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Melittin, a major peptide component of bee venom, and its conjugates in cancer therapy

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Abstract

Melittin (MEL), a major peptide component of bee venom, is an attractive candidate for cancer therapy. This agent has shown a variety of anti-cancer effects in preclinical cell culture and animal model systems. Despite a convincing efficacy data against variety of cancers, its applicability to humans has met with challenges due to several issues including its non-specific cytotoxicity, degradation and hemolytic activity. Several optimization approaches including utilization of nanoparticle based delivery of MEL have been utilized to circumvent the issues. Here, we summarize the current understanding of the anticancer effects of bee venom and MEL on different kinds of cancers. Further, we also present the available information for the possible mechanism of action of bee venom and/or MEL.

Keywords

Bee venom; Melittin; Melittin conjugates; Cancer management; Anti-cancer effects

Introduction

Cancer is one of the major ailment effecting humankind and remains as one of the leading causes of mortality worldwide. The current available data suggests that over 10 million new patients are diagnosed with the disease every year and over 6 million deaths are associated with it representing roughly 12% of worldwide deaths. Fifteen million new cancer cases are anticipated to be diagnosed in the year 2020 [1] which will potentially increase to over 20 million by 2025 [2] and more in years to come. It is also anticipated that the growth and aging of the population may increase the new cancer cases to 21.7 million with about 13 million cancer deaths by the year 2030 [3].

Cancer development and progress are multifactorial process [4], either external factors such as tobacco, infectious organisms, environmental pollutants and an unhealthy diet or internal

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Conflict of interest
None.

factors such as inherited genetic mutations, hormones, and immune conditions may act together or in concert to cause the onset of this disease [5]. Since cancer is associated with such high morbidity and mortality worldwide, there is an urgent need to determine ways of management of this ailment. The current treatment modalities are mainly comprised of surgery, radiation based therapy, chemotherapy, gene therapy and/or hormonal therapy [5, 6]. All of these procedures utilized in mainstream medicine are almost always associated with significant unforeseen effects which pose challenge in its management. There has been an intense rush to devise alternative therapeutic approaches that have the potential for circumventing the usual side effects associated with mainstream medicines. We and others have suggested a concept of dietary intervention which has gained popularity and wide acceptance [7–12]. Another approach that has gained importance is the use of biotoxins such as animal venoms as cancer therapeutic agents [13–17]. These biotoxins are produced by living organisms as a defense mechanism against predators and are known to have both toxicological as well as pharmacological effects [18]. Current data suggests that toxin from bee venom (BV) has some potential as anti-tumor agent [19]. On the other hand, apitherapy, the medical uses of honey bee products range from royal jelly to BV, has been introduced as a natural therapeutics in cancer chemotherapy [20].

BV is a biotoxin or api-toxin synthesized and secreted by a gland that is present in the abdominal cavity of the bee and is composed of complex mixture of several biologically active peptides including Mellitin (MEL), enzymes, bioactive amines, and non-peptide components (Table 1) that has a variety of pharmaceutical properties [21]. Bee venom therapy (BVT), has been used in traditional medicine to treat diseases such as arthritis, rheumatism, pain, tumors, and skin diseases [22]. Studies have linked BV to variety of cancer management effects including induction of apoptosis, necrosis, cytotoxicity and inhibition of proliferation in variety of cancer types of cancer cells, including prostate, breast, lung, liver and bladder [23]. Overall, BV and its selective components are considered promising agents for cancer management [19]. In addition, BV has also been linked with management of the side effects of cancer chemotherapy including a study where BV pharmacopuncture or MEL were used as a symptom-control therapy for chemotherapy-induced peripheral neuropathy [24]. However, the efficacy of BV appears to be due to the synergetic effect of MEL and this anti-cancer peptide might be the better choice than BV in native form [19].

MEL is the main active pharmacological component of BV, accounting for 40–50% of its total dry weight. It is a water-soluble, linear, cationic, hemolytic and amphipathic peptide weighting 2840 Da [25] and consisting of 26 amino acid (Fig. 1) with a chemical formula $C_{131}H_{229}N_{39}O_{31}$, the N-terminal region is mainly hydrophobic due to +4 charges while the C-terminal region is hydrophilic because of +2 charges hence the total is +6 charges at physiological pH [26].

Previous studies suggested the biological effects of MEL as antiviral, antibacterial, antifungal, anti-parasitic and anti-tumor and proposed the basis of MEL action as a non-selective cytolytic peptide which physically and chemically disrupts all prokaryotic and eukaryotic cell membranes [27–30]. MEL binds to negatively charged membrane surface (Fig. 2) and then disturbs the integrity of phospholipid bilayers by pore formation

accompanied by the leakage of atomic ions and molecules and the enhancement of permeability that ultimately leads to cell lysis [31]. MEL was considered an attractive candidate for cancer chemotherapy causing more damage to the tumor cell membranes since its membrane potential is higher and cells are less likely to develop resistance to a membrane pore formation [32, 33]. Although the potential applicability of MEL as a cancer chemotherapeutic agent has long been recognized, its rapid degradation in the blood and its nonspecific cellular lytic activity poses significant challenges [34]. MEL when injected intravenously causes severe toxic reactions such as hemolysis [35] which is a limiting factor for its widespread use for cancer therapy. Recently, it has been made clear that MEL and/or its conjugates can work in conjunction with hormone receptors [36], gene therapy [37] or as nanoparticles [33, 34] for targeted therapies of some cancer types.

This review summarizes the current available literature about recent application of BV, MEL and different conjugates of MEL against several cancers both *in vitro* and *in vivo* (Table 2).

Anticancer effects of BV and its-conjugates

Effects on apoptosis

Apoptosis is an ordered and orchestrated cellular process that occurs in physiological and pathological conditions [38]. It is the main event that is known to regulate the occurrence and/or spread of cancer. Several studies have suggested that BV has potential anticancer effects against breast [39], hepatocellular carcinoma (HCC) [40], ovarian [41], prostate [42], melanoma [43], lung [44], leukemia [45] and cervical [46] cancers. It has been suggested that BV inhibits proliferation of the cancer cells via induction of apoptosis through multiple investigated mechanisms. In HCC, BV was shown to induce cytotoxic, genotoxic and mutagenic potential against HepG2 cells within three hours however it did not affect the mutagenicity induced by methyl methanesulfonate [47]. In another study, possible growth-inhibiting effects of BV applied alone or in combination with a cytotoxic drug bleomycin on HeLa and V79 cells was tested *in vitro*. Apoptosis, necrosis, and lysis were presumed as possible mechanisms by which BV inhibited growth and clonogenicity of V79 cells. HeLa cells, on the other hand, showed greater resistance to BV [46]. Another study investigated the mechanisms by which BV inhibits K1735M2 melanoma cells *in vitro* and B16 melanoma in C57BL/6 mice, *in-vivo* [48] Apoptosis was suggested as the possible mechanism by which BV inhibited cell proliferation and induced K1735M2 cell differentiation. The *in vivo* results showed that systemic administration of 1.0 and 3.0 mg/kg of BV resulted in significant inhibition of B16 melanoma growth with the relative tumor inhibition being 20 and 53% respectively. In another study, it was demonstrated that NCI-H1299 lung cancer cells treated with BV exhibit several features of apoptosis. In addition, expression of COX-2 mRNA and synthesis of PGE2 were inhibited by BV [49].

Choi et al. [44] reported in a study that BV induces apoptotic cell death in A549 and NCI-H460 lung cancer cells through the enhancement of death receptor 3 (DR3) expression and inhibition of NF- κ B pathway. A combination treatment of TNF-like weak inducer of apoptosis, docetaxel and cisplatin, with BV synergistically inhibited both A549 and NCI-H460 lung cancer cell growth with further down regulation of NF- κ B activity. In a parallel study, the authors used BV treated NK-92MI cells to co-culture with NSCLC cells and

found that there is a further decrease in cell viability up to 70 and 75% in A549 and NCI-H460 cell lines respectively. Further, the DNA binding activity and luciferase activity of NF- κ B was also inhibited after co-culture with BV treated NK-92MI cell lines. The knock down of death receptors with siRNA was observed to reverse the decrease in cell viability and NF- κ B activity after co-culture with BV treated NK-92MI cells [50]. Similar effects of death receptor mediated BV activity was observed by Jo et al. [41], where they suggested that BV and MEL induces apoptotic cell death in SKOV3 and PA-1 ovarian cancer cells through induction of death receptors and inhibition of JAK2/STAT3 pathway.

A study reported that BV induces apoptosis in leukemic U937 cells through downregulation of ERK and Akt signaling pathway [45]. Further, PD98059 (an inhibitor of ERK) or LY294002 (an inhibitor of Akt) significantly decreased cell viability and increased LDH release. In another study it was observed that BV induces apoptosis in A2058 melanoma cells but not in normal skin fibroblast Detroit 551 cells and that the apoptosis was induced via a caspase independent pathway. In this study the authors observed that JNK and ERK were rapidly activated after a 5 min incubation with BV, while p38 and AKT were inactivated after 30 min administration of BV [43].

BV has been observed to inhibit prostate cancer under *in vitro* and *in vivo* conditions and these effects were suggested to be mediated through activation of caspase via inactivation of NF- κ B pathway. In this study both BV and MEL inhibited cancer cell growth through induction of apoptotic cell death in LNCaP, DU145, and PC-3 human prostate carcinoma cells. These effects were mediated by the suppression of constitutively activated NF- κ B. Further, BV administration to nude mice implanted with PC-3 cells resulted in inhibition of tumor growth and activity of NF- κ B accompanied with apoptotic cell death [42]. Similar effects on BV were observed in colon cancer cells where activation of death receptors and inhibition of nuclear factor kappa B were observed to be regulating the cancer death [51]. The study demonstrated that BV inhibited growth of colon cancer cells through induction of apoptosis without any effect on FHC colon epithelial normal cells. The expression of death receptor (DR) 4, DR5, p53, p21, Bax, cleaved caspase-3, cleaved caspase-8, and cleaved caspase-9 were increased by BV treatment in a dose dependent manner. Further, the DNA binding activity of nuclear factor kappa B (NF- κ B) was also inhibited by BV treatment. In addition, BV significantly suppressed tumor growth *in vivo* [51].

Effects on invasion, migration and metastasis

Cancer begins as a localized disease however as it progresses, the tumor cells begin to invade into the surrounding tissues and ultimately into other organs as distant metastases. Invasion and metastasis govern, to a large extent, the severity of the disease and is considered a pressing goal in management of the disease. Agents that could induce effects on one or both of these factors could result in an effectual therapy of human cancer(s). In a study, possible tumor growth- and metastasis-inhibiting effects of BV were studied in mice and in tumor cell cultures. The collected data suggested that intravenous administration of BV to mice significantly reduced the number of metastases of mammary carcinoma cells to the lung [52]. Further, the study proposed that BV has an indirect mechanism of tumor

growth inhibition and promotion of tumor rejection that is based on stimulation of the local cellular immune responses in lymph nodes.

Another study evaluated the cytotoxic effect of BV alone and its synergistic cytological effects in combination with cisplatin on ovarian cancerous cisplatin resistant A2780cp cells. The results clearly suggested that BV exerts an anti-tumor effect on human ovarian cancer and has the potential for enhancing the cytotoxic effect of cisplatin [53]. While, in another study, BV was observed to inhibit PMA-induced MMP-9 expression and activity by inhibition of NF- κ B via p38 MAPK and JNK signaling pathways in MCF-7 cells [54]. In addition, MMP-9 inhibition by MEL, apamin and phospholipase A2 (PLA2), representative single component(s) of BV were also tested. PMA-induced MMP-9 activity was significantly decreased by MEL, but not by apamin and PLA2 [54]. In another study, the inhibitory effects of BV and its constituents MEL and apamin were confirmed on the EGF-induced invasion and migration of breast cancer cells [39]. Further, MEL inhibited the EGF-induced MMP-9 expression via blocking the NF- κ B and PI3K/Akt/mTOR pathway in these cells. In addition, MEL significantly suppressed the EGF-induced FAK phosphorylation through inhibition of mTOR/p70S6K/4E-BP1 pathway [39].

Mellitin anticancer effects

Two mellitin (MEL) isoforms (Fig. 1) have been shown to exhibit anti-cancer effects, i) MEL derived from BV of *Apis mellifera* and ii) MEL derived from BV of *Apis cerana* (MEL-N). MEL from *A. mellifera* is the most used in cancer research as a pharmacological peptide rendering stronger anti-cancer activities than MEL-N with only one study demonstrating MEL-N cytotoxicity and cell proliferation inhibition in SKOV-3 and PA-1 human ovarian cancer cells [55]. Current available literature involving both *in vitro* and *in vivo* studies suggest that MEL affects signal transduction and regulatory pathways leading to multiple cancer death mechanisms including inhibition of proliferation, induction of apoptosis, inhibition of angiogenesis, cell cycle arrest, and inhibition of cancer motility, migration, metastasis and invasion etc. which are being discussed below.

Effects on apoptosis

MEL has been studied extensively for its effects on regulation of apoptosis and different factors that regulate the induction of apoptosis in variety of cancer types. MEL was observed to activate caspases in different cancers such as leukemia U937 [56] and Jurkat [57], melanoma A2058 [43], HCC (SMMC-7721, Hep3B, HepG2 and BEL-7402) [57] cells, prostate PC-3, LNCaP and DU-145 [42] and cervical HeLa [57] cells. Similarly, MEL was shown to activate a death receptor-induced apoptotic cell death pathway in ovarian cancer SKOV3 and PA-1 cells through enhancement of DR3, DR4, and DR6 expression and inhibition of JAK2/STAT3 pathway [41]. Another study reported that MEL induces apoptosis in leukemic U937 cells through downregulating Akt signal pathways [56]. Furthermore, in this study, MEL-induced apoptosis was also accompanied by downregulation of Bcl-2, activation of caspase-3, downregulation of the inhibitor of apoptosis protein family proteins. Treatment of U937 cells with the caspase-3 inhibitor, z-DEVD-fmk, was capable of significantly restoring cell viability in MEL-treated cells. Additionally, the caspase-3 mediated apoptotic response was significantly attenuated in

Bcl-2-overexpressing U937 cells treated with MEL. Overall, the results of this study indicated that key regulators in MEL-induced apoptosis in human leukemic U937 cells include Bcl-2 and caspase-3, which are controlled through the Akt signaling pathway [56].

Another study reported that MEL can induce apoptosis of HCC cells by activating Ca^{2+} /calmodulin-dependent protein kinase, transforming growth factor-beta-activated kinase 1 (TAK1), and JNK/p38 MAPK. MEL-induced apoptosis was inhibited by calcium chelator, by inhibitors for Ca^{2+} /calmodulin-dependent protein kinase, JNK and p38, and by dominant negative TAK1. Further, in the presence of MEL, TRAIL-induced apoptosis was significantly increased in TRAIL-resistant HCC cells. Overall the data suggested that MEL can synergize with TRAIL in the induction of HCC cell apoptosis by activating the TAK1-JNK/p38 pathway but inhibiting the $\text{I}\kappa\text{B}\alpha$ kinase-NF κB pathway [57]. Another study tested the efficacy of MEL in gastric cancer and observed that the agent induces apoptosis in SGC-7901 cells [58]. The accumulated data suggested that MEL induces early apoptosis, induces ROS levels, and induced caspase-3 activity. Further, with the addition of the caspase-3 inhibitor, caspase-3 activity was significantly decreased compared to the control group. The expression of the Cyt C, Endo G, and AIF proteins in SGC-7901 cells was significantly higher than those in the control, while the expression of the Smac/Diablo protein was significantly lower.

In osteosarcoma MG63 cells, MEL induced a $[\text{Ca}^{2+}]_i$ increase by causing Ca^{2+} entry through L-type Ca^{2+} channels in a manner independent of protein kinase-C and phospholipase A(2) activity; and this $[\text{Ca}^{2+}]_i$ increase subsequently caused apoptosis [59]. Another study explored the effects of MEL on apoptosis in osteosarcoma and fetal osteoblast cells and the mechanism that induced MG63 cell growth was also explored. The results indicated that the expression or incubation of MEL in the MG63 cells triggered apoptosis and the inhibition of proliferation. One protein from the ER stress unfolded protein response pathway, IRE- α , was involved in the MEL-induced apoptosis in MG63 cells. MEL was noted to be serving as an effective factor that inhibits the proliferation of MG63 cells via activating the ER stress-mediated apoptosis pathway. Further, this activation was triggered by the IRE- α pathway mediated by inducing CHOP protein expression [60].

Effects on cell cycle regulation

Cell cycle is the process by which cells progress and divide and normally it is regulated by a series of signaling pathways by which a cell grows, replicates its DNA and divides. This process also includes mechanisms to ensure errors are corrected, and if not, the cells undergoes apoptosis. In cancer however this regulatory process malfunctions which results in uncontrolled cell proliferation and ultimately growth and progression of the tumor. Some evidences are available where MEL has been shown to regulate the cell cycle machinery. MEL inhibited HCC SMMC-7721 cells proliferation by down-regulation of MeCP2 *in vitro* through blocking of Shh signaling pathway and induction of G0/G1 cell cycle arrest [61]. Suppression of Rac1-dependent pathway was demonstrated by MEL in seven HCC cells leading to metastasis prevention in nude mouse models via reduction of motility and migration [62]. On the other hand, Jeong et al. [39] concluded the inhibitory effects of MEL against two breast cancer cell lines MDA-MB-231 and MCF-7. The authors suggested that

MEL inhibited the EGF-induced MMP-9 expression via blocking the NF- κ B and PI3K/Akt/mTOR pathway. They also stated that MEL significantly suppressed the EGF-induced FAK phosphorylation through inhibition of mTOR/p70S6K/4E-BP1 pathway. The presented data clearly suggested that the inhibitory effects of MEL on breast cancer motility and migration may be related to the inhibition of mTOR pathway [39].

Another study found that MEL inhibits cellular proliferation *in vitro* and significantly downregulated the expressions of CyclinD1 and CDK4. Further, MEL was also capable of upregulating the expression of PTEN and attenuating HDAC2 expression. In addition, treatment with MEL caused a downregulation of Akt phosphorylation, while overexpression of HDAC2 promoted Akt phosphorylation. These findings suggested that the inhibition of cellular growth by MEL might be led by HDAC2-mediated PTEN upregulation, Akt inactivation, and inhibition of the PI3K/Akt signaling pathways [63].

Effects on angiogenesis, invasion and necrosis

MEL has demonstrated potential efficacy in inhibiting angiogenesis and invasion markers in a variety of human cancers tested under preclinical model systems. MEL was observed to suppress HIF-1 α /VEGF expression through inhibition of ERK and mTOR/p70S6K pathway in human cervical carcinoma CaSki cells [64]. MEL was found to decrease the EGF-induced HIF-1 α protein and significantly regulated angiogenesis and tumor progression. Further, the inhibition of the HIF-1 α protein level was thought to be due to the shortened half-life by MEL. MEL specifically inhibited the EGF-induced HIF-1 α expression by suppressing the phosphorylation of ERK, mTOR and p70S6K and also blocked the EGF-induced DNA binding activity of HIF-1 α and the secretion of VEGF. In a subsequent study Yang et al. suggested that MEL enhances radiosensitivity of hypoxic head and neck squamous cell carcinoma by suppressing HIF-1 α [65].

MEL isolated from Iranian honey bee venom using reversed-phase HPLC exhibited toxicity on gastric cancer AGS cells was determined. MEL was observed to induce necrosis in these cells as determined by morphological evaluation, DNA fragmentation assay, and flow cytometric analysis [66]. Similar observations were made in HCC N1S1, BEL-740261 and McA-RH7777 cells where MEL increased calpain activity and cell necrosis. Further, MEL-induced cell necrosis was ameliorated by a calpain protease inhibitor [67].

The antitumor effect of MEL was compared with that of NS398, a COX-2 inhibitor, *in vivo* and *in vitro*. MEL suppressed the VEGF-A transfected highly metastatic Lewis lung cancer (VEGF-A-hm LLC) tumor growth. In addition, MEL significantly inhibited the number of vessels around VEGF-A-hm LLC cells. The results were superior to those obtained in the mice treated with NS398. Additionally MEL dose-dependently inhibited proliferation and tube formation in human umbilical vein endothelial cells (VEGF-A-HUVECs), without affecting cell viability in native HUVECs. MEL also decreased the expression of VEGF receptor-2, COX-2, and prostaglandin E2 in VEGF-A-transfected HUVECs. These effects were accompanied by a reduction of the phosphorylation of extracellular signal-regulated kinase 1/2 and c-jun N-terminal kinase, whereas it increased the phosphorylation of p38 MAPK [68].

Park et al. [69], examined the inhibitory effect of BV and its major peptides, MEL and apamin, on PMA-induced invasion induced by MMP-9 expression in Caki-1 renal cancer cells. BV and MEL significantly suppressed the PMA-induced invasion by inhibiting MMP-9 expression in Caki-1 cells. Furthermore, as evidenced by MMP-9 promoter assays, MEL inhibited MMP-9 gene expression by blocking the PMA-stimulated activations of AP-1 and NF-kappaB. In addition, MEL suppressed the PMA-induced phosphorylations of ERK and JNK [69]. Similarly, MEL was also reported to induce NFκB inactivation in HCC BEL-7402 [70] and prostate cancer cell lines, PC-3, LNCaP and DU-145 [42].

Wang et al. [71] evaluated the synergistic interaction of MEL and 5-Fu, DDP, and TXT on human gastric cancer cell line BGC-823 and further explored their possible mechanism of action. Both MEL and the chemotherapeutic agents inhibited the growth of BGC-823 and showed synergism in the combinations. Further, the gene expression of chemotherapeutic agent-associated genes such as thymidylate synthetase, excision repair cross-complementing gene 1, breast cancer susceptibility gene 1, beta-tubulin III, and microtubule-associated protein tau were observed to be suppressed [71].

Anticancer effects of MEL-conjugates

Application of MEL in cancer thereby has met with limited success due to several issues including toxicity, non-specificity, degradation, inefficient systemic delivery, limited bioavailability and hemolysis [33, 34, 72, 73]. To circumvent the issue with the use of MEL for cancer therapy multiple approaches have been utilized. Nanotechnology, gene therapy and immunoconjugation are currently being utilized to enhance the efficacy, selectivity and specificity in order to improve the outcome of MEL in cancer therapy under *in vitro* and in animal model systems.

Sun et al. [74] synthesized and tested a fused toxin, composed of disintegrin, uPA-cleavable linker, and MEL. The DLM (disintegrin-linker-Melittin) linker was uPA-cleavable, enabling DLM to release MEL. The study reported that DLM had less binding activity than the native form. Treating tumors expressing uPA with DLM enhanced tumor cell killing as well as reduced toxicity to erythrocytes and other non-cancerous normal cells. DLM showed a dose-dependent cytotoxicity against BT-549, MDA-MB-231, SMMC-7721, MCF-7, and SKOV-3 cells without showing any significant cytotoxicity against normal MCF-10, L-02 and HEK293 cells. Data revealed tumor cell necrosis as the mechanism of cell death, and the fused DLM toxin with an uPA-cleavable linker enhanced tumor selectivity and killing ability [74].

Liu et al. produced a novel fusion protein (Melittin-mutant human interleukin 2, Melittin-MhIL-2) comprising a mutant human interleukin 2 genetically linked to MEL [75]. The fusion protein directly inhibited the growth of human ovarian cancer SKOV3 cells *in vitro* and inhibited tumor growth in ovarian cancer mice. Later, in a separate study the authors assessed the antitumor immune response and antitumor effect of the conjugate against cancers of different tissue origins both *in vitro* and *in vivo* [76]. The Melittin-MIL-2 was very effective in inducing T cell and NK-cell cytotoxicity and the fusion protein significantly increased IFN-γ production in PBMCs. *In vitro*, the Melittin-MIL-2 mediated immune cells killing or directly killed the cancer cell lines of different tissue origins. *In vivo*, the fusion

protein exhibited stronger inhibition on the growth of transplanted human tumors compared to rIL-2. Furthermore, the fusion protein reduced lung metastasis of breast cancer [76].

A study constructed a triple-controlled cancer-selective oncolytic adenovirus, QG511-HA-Melittin, carrying MEL gene, in which hypoxia-response element (HRE)-AFP promoter was used to control viral E1a expression targeting AFP-positive cancer cells in hypoxia microenvironment, and the E1b-55 kDa gene was deleted in cancer cells with p53-deficiency. QG511-HA-Melittin had a strong inhibition effect on AFP-positive hepatocellular carcinoma cell proliferation, such as Hep3B and HepG2, whereas, there was low or no inhibition effect of QG511-HA-Melittin on AFP-negative cancer cells SMMC-7721 and normal cells L-02. In the *in vivo* experiment QG511-HA-Melittin significantly inhibited the growth of HCC xenografts [37]. Wang et al. [77] generated a protein containing VEGF165 fused to MEL. The activity of VEGF165-Melittin fusion protein was compared with MEL for its ability to suppress the growth of tumor cell line. The fusion toxin selectively inhibited growth of human hepatocellular carcinoma HepG-2 cell line with high expression of VEGFR-2. In an *in vivo* initial experiment, the fusion protein inhibited tumor growth in xenografts assays. Furthermore, successful expression and characterization of the fusion protein demonstrated its efficacy for use as a novel treatment strategy for cancer. In another study recombinant adenoviruses carrying the MEL gene and alpha-fetoprotein (AFP) promoter (Ad-rAFP-Mel) were constructed through a bacterial homologous recombinant system. The MEL mRNA was transcribed in BEL-7402 hepatocellular carcinoma cells transduced by Ad-rAFP-Mel. The efficiency of adenovirus-mediated gene transferred to BEL-7402 cells was 100% when the multiplicity of infection of Ad-rAFP-Mel was 10 *in vitro*, and was also high *in vivo*. The inhibitive rates of recombinant adenovirus Ad-rAFP-MEL for SMMC7721 cells, BEL7402 cells and L-02 cells were about 16.1%, 66.2% and 7.5%, respectively, similarly, the inhibitive rates for recombinant adenovirus Ad-CMV-MEL for the same cells were about 65.9%, 58.9% and 31.7%, respectively whereas a significant antineoplastic effect was observed *in vivo* by intratumoral injection of Ad-rAFP-MEL [78, 79].

The ability of MEL to kill HepG2 cells *in vitro* was increased after being incorporated into AM-2 [80] or EGFP [81]. Results of cell growth inhibition tests confirmed that the affinity of MEL was increased after being incorporated into AM-2, and AM-2-Melittin specifically targeted and killed HepG2 cells *in vitro* [80].

Nanotechnology and gene therapy are introduced together to provide another relatively safe, highly effective MEL-conjugate strategy in HCC treatment. A non-viral vector (pSURV-Mel), encoding MEL gene, was developed to evaluate its anti-tumor effect in HCC cell lines and *in vivo* in a human HCC xenograft tumor. The accumulated data showed that the survivin promoter was specifically activated in tumor cells, and the pSURV-Mel plasmid expressed MEL selectively in tumor cells and also induced cytotoxicity. The intratumoral injection of pSURV-Mel significantly suppressed the growth of xenograft tumors [82].

In another study a recombinant immunotoxin was constructed by which MEL was fused to an anti-asialoglycoprotein receptor (ASGPR) single-chain variable fragment antibody (C1), and the targeting ability and cytolytic efficacy of the fusion protein were studied. The data suggested that the recombinant protein C1M was expressed in *Escherichia coli* as a soluble

style. Binding of C1M to the surface of hepatocellular carcinoma (HCC) cells was also confirmed. C1M kept the hemolytic activity of MEL and exhibited cytolytic capacity to HepG2 cells and the effects were greatly inhibited by co-administration with asialoorosomucoid, a natural ligand for ASGPR [83].

Liu et al. [84] constructed a novel fusion protein, sTRAIL-Melittin, containing a small ubiquitin-related modifier (SUMO) tag and expressed this fusion protein in *E. coli* to ameliorate the cytotoxicity of MEL on cells and to enhance the activity of TRAIL. The results demonstrated that sTRAIL-Melittin had cytotoxic and apoptotic activity in K562 leukemia cells and HepG2 liver carcinoma cells, while it had only a minimal effect on erythrocytes and normal HEK293 cells. Furthermore, sTRAIL-Melittin also showed antibacterial activity to *Staphylococcus aureus* [84]. Huang et al. [85] designed a hybrid cytolytic peptide, α -Melittin, in which the N-terminus of MEL was linked to the C-terminus of an amphipathic α -helical peptide via a GSG linker and developed its lipid nanoparticles. The collected data confirmed that α -Melittin peptides were efficiently released from the nanoparticles and were cytotoxic to the melanoma cells. Further, under *in vivo* conditions the growth of melanoma cells was blocked by the α -Melittin-NPs, with an 82.8% inhibition rate relative to the PBS-treated control group [85].

Holle et al. [86] utilized a different approach of recombinant adenovirus with an MMP2 cleavable fusion gene between LAP and MEL. When delivered through recombinant adenovirus, this latent fusion protein was able to specifically target tumor cells both *in vitro* and *in vivo*. The *in vitro* studies showed that the MEL-MMP2-LAP recombinant adenovirus can be activated by MMP2 and leads to the release of MEL to lyse the target cells. *in vivo* studies also showed a 70% decrease in B16 tumor volume in MEL-MMP2-LAP recombinant adenovirus-treated mice as compared to the control mice. Further, no significant systemic toxicity was observed in the treated mice [86].

In another study, the efficacy of immunoconjugates containing a synthetic analogue of MEL was determined against human prostate cancer. In this study antibodies which recognize human prostate cancer cells, were cross-linked to synthetic MEL and tested in tumor xenografts. Systemic or intratumoral injection of immunoconjugates inhibited tumor growth in mice relative to carrier alone, unconjugated antibody and nonspecific antibody-peptide conjugates and improved survival for treated mice [87].

The applicability of fusion biotoxin combining pore-forming toxin, MEL and gelonin, a ribosome-inactivating protein, for the anti-cancer treatment was tested under *in vitro* assays and *in vivo* animal studies. The conjugate exhibited higher cellular uptake and significantly enhanced cytotoxic activity in Hela, colon CT26 and LS174T and malignant glioma 9L and U87 cancer cells over each agent alone or their physical mixture. Further, it also exhibited superior anti-tumor efficacy in HeLa tumor implanted on athymic nudes [88].

LeBeau et al. [89] evaluated Fibroblast-Activation Protein- α (FAP) as a tumor-specific target by constructing putative FAP-selective peptide protoxins through modification of the prodomain of MEL. Peptide protoxins were identified that were efficiently activated by FAP and selectively toxic to FAP-expressing cell lines. Intratumoral injection of these FAP-

activated protoxin produced significant lysis and growth inhibition of human breast and prostate cancer xenografts with minimal toxicity to the host animal [89].

In a study Su et al. [90] took advantage of Urokinase plasminogen activator (uPA)'s EGF-domain specific binding to uPAR and the anti-tumor effects of MEL to design and express fusion protein that contained uPA amino acids and MEL. The fusion protein was designed to compete with uPA for binding to uPAR and reduce the toxicity of MEL on normal tissues. The recombinant protein was able to suppress the growth, induce cell cycle arrest and apoptosis in SKOV3 cells without any obvious toxicity on normal tissues. In a similar type of study these authors constructed a pPICZαC-ATF-Melittin eukaryotic expression vector and the recombinant ATF-mellitin (rATF-MEL) inhibited the growth of SKOV3 cells and had no cytotoxicity on normal cells [91]. In another study, an MMP2 cleavable Melittin/avidin conjugate was designed and tested in prostate and ovarian cancer cells [92]. *in vitro* the Melittin/avidin conjugate demonstrated a strong cytolytic activity against cancer cells with high MMP2 activity viz. DU 145 and SK-OV-3 while exhibiting very little activity against normal L-cells that display low MMP2. *in vivo* the Melittin/avidin conjugate inhibited the tumor growth and the tumor size was significantly smaller in the group injected with the complex [92]. Winder et al. in a study [93] suggested that vector mediated delivery of MEL to tumor cells may prove useful for cancer gene therapy. The study utilized expression constructs carrying cecropin or MEL introduced into a human bladder carcinoma derived cell line and the resultant cell clones analyzed for tumorigenicity in nude mice. Expression of cecropin resulted in either a complete loss of tumorigenicity in some clones or reduced tumorigenicity, as measured by latency of tumor formation.

Nanotechnology for bee venom and Melittin

Nanotechnology mediated approaches to develop drugs have attracted intense attention in cancer prevention and therapy research. This technology appears to hold great promise in the field of cancer management because of the unique physicochemical properties of nanoparticles including nanometer size, large surface area-to-mass ratio, and efficient interaction with cells. Several nanotechnology mediated conjugates of MEL have already been successfully synthesized and tested in a variety of human cancers in preclinical models F. Hu et al. [94] prepared and tested the *in vitro* tumor cells selectivity of sterically stabilized immunoliposomal peptides in BV. The sterically stabilized liposomal peptide for bee venom (PBV-SL) was prepared using soybean phosphatidylcholine, cholesterol, and cholesterol-PEG-COOH and humanized anti-hepatoma disulfide-stabilized Fv (hdscFv25) was coupled to sterically stabilized liposomes. The study determined that the hdscFv25-immunoliposomes (SIL[hdscFv25]) were immunoreactive and these further showed higher tumor cells selectivity. PBV-SIL[hdscFv25] were able to kill SMMC-7721 cells *in vitro* with higher efficiency than non-targeted liposomes. However, no significant differences were observed between PBV-SIL[hdscFv25] and PBV-SL in HeLa cells [94].

An *in-silico* to *in-vitro* approach was utilized to develop well-defined, self-assembled, rigid-cored polymeric nano-conjugates for controlled delivery of MEL. The study utilized rigid core micellar systems stabilized by amphiphilic PS67-b-PAA27 (poly-styrene-b-polyacrylic acid) or by phospholipids encapsulation. MEL-PS67-b-PAA27 Polymer improved cell

proliferation inhibition of breast cancer cells either estrogen-negative MDA-MB-231 cells or estrogen-positive MCF-7 cells [95].

Perfluorocarbon nanoemulsion vesicle was used to deliver MEL *in vivo*. The nanovehicle carriers were synthesized as an oil-in-water emulsion composed of a liquid perfluorooctyl bromide (PFOB) core having a monolayer of phospholipid forming a stabilizing interface with the aqueous media. MEL was delivered using the nanoconjugates to target and kill syngeneic (B16F10 mouse melanoma), xenograft (MDA-MB-435 human breast cancer) and precancerous lesions in K14-HPV16 mice with squamous dysplasia and carcinoma [72]. The study demonstrated that the favorable pharmacokinetics of the nanocarrier allows accumulation of MEL in murine tumors *in vivo* and a dramatic reduction in tumor growth without any apparent signs of toxicity. In addition, direct assays demonstrated that molecularly targeted nanocarriers selectively delivered MEL to multiple tumor targets, including endothelial and cancer cells, through a hemifusion mechanism. Later the same group, using similar Perfluorocarbon nanoparticles, demonstrated that intravenous administration of MEL prodrug-loaded nanoparticles in a mouse model of melanoma significantly decreased tumor growth rate. Treatment with prodrug-loaded nanoparticles resulted in a significant decrease in tumor growth rate compared to saline and blank nanoparticle treatment [96].

MEL has issue with solubility and stability so a study modified it with an anionic agent, sodium dodecyl sulfate by hydrophobic ion-pairing. The formed complex was found to be soluble in organic solvents. The complex was formulated in poly(D,L-lactide-coglycolide acid) nanoparticles by emulsion solvent diffusion method. The nanoparticles were about 130 nm in size with a high encapsulation efficiency. However, the growth inhibitory effects of modified MEL and MEL-loaded nanoparticles were not changed in MCF-7 cells as compared to free MEL [97]. Another group developed an environment-sensitive MEL delivery system, dual secured nanosting (DSNS), through the combination of a zwitterionic glycol chitosan and disulfide bonds to safely deliver MEL to cancer cells. MEL loaded DSNS were highly efficient in killing MCF-7, HCT-116, SKOV-3, and NCI/ADR-RES cells at 5 μ M while not showing any hemolytic effects [73].

A study targeted HER2-overexpressing human breast cancer cells with pegylated immunoliposomes bearing trastuzumab and MEL. Using a panel of human breast cancer cells with different HER2 expression levels the study demonstrated that these immunoliposomes decreased cancer cells viability in a dose-response manner and in correlation to their level of HER2 expression. The morphological changes observed in the treated cells suggested a cytolytic process suggesting effective strategy for the treatment of HER2-overexpressing tumors [98].

Conclusions and future prospects

The accumulated data so far clearly suggest that both BV and its individual constituents especially MEL has potential for cancer therapy. The anti-cancer properties of MEL should be further studied and developed as an alternative approach of cancer therapy. If successful, this approach could be of tremendous value in many parts of world where expensive

chemotherapeutic drugs are not available in the healthcare system. As other chemotherapeutic agents do, both BV and MEL have shown significant efficacy of inducing apoptosis, necrosis, mitochondrial disruption, blocking of angiogenesis, cell cycle arrest and inhibition of cancer cell metastasis and invasion (Fig. 3). According to the studies presented in this review article, over 60 different cancer cells have been investigated for their response to BV and MEL alone. BV and its main peptide MEL are attractive candidates for cancer therapy however some non-specific cytotoxicity along with its *in vivo* lysis property restricted the therapeutic potentiality in clinical applications. Consequently, gene, immune and nanotechnology strategies were utilized to improve the outcome of MEL in cancer therapy. Based on the available evidence, we believe that, the use of nanotechnology is presently the best optimization strategy for circumventing the issue associated with the use of BV and MEL. The current literature clearly suggests that the use of nanotechnology mediated delivery of MEL can enhance the therapeutic efficacy of MEL in addition to providing significant systemic delivery to target cancer cells with minimal or none hemolytic effect [72, 73, 85, 96, 97, 99]. We believe that several further refinements are still needed to further improve the outcome of BV and MEL in cancer therapy. The clinical translation of BV or MEL is still a long way to be achieved but we believe that the ongoing work on the subject will ultimately allow these agents to be considered as a potential anti-cancer therapy in the years to come.

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Fig. 1. Mellitin chemical composition
(A) Molecular structure, and (B) Amino acid sequences of two Mellitin isoforms, *Apis mellifera* and *Apis cerana*.

Fig. 2. Schematic drawing of lytic mechanism for melittin
(A) Melittin and membrane bilayer, (B) Pore formation, and (C) membrane lysis.

Fig. 3. Schematic drawing of the mechanism of action of melittin

This cartoon is based on the available literature about the anticancer effects of melittin.

Table 1

Dried bee venom composition.

Molecular group	Component	MW	Percent in dry venom
Enzymes	Phospholipase A2	19,000	10–12
	Phospholipase B		1
	Hyaluronidase	38,000	1.5–2
	Acid phosphomonoesterase	55,000	1
	α -Glucosidase	170,000	0.6
	Phosphatase		1
	Lysophospholipase		1
Peptides	Melittin	2840	40–50
	Apamine	2036	2–3
	MCD	2588	2–3
	Secapine	3000	0.5–2
	Pamine		1–3
	Minimine	6000	2–3
	Adolapine	11,500	1
	Procamine A, B	600	1.4
	Protease inhibitor	9000	<0.8
	Tertiapine	2500	0.1
	Cardiopep	2500	<0.7
	Melittin F		0.01
Phospholipids		700	1–3
Amines	Histamine	307.14	1.5
	Dopamine	189.64	0.13–1
	Noradrenalin	169.18	0.1–0.7
	Neurotransmitters		0.1–1
Amino acids	γ -aminobutyric acid	189.64	0.13–1
	α -amino acids	169.18	0.1–0.7
Carbohydrates	Glucose	180	2–4
	Fructose		
Pheromones	Iso-pentyl acetate; n-butyl acetate;	200	4–8
	iso-pentanol; n-hexyl acetate;		
	n-octyl acetate; 2-nonanol;		
	n-decyl acetate; benzyl acetate;		
	benzyl alcohol; (2)-11-eicosen-1-ol		

Table 2

Anticancer effects of MEL, BV and their conjugates.

Cancers	Cell lines	MEL	BV	Dose	IC ₅₀	Biological effects
Conjugates						
Hepatocellular carcinoma	SMMC-7721	MEL		1, 2 and 4 µg/mL [55]; 0.5, 1, 2, 4, 8, 16 and 32 µg/mL (<i>in vitro</i>); 80 µg/kg (<i>in vivo</i>) [62]; 1, 5 and 10 µg/mL (<i>in vitro</i>); 50 and 100 µg/kg (<i>in vivo</i>) [57].	–	Cell proliferation inhibition; Induction of G0/G1 cell cycle arrest [61]. Cancer cell metastasis, motility and migration inhibition [62]. Apoptosis induction; Activated CaMKII-TAK1-MKK-JNK/p38 pathway; Inhibited TAK1-mediated activation of IKK-NFκB pathway; Inhibition of TRAIL-induced activation of IKK-NFκB [57].
		DLM		0.041, 0.082, 0.164, 0.328, 0.656, 1.313, and 2.626 µM.	130 nM	Cell necrosis induction [74].
		MEL-MIL-2		50, 100, 150 and 200 µg/mL (<i>in vitro</i>); 200 µM (<i>in vivo</i>).	–	Cell proliferation inhibition; Inhibition of transplanted human tumor growth [76].
		Ad-QG511-HA-MEL		A series of MOIs (MOI = 0, 0.1, 0.5, 1, 2, 5, 10, 20, 50, and 100).	–	Strong inhibition effect on AFP-positive hepatocellular carcinoma cell proliferation; Inhibition of HCC xenografts growth [37].
		ASGPR-MEL		1.5 or 0.15 µg/mL	–	Cellular death induction [83].
		Ad-rAFP-MEL		–	–	Cellular proliferation inhibition of AFP-producing human HCC <i>in vitro</i> and <i>in vivo</i> ; Significant antineoplastic effect <i>in vivo</i> [78, 100].
		PBV-SIL [hdscFv25]		–	–	Higher cancer cells selectivity; Cancer cells growth inhibition [94].
		Ad-CMV-MEL		–	–	Tumor growth inhibition [79].
		MEL		1,4 and 8 µg/mL [59]; 0.5, 1, 2, 4, 8, 16 and 32 µg/mL (<i>in vitro</i>); 80 µg/kg (<i>in vivo</i>) [56]; 1, 5 and 10 µg/mL (<i>in vitro</i>); 50 and 100 µg/kg (<i>in vivo</i>) [58].	–	Cell proliferation inhibition; Downregulation of CyclinD1 and CDK4 [63]. Tumor cell metastasis, motility and migration inhibition [62]. Apoptosis induction; Mitochondrial permeability disruption [57].
		BV		0.01, 0.05, 0.1, 0.5, 1, 5, 10, 20, 40, 80 and 160 µg/mL.	–	Induced cytotoxic, genotoxic and mutagenic potentials to cancer cells [47].
		Ad-QG511-HA-MEL		A series of MOIs (MOI = 0, 0.1, 0.5, 1, 2, 5, 10, 20, 50, and 100)	–	Strong inhibitory effect on AFP-positive cell proliferation [37].
		sTRAIL-MEL		1, 2, 3 µM	3 µM	Cellular apoptosis [84].
		AM-2-MEL		40 µM	–	Cell growth inhibition [80].
		ASGPR-MEL		1.5 or 0.15 µg/mL	–	Cell death [83].
MEL-EGFP		–	–	Cell proliferation inhibition [81].		
Nano-(pSURV-MEL)		–	–	Tumor xenograft growth inhibition; Induction of cell death and apoptosis [82].		

Cancers	Cell lines	MEