinal cord. These results suggest that the BV pretreatment has both anti-nociceptive and anti-inflammatory effects against carrageenan-induced inflammatory pain. This also suggests that BV may be useful in treating the pain and edema associated with chronic inflammatory diseases. The anti-nociceptive effect of the BV pretreatment on the formalin-induced pain behavior and its associated spinal cord Fos expression in rats was also demonstrated (Kim et al., 2003). Adult Sprague-Dawley rats were injected with BV directly into the Zusanli (ST36) acupoint, which resulted significantly less paw-licking time in the late phase of the formalin test and the inhibition of the formalin-induced expression of Fos in the spinal cord.

Kwon et al. (2001b) also compared the anti-nociceptive effects of apipuncture with non-acupoint stimulation because the alternative forms of acupoint stimulation including electroacupuncture, moxibustion, and acupressure appear to have more potent analgesic effects than manual needle acupuncture. Different doses of BV were injected into either an acupoint or non-acupoint 30 min before either an intraplantar formalin injection or intraperitoneal acetic acid injection. Using the abdominal stretch assay, they found that high doses of BV had a potent anti-nociceptive effect, irrespective of the site of the BV injection. In an abdominal stretch assay, an injection of BV into an acupoint (Zhongwan, Cv. 12) produced significantly greater anti-nociception than an injection into a non-acupoint injection (10 mm from Zhongwan, Cv. 12). Similarly, in the formalin test, an injection of BV into an acupoint (Zusanli, St. 36) produced more potent anti-nociception than an injection into a non-acupoint (gluteal muscle). In contrast, a BV injection into an arbitrary non-acupoint site on the back did not produce any anti-nociception in either the writhing or formalin tests. This suggests that an injection of BV directly into an acupoint can produce a potent anti-nociceptive effect. It was also reported that a BV pretreatment directly into the Zusanli point decreased the paw-licking time and markedly inhibited the level of Fos expression in the spinal cord in the late phase of the formalin test. This indicates that a pretreatment with BV into the Zusanli point has an anti-nociceptive effect on the formalin-induced pain behavior. Kwon et al. (2001c) also reported the efficacy of BVA using both pain relief scores and computerized infrared thermography (IRT) after 4 weeks treatment with BVA in patients with knee OA. They found that a significantly higher proportion of subjects receiving the BVA reported substantial

pain relief compared with those receiving traditional needle acupuncture therapy. Furthermore, the IRT score was significantly improved and showed a strong correlation with the level of pain relief. From these studies, they suggested that a BV injection directly into an acupoint can have a potent anti-nociceptive effect, and that this alternative form of acupoint stimulation (acupuncture) might be a promising method for pain relief. They further showed that a subcutaneous BVA injection of water-soluble fraction into the Zusanli acupoint dramatically inhibited the paw edema and radiological changes (i.e., new bone proliferation and soft tissue swelling) caused by Freund's adjuvant injection. The BVA treatment also decreased the level of serum IL-6 that was increased by RA to the levels observed in non-arthritic animals. In addition to the significantly reduced arthritis-induced nociceptive behaviors (i.e., nociceptive scores for mechanical hyperalgesia and thermal hyperalgesia), the BVA treatment also significantly suppressed the adjuvant-induced Fos expression in the lumbar spinal cord 3 weeks after the adjuvant injection. However, a treatment with an ethylacetate soluble fraction of BV (0.05 mg/kg/day) had no anti-inflammatory or anti-nociceptive effects on RA. However, it is unclear which constituent of the BVA fraction is directly responsible for these anti-arthritic effects. Nonetheless, adolapin had a potent analgesic effect, as demonstrated by the "writhing" test (ED_{50} 0.016 mg/kg) and the Randall-Sellito's test (ED_{50} 0.013 mg/kg). The anti-inflammatory activity of adolapin was most significant with regard to carrageenan, PG, and adjuvant rat hind paw edema and adjuvant polyarthritis. The effects of adolapin are presumably due to its capacity to inhibit the PG synthesis system.

4.5. Possible mechanism of the anti-nociceptive effect of bee venom

The precise anti-nociceptive mechanism(s) of BV are unclear but several mechanisms have been suggested (Table 2). Adrenergic receptor activation in the locus coeruleus (LC) and spinal dorsal horn causes the depression of nociceptive transmission from the primary afferent fibers to the second order nociceptive neurons, thereby inhibiting the signaling to the higher brain regions. The descending noradrenergic system modulates the transmission of nociceptive information at the spinal cord level (Kuraishi et al., 1979; Glynn et al., 1986). The systemic administration of α_2-adrenoceptor agonists elevates the nociceptive threshold, which is mediated by the activation of the α_2-adrenoceptors in the LC and dorsal horn of the spinal cord (Yaksh, 1986). The activation of the adrenergic pathway might be associated with the anti-nociceptive effect of BV. Kwon et al. (2001a) reported the dose-dependent suppression of acetic acid-induced abdominal stretching and acetic acid-induced Fos expression in the spinal cord and nucleus tractus solitarii in ICR mice that had received a BV injection into the Zhongwan acupoint (CV12) 30 min before an intraperitoneal injection of acetic acid. This effect was completely blocked by pretreatment with yohimbine, an α_2-adrenoceptor antagonist, suggesting that stimulation of an acupoint with BV can produce visceral anti-nociception that is associated with the activation of the α_2-

adrenoceptors. Mediation of the α_2-adrenoceptor was also observed in the anti-nociceptive effect of BV Zusanli (ST36) acupuncture on inflammatory pain in the rat model of CIA (Baek et al., 2006). Lee's group (Roh et al., 2004, 2006) also demonstrated the potential antihyperalgesic and antiallodynic effects of BVA as well as the relevance of the α_2-adrenoceptors in a rat neuropathic pain model. An injection of BV into the Zusanli acupoint 2 weeks after a chronic constrictive injury (CCI) to the sciatic nerve showed a significant decrease in thermal hyperalgesia induced by a CCI. This effect was reported to be associated with the activation of the spinal opioid receptors and/or α_2-adrenoceptors. This is because a pretreatment with idazoxan, an α_2-adrenoceptor antagonist, completely blocked the effect of acupuncture but did not involve the opioid receptor. They also reported that BV reduces the visceral pain behavior through the spinal α_2-adrenergic activity but not through the opioid and serotonin receptors in mice (Kwon et al., 2005). They suggested that acupuncture is an effective alternative therapy for patients with painful peripheral neuropathy, particularly for those who respond poorly to opioid and serotonegic analgesics. The same group also demonstrated that acupuncture-induced anti-nociception is produced by the activation of the α_2-adrenergic and serotonergic components of the descending pain inhibitory system in the rat formalin pain model. However, the opioid receptor, alpha1 adrenoceptor and beta adrenoceptor are not involved (Kim et al., 2005). Baek et al. (2006) also demonstrated the involvement of the α_2-adrenergic receptor but not the opioid receptor signal in the anti-nociceptive effect of BV in inflammatory pain, particularly in the rat model of CIA model. It was demonstrated that an injection of BV into the Zusanli acupoint (ST36) had an anti-nociceptive effect. Furthermore, a pretreatment with yohimbine (an α_2-adrenergic receptor antagonist) blocked the anti-nociceptive effect of BV, but a pretreatment with naloxone (a mu-opioid receptor antagonist) had no such effect.

An injection of BV into the Zusanli point 10 min before an intraplantar formalin injection dose-dependently attenuated the nociceptive behavior associated with the second phase of the formalin test. The destruction of the CSPA by a pretreatment with resiniferatoxin (RTX) selectively decreased the BV-induced spinal Fos expression but did not affect the BV-induced anti-nociception. Furthermore, the BV injection increased the level of Fos expression in the tyrosine hydroxylase immunoreactive neurons in the locus caeruleus, which was unaltered by a RTX pretreatment. Finally, the anti-nociception of BV was blocked by an intrathecal injection of idazoxan. This effect was not modified by a RTX pretreatment. This suggests that BV stimulation of the Zusanli point activates the central catecholaminergic neurons through the capsaicin-insensitive primary afferent (CIPA) fibers without inducing nociceptive behavior. This in turn activates the spinal α_2-adrenoceptors, which ultimately reduces the formalin-evoked nociceptive behaviors. This demonstrates that BVA produces significant anti-nociception without any nociceptive behavior in rodents. This anti-nociception is mediated by the CIPA and involves the activation of the central adrenergic circuits. Overall, these reports suggest that the α_2-adrenoceptor is the major pathway in the anti-

nociceptive effect of BV but the opioid receptor signals are not involved.

The above studies suggest that an intraplantar injection of BV and its major constituent, melittin, produces robust nociceptive behavior and hypersensitivity in rodents. On the other hand, a BV injection into the Zusanli acupoint produces very little nociceptive behavior but rather has a significant anti-nociceptive effect in a variety of animal pain models. Despite the apparent contradictory data in the literature regarding the consequences of a BV injection, there are no explanations for the possible relationship between the nociceptive and anti-nociceptive effects of BV, particularly with respect to a BV injection. Dr Lee's group carried out most of the studies showing an anti-nociceptive effect of BV. They reported that an intraplantar injection of BV produces a set of nocifensive behaviors including licking, biting, and flinching for a period of ~ 1 hr after the injection. In contrast, they failed to detect any observable nocifensive behavior when different doses of BV were injected into the Zusanli acupoint. They suggested that this difference might be due to the different injection sites of BV. They also speculated that the subcutaneous tissue of the hind paw has a greater innervation density than the area near the stifle joint where the Zusanli acupoint is located. In addition, they also indicated the possibility of anatomic and functional differences between the intraplantar glabrous skin and hairy skin in rodents where the Zusanli point is located. Indeed, they failed to find a relationship between the spontaneous nociceptive behavior and Fos expression in the spine, which is generally increased by nociceptive stimuli in the dorsal horn, after injecting BV into the Zusanli point. The threshold of neuronal activation to stimulation differs according to the type of primary afferent fiber (Aβ, Aδ, or C). Moreover, chemical stimulation of the Zusanli acupoint by BV activates the peripheral CIPAs fibers, which in turn causes catecholaminergic neuronal activation in the LC region. Overall, activation of the peripheral capsaicin insensitive primary afferents fibers followed by activation of the central catecholaminergic pathway might be important events in the anti-nociceptive effect of BV. In addition to the triggering the endogenous pain inhibitory system, modification of the local release in neurotransmitters and neuropeptides, such as substance P might be associated with the anti-nociceptive effects of BV. A further study will be needed to clarify this issue.

5. Anti-cancer effect of bee venom

Havas (1950) first reported the effects of BV on a colchicines-induced tumor. Almost 30 year later, 2 interesting but conflicting reports were published. Mufson et al. (1979) 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. They also reported that melittin like phorbol ester (12-*O*-tetradecanoyl phorbol-13-acetate, TPA) which is a well known

Table 3
Cancer cell growth inhibition and the in vitro anti-cancer effect of BV and its components

Treatment condition	Cancer cells	Results	References
Melittin			
Melittin	L1210, Leukemic lymphocyte, SK-OV, Ovarian carcinoma cell, A253, Squamous carcinoma cell IRSC-10M, A549, Lung tumor cells	Inhibition of cell growth and clonogenicity SK-OV, Ovarian carcinoma cell, Cell cytotoxic effect	Hait et al., 1985; Hait & Lee, 1985; Lazo et al., 1985, 1986; Killion & Dunn, 1986; Zhu et al., 1991
	U937, HL60, KG1a, CEM, CEM/VLB100, Leukemic cells, Ras transformed cells, N1S1, McA-RH7777, Hepatocellular carcinoma cells	Activation of $cPLA_2$ and enhancement calapin activity	Sharma, 1993; Hanada et al., 1995; Wu et al., 1998
	MG63, U2 OS, Osteosarcoma cells C6, Astrocytoma cells	Inhibition of cell proliferation and induction of apoptosis Activation of Fas signal Enhancement of PLA_2-independent Ca^{2+} entry	Lee & Hait, 1985; Chen et al., 2004; Chu et al., 2007
Synthetic melittin			
Synthetic melittin	U937, Leukemia cells	Inhibition of several acylhydrolases PLD activation	Saini et al., 1999
Hecate, hecate-betaCG and LHRH-hecate	Porcine granulose, luteal cell, leydig cancer cells (BLT-1), MCF-7, MDA-MB-231, MDA-MB-435S Breast cancer cells BLT-1, Leydig tumor cells	Destruction of cancer cells expressing lutropin/choriogonadotropin receptors	Zaleska et al., 2003; Bodek et al., 2003; Leuschner et al., 2003b
*Hecate-CGbeta conjugate	Mammary gland tumor	Growth repression of rat mammary gland tumor	Zaleska et al., 2004
Recombinant adenovirus carrying melittin gene (Ad-rAFP-Mel)	BEL-7402, Hepatocellular carcinoma cells	Induction of morphological changes and apoptosis	Li et al., 2004, 2006
**Melittin/avidin conjugate, Immunoconjugates (containing peptide 101, BV first 22 amino acid)	DU-145, LNCaP-LN3, BLCA-38, Prostate cancer cells	Induction of apoptosis Induction of tumor growth in vivo through MMP-2 expression	Holle et al., 2003; Carter et al., 2004; Russell et al., 2004
Melittin fragment			
**hecate and hecate β hCG	OVCAR-3, Ovary cancer cells	Disruption of ovarian cancer cells in vitro and reduction of tumor volume and burden	Gawronska et al., 2002
**LHRH-hecate	LNCaP, PC-3, DU145, BRF-41T, Prostate cancer cell	Peptide conjugate LHRH of killing androgen dependent and independent prostate cancer cells in vitro and in vivo	Leuschner et al., 2003a
Humanized anti-hepatoma disulfide-stabilized Fv (hdsFv25)	SMMC-7721, Hepatoma cells	Induction of apoptosis	Hu et al., 2006a
*Premelittin construct	EJ, Human bladder derived-carcinoma cells	Complete loss of tumorigenicity (in vivo)	Winder et al., 1998
BV			
**BV	K1735M2, B16, Melanoma cells,	Inhibitions of cell proliferation, and induction of apoptosis	Liu et al., 2002; Jang et al., 2003; Hu et al., 2006b; Moon et al., 2006; Putz et al., 2006
	SMMC-7721, Hepatoma cells,	Inhibition of COX-2 mRNA expression and PGE_2 synthesis	
	NCI-H1299, Lung cancer cells, U937, Leukemic cells, Renal cancer cells	Inhibition of solid tumor growth Induction of Bcl-2 and caspase-3 Down-regulation of the ERK and Akt signal pathway Disruption of membrane integrity and generation of cytotoxic lyso-Ptdlns(3,4)P2	
*BV (in vivo, 150-600 μg/mouse)	Mammary carcinoma cells, Renal cancer cells	Reduction of metastases	Orsolic et al., 2003; Putz et al., 2006

LH/CG, lutropin/choriogonadotropin; LHRL, luteinizing hormone releasing hormone.
* in vivo, ** in vivo and in vitro.

tumor promoter, inhibits the differentiation of mouse melanoma cells, enhances the anchorage-independent growth of adenovirus-transformed rat embryo cells, and induces the release of arachidonic acid and PG synthesis from C3H/10T½ mouse embryo fibroblasts. McDonald et al. (1979) also evaluated the carcinogenic effects of BV in a mortality study of 580 occupationally exposed beekeepers. The subjects were identified from the obituary notices published between 1949 and 1978 in 3 journals of the U.S. beekeeping industry. The death certificates were examined for the causes of death, and the proportionate mortality ratios were compared with those of the general U.S. population. The incidence of death from cancer in Beekeepers was slightly lower than expected. The lower incidence of lung cancer in male beekeepers was significant. The frequencies of the other cancers were similar to expectation. Mortality from diseases other than cancer was similar to the general population. This suggests that BV might have a chemopreventive effect. Since then, several studies have reported the anti-tumor effect of BV, particularly melittin (Table 3). Fisher et al. (1980) reported that the melittin-like epidermal growth factor elicits the progression of the transformed phenotype, that is the temporal acquisition of anchorage-independent growth in an adenovirus-transformed clone model, which are the cell culture systems that respond to the combined action of initiating chemical carcinogens, tumor promoters, and transforming viruses. Pippia et al. (1989) also reported the promoting activity of melittin in rat fibroblasts. They also demonstrated that the tumor promoting effect of melittin is likely to be due to the increased intercellular adhesion of neoplastic cells. However, Seif (1980) reported that melittin does not increase the frequency of cellular transformation caused by the polyoma virus while other tumor promoters such as griseofulvin, TPA, epidermal growth factor, vinblastine, cytochalasin B, podophyllotoxin, colcemid, and colchicines increased the frequency of cell transformation by the polyoma virus from between 8- and 40-fold.

5.1. Cell cytotoxic effect of melittin

Hait et al. (1985) first demonstrated the inhibitory effect of melittin in vitro. They showed that a calmodulin inhibitor, melittin, inhibited the growth and clonogenicity of human and murine leukemic cells, and their potency reflected their activity as inhibitors of calmodulin. Melittin is also a more potent inhibitor of cell growth and clonogenicity than the phenothiazine metabolite, chlorpromazine sulfoxide. Lee and Hait (1985) also 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. (1985) reported a similar mechanism for the cytotoxic effect of melittin in leukemic L1210 cells. They also found that melittin increased the toxicity of bleomycin to human granulocyte/macrophages and erythroid stem cell colonies (Lazo et al., 1986). However, they found that all agents such as etoposide or X-irradiation, which cause breaks in DNA, do not share the calmodulin-dependent mechanism. However, the Hait group demonstrated that calmodulin antagonists can significantly increase the lethality of bleomycin

to some but not all human tumor cells, and that nonmalignant hematological human cells may also be affected by this combination. Hait and Lee (1985) reported that the cytotoxic effect of melittin is proportional to the antagonistic effect of calmodulin. These studies support the pharmacological role of calmodulin, as a potential intracellular target for the antiproliferative effect of melittin.

Killion and Dunn (1986) 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. The lysis of the normal cells was abolished when galactosamine, glucosamine, or beta-lactoglobulin was added to the melittin cell reaction, but the lysis of the leukemia cells was unaffected. 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. Zhu et al. (1991) 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-10M 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. In this regard, it is noteworthy that a 26-amino acid, amphipathic peptide from BV, is particularly active against cells in culture that expresses high levels of the ras oncogene (Sharma, 1992). In this study, it was demonstrated that the acquisition of resistance to increasing concentrations of melittin is accompanied by corresponding decreases in the levels of ras oncoprotein expression and the number of copies of the ras gene. This results in a concomitant reversion of transformed cells into a normal morphology in a strict dose-dependent manner. Melittin also preferentially hyperactivates PLA_2 in ras oncogene-transformed cells, resulting in their selective destruction. This suggests that the hyperactivation of PLA_2 by melittin might be a target for the cytotoxic effect of melittin against cancer cells.

5.2. Activation of phospholipase A_2 as a target molecule of anti-cancer effect of melittin

Consistent with Sharma's initial finding, the involvement of PLA_2 in melittin-induced cytotoxic effect against cancer cells was also demonstrated that melittin induces the hyperactivation of PLA_2 activity and calcium influx in ras-transformed cells (Sharma, 1993). Since then, several studies have demonstrated the role of the PLA_2 activity and/or related target molecules in the melittin-induced cytotoxic effect against a number of cancer cells. PLA_2 from a human pancreas, which is designated hPLA2-I, functions as a digestive enzyme. Interestingly, Hanada et al. (1995) reported that the mature form of $hPLA_2$-I stimulated the growth of a human pancreatic cancer

cell line MIAPaCa-2, whereas the pro-form was ineffective. PLA$_2$s from the *Laticauda semifasciata* fraction I, *Crotalus adamanteus* venom, *Streptomyces violaceoruber* and BV had no proliferative effect on the growth of MIAPaCa-2 cells. Scatchard plot analysis revealed that the MIAPaCa-2 cells possessed a specific binding site for mature hPLA$_2$-I. This suggests that the mature hPLA$_2$-I, but not the pro-form, functions as a growth factor for pancreas carcinoma through the specific binding site. Arora et al. (1996) also reported that melittin, a PLA$_2$ 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 PLA$_2$ is an important component of the signaling pathway leading to cell death. Wu et al. (1998) 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 cPLA$_2$ activity in the KG1a cell line. Saini et al. (1999) also reported that in human monocytic leukemia cells (U937), synthetic melittin caused the cytolysis of U937 cells within 10–15 min. Cellular hypertrophy (5 min) and aggregation (1 min) preceded cytolysis. Therefore, the transient activation of PLA$_2$ by melittin causes cytolysis at the point of initiation. This suggests that melittin rather than BV has a cytotoxic effect against several cancer cell lines, and its activation effect of PLA$_2$ might be a target of melittin, suggesting that PLD plays a role in melittin-mediated membrane disruption/cytolysis through an uncharacterized signal transduction mechanism.

The activation of PLA$_2$ might have a cytotoxic effect on cancer cells through several subsequent cellular changes. The synergistic effect of BV sPLA$_2$ (bv-s PLA$_2$) and phosphatidylinositol-(3,4)-bisphosphate (PtdIns(3,4)P2) in inducing cell death has attracted considerable interest. Putz et al. (2006) demonstrated that the cooperation of bv-sPLA$_2$ 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-sPLA$_2$ 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-sPLA$_2$ 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. They further demonstrated that bv-sPLA$_2$ and PtdIns(3,4)P2 synergistically generate tumor lysates that enhance the maturation of immunostimulatory human monocyte-derived dendritic cells. Such tumor lysates, which represent complex mixtures of tumor antigens showing potent adjuvant properties, meet all the requirements of a tumor vaccine (Putz et al., 2007). Chu et al. (2007) also reported the PLA$_2$-independent involvement of Ca^{2+} in BV-induced apoptosis. Melittin, at concentrations >0.075 μM, increased the intracellular Ca^{2+} in MG63 human OA cells in a concentration-dependent manner. At concentrations of 0.5 and 1 μM, melittin

killed 33% and 45% of the cells through apoptosis, respectively. They also revealed through a treatment with antagonists that melittin induced an increase in intracellular Ca^{2+} by causing Ca^{2+} entry through the L-type Ca^{2+} channels in a manner independent of PKC and PLA$_2$ activity.

5.3. Enhancing chemotherapeutical efficacy and specificity of melittin through the delivery system

The major issues of cancer chemotherapy are related to the therapeutic concentrations of agents that cause severe side effects. The development of desirable drug delivery systems has great potential in enhancing the chemotherapeutical efficacy and specificity. Most cell lytic peptides produced by insects, amphibians, and mammals 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. Melittin, a 26 amino acid, and hecate-1, a 23 amino acid analog of melittin, which are cationic and amphipathic, might be desirable therapeutic peptides. 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. (2002) reported that the melittin fragment (hecate), and conjugated to a 15-amino acid beta-chain of human chorionic gonadotropin (hCG), destroyed ovarian cancer cells (NIH: OVCAR-3) in a dose-dependent manner. The removal of steroids from the culture medium reversibly reduced the sensitivity of the OVCAR-3 cell line to the hecate-hCG. In vivo studies have shown a reduction in the average tumor volume and tumor burden in lytic peptide-treated animals. Gawronska et al. (2002) also reported the expression of the luteinizing hormone (LH)/hCG receptor protein in OVCAR-3 cells and tumor tissues. They (Leuschner et al., 2003a; Zaleska et al., 2003) also found that injections of a luteinizing-hormone-releasing-hormone (LHRH)-hecate conjugate resulted in tumor growth arrest and a marked decrease in the tumor burden and tumor viability in PC-3 cells and a granulosa cancer cell line (KK-1) possessing LH/CG receptors. Therefore, LHRH-hecate might be effective in treating hormone-dependent cancers. Hecate-betaCG selectively kills the cells expressing LH/CG receptors (Leuschner & Hansel, 2005). Its toxicity is dependent on the number of binding sites for LH/CG. Similar effects were also observed in breast cancer cell lines (MCF-7; MDA-MB-231) and a mouse Leydig tumor cell line (BLT-1) (Bodek et al., 2003). The ability of hecate-betaCG to destroy xenografts of human breast cancer cells (MDA-MB-435S) in nude mice was also demonstrated (Leuschner et al., 2003b). It was also found that a Hecate-CGbeta conjugate can repress mammary gland tumor growth in mammary gland carcinogenesis model using combined prenatal exposure to synthetic diethylstilbestrol (DES) followed by postnatal exposure to dimethylbenz[*a*]anthracene (DMBA) (Bodek

et al., 2005a, 2005b). This effect was observed even in tumor tissues that lack or have very low levels of LHR (Zaleska et al., 2004). This suggests that the 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. 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.

The apoptosis of cancer cells by BV has been reported both in-vitro and in-vivo. Liu et al. (2002) reported that BV inhibits the proliferation of K1735M2 mouse melanoma cells in-vitro, as well as B16 melanoma, which is a transplantable solid melanoma in C57BL/6 mice in vivo. In the in vivo experiments, BV was injected intraperitoneally into the mice 24 hr after the mice had been inoculated with B16 cells. The apoptosis of the K1735M2 cells was suggested to be the possible mechanism by which BV inhibited cell proliferation and induced K1735M2 cell differentiation in vitro. The apoptosis of NCI-H1299 lung cancer cells through the inhibition of COX-2 (Jang et al., 2003), and the osteosarcoma cell line, U2 OS, through the up-regulation of Fas expression by BV (Chen et al., 2004) were also reported. Holle et al. (2003) 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. Moon et al. (2006) reported the molecular mechanisms of BV-induced apoptosis in leukemic U937 cells through the down-regulation of the ERK and Akt (protein kinase B) signal pathway. Furthermore, BV-induced apoptosis was accompanied by the down-regulation of B-cell leukaemia/lymphoma-2 (Bcl-2), the activation of caspase-3 and the subsequent poly(ADP-ribose)polymerase (PARP) cleavage. The induction of apoptosis also was accompanied by the down-regulation of the inhibitor of the apoptosis protein (IAP) family of proteins. This indicates that the down-regulation of Bcl-2 plays an important role as an activator of a caspase-3 involved in BV-induced apoptosis. BV also triggered the activation of p38 MAPK and JNK, and the down-regulation of ERK and Akt. These results showed that the induction of apoptosis might be an important role in the anti-tumor activity of BV (or melittin), even though the molecular mechanisms for the induction of apoptosis are not completely understood.

Gene therapy based on putative killer-suicide genes is an alternative approach to the selective killing of cancer cells. When a vector carrying the killer gene is transfected into tumor cells, the corresponding prodrug selectively kills the cancer cells. This is particularly important in suicide gene strategies, in which the low-level expression of toxic genes in normal tissues may lead to severe toxicity. Therefore, further safeguards are needed in order

to ensure that gene delivery to these tissues does not result in significant gene expression and the resulting toxicity. One attractive approach to this problem relies on the ability to control gene expression very tightly at the transcriptional level. Some biotoxins, including the diphtheria toxin and ricin, can be used for gene therapy. With regard to melittin, the gene sequence of encoded melittin protein is short (78 bp). Hence, its synthesis and transfection is relatively easy for targeted gene therapy, and has accordingly attracted considerable attention as gene therapy. 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. (1998) 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 expression of melittin resulted in either the complete loss of tumorigenicity in some clones or reduced tumorigenicity, as measured by the latency of tumor formation. The recombinant adenoviruses carrying the melittin gene and α-fetoprotein (AFP) promoter (Ad-rAFP-Mel) were constructed by Ling et al. (2004). They reported that the mRNA of the melittin gene was transcribed in HepG2 hepatocellular carcinoma cells transduced by Ad-rAFP-Mel. The inhibitive rate of Ad-rAFP-Mel for BEL7402 cells was 66.2% according to a MTT assay. The rates of Ad-CMV-Mel inhibition in BEL7402, SMMC7721 and L02 cells were 58.9%, 65.9% and 31.7%, respectively. The tumorigenicity rates of hepatocarcinoma cells transfected with Ad-rAFP-Mel were lower. A significant antineoplastic effect was detected in the transplanted tumor in nude mice after an intratumoral injection of Ad-rAFP-Mel. This suggests that Ad-rAFP-Mel can inhibit the proliferation of AFP-producing human hepatocarcinoma cells both in vitro and in vivo. Li et al. (2004) examined the apoptotic ability of a recombinant adenovirus carrying the melittin gene. Ling et al. (2005) also reported lower tumorigenicity rates of hepatocarcinoma cells transfected with Ad-rAFP-Mel. A significant antineoplastic effect was detected on the transplanted tumor in nude mice after an intratumoral injection of Ad-rAFP-Mel. Ad-rAFP-Mel can inhibit the proliferation of AFP-producing human hepatocarcinoma cells both in vitro and in vivo through the induction of apoptosis. Li et al. (2006) further showed that an Ad-rAFP-Mel infection markedly induces cellular apoptosis, and Fas expression on Bel-7402 cells. They suggested this to be a possible molecular mechanism for the anti-tumorigenecity of Ad-rAFP-Mel even though more study will be needed. This suggests that the animal toxin gene might be an interesting antitumor gene.

Other delivery systems have been examined for their suitability as delivery systems for melittin in order for it to be selectively cytototic against cancer cells. The radiolabeled immunoconjugates and antibodies with immunoconjugates showed similar cytotoxic effects in xenograft tropism. A systemic or intratumoral injection of immunoconjugates containing peptide 101 was designed around the first 22 amino acids of melittin to maintain the amphipathic helix, to enhance water solubility, to increase the hemolytic activity of human prostate cancer (DU-145, LNCaP-LN3 and BLCA-38) and inhibit tumor growth in mice (Russell et al., 2004; Carter et al., 2004). Immunoconjugate delivery has beneficial effects in being able to increase the

cytotoxicity. The peptides in BV have attracted considerable attention as a possible anti-cancer treatment. Hu et al. (2006a) reported the efficacy of sterically stabilized liposomal peptide in BV using soybean phosphatidylcholine, cholesterol, and the cholesterol derivatives of poly ethylene glycol with terminal COOH groups coupled with the humanized anti-hepatoma disulfide-stabilized Fv (hdsFv25) as well as the N-hydroxy-succinimide ester method (SIL[hdscFv25]). The hdsFv25 and SIL[hdscFv25]-immunoliposomes have a strong affinity and specificity to SMMC-7721 cells in vitro (Hu et al., 2006b). Peptide of BV-loaded sterically-stabilized liposomes modified with the hdsFv25 and SIL[hdscFv25] can kill SMMC-7721 cells in vitro with a higher efficiency than the non-targeted liposomes of melittin. Hu et al. (2006c) also reported the in vivo efficacy of hdsc Fv25 and showed that a treatment with BV resulted in the significant retardation of SMMC-7721 cell growth in Balb/c nude mice. In addition, they emphasized the importance of apoptosis in the inhibition of cancer cell growth. These results suggest that this strategy might be applicable to immunotherapy in other cancers. Orsolic et al. (2003) reported that the intravenous administration of BV to mice significantly reduced the number of metastases to the lung. However, the subcutaneous administration of BV had little effect on the number of lung metastases, indicating that the antitumor effect of the BV is dependent on the injection route as well as close contact between the components of the venom and the tumor cells, as evidenced by other studies showing the anti-nociceptive effects.

6. Perspective

In vitro and in vivo as well as clinical trials have demonstrated that BVT may be an important traditional medicine to treat a variety of conditions such as arthritis and rheumatism, pain and cancer. Since BV contains at least 18 active components including enzymes, peptides and biogenic amines, which have a wide variety of pharmaceutical properties, the identification of a single constituent, the possible mechanisms, and a justification of the applicable route and formulation are needed. Recent studies have demonstrated diverse mechanisms, and the anti-arthritis and anti-inflammatory effects of BV and its constituents. The inhibitory ability of BV and its constituent on the expression of inflammatory genes such as COX-2 and PLA$_2$ expression, as well as on the generation of mediators such as TNF-α, IL-1, IL-6, NO and ROS could be important for understand the anti-arthritis effect of BV and its constiteunts (Murakami et al., 1997; Pelletier et al., 1998; Amin et al., 1999; Yang et al., 1999; Cernanec et al., 2002; Park et al., 2004, 2007). Consideration of the side effects of several nonsteroid antiinflammatory drugs, BVT might be an attractive alternative treatment for inflammatory diseases, such as arthritis rheumatism. Interestingly, apamin, a SK blocker, significantly inhibited both the ovalumine-induced tracheal contraction and histamine release from lung tissues (Ichinose et al., 1995). The MCD peptide has an anti-allergic activity by inhibiting release of histamine from mast cells (Buku, 1999, 2001), suggesting that these compounds can be applicable for allergy diseases.

The anti-nociceptive effects of BV in the thermal, visceral and inflammatory pain responses in experimental conditions have been demonstrated. Apcupoint stimulation into subcutaneous (acupuncture) therapy may be important for the anti-nociceptive effects of BV. Subcutaneous acupuncture therapy of BV reduces visceral nociceptive effects (Kwon et al., 2001a, 2005), mechanical and thermal hyperplasia (Kwon et al., 2001b; Lee et al., 2001), and formalin-induced pain behavior (Kim et al., 2003, 2005; Roh et al., 2006). Using specific antagonists, it has been reported that BV selectively alleviated the mechanical, visceral, inflammatory pain responses through differential modulation of the central or spinal α_2-adrenergic, serotonergic, opiod receptors activity (Kwon et al., 2001a, 2001b, 2001c; Kim et al., 2003, 2005; Kwon et al., 2005). These results suggest that acupuncture is also an effective alternative therapy for patients with painful peripheral and central neuropathy, particularly for those who respond poorly to α_2-adrenergic, opioid and serotonergic analgesics. BV also reduced the collagen-induced arthritic (Baek et al., 2006) knee OA-related pain (Kwon et al., 2001c). These results suggest that BV can be used clinically to relieve the pain involved in the development or progression of inflammatory diseases, such as arthritis.

BV also has anti-cancer activity (Liu et al., 2002; Hu et al., 2006c; Putz et al., 2006; Moon et al., 2006). Several cancer cells including renal, lung, liver, prostate, bladder, and mammary cancer cells as well as leukemia cells can be targets of melittin, a major constituent of BV. In contrast to normal eukaryotic cells with a low membrane potential, the cell membranes of prokaryotic and cancer cells maintain a large membrane potential. Hence, many lytic peptides selectively disrupt the cancer cell membranes rather than those of normal cells. The cell cytotoxic effect through the activation of PLA$_2$, caspase and MMP, which destroy cancer cells, is suggested as an important basic mechanism for the anti-cancer activity of BV (Holle et al., 2003; Moon et al., 2006). 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 (Li et al., 2004; Russell et al., 2004; Li et al., 2006; He et al., 2006). Therefore, melittin, a cationic and amphipathic peptide might be a desirable selective therapeutic peptide. It should be noted that melittin is particularly active against cells in culture that expresses high levels of the ras oncogene (Sharma, 1992). Moreover, melittin preferentially hyperactivates PLA$_2$ in ras oncogene-transformed cells resulting in their selective destruction. This suggests that the hyperactivation of PLA$_2$ by melittin might be a target for the cytotoxic effect of melittin against cancer cells.

Kim et al. (2004) reported the general pharmacological profiles of BV in rodent models. Subcutaneous injections of BV and its fractions did not have any significant side effects on the general physiological functions of the central nerves, cardiovascular respiratory and gastrointestinal functions at the highest dose tested (200 times and 100 times higher doses than that used clinically, respectively). This suggests that the doses of BV in the therapeutic range or higher are safe in clinical studies, indicating

that BVT is, even though a large number of subjects and clinical studies will be needed to determine the effectiveness of BV for practical indications. In summary, the data from experimental and clinical studies have shown that BVT is can be used as an alternative medicine to treat various diseases such as arthritis, pain, and cancerous tumors. However, several concerns such as the injection route, dose, delivery system and side effects need to be considered carefully before treatment BV clinically.

Acknowledgment

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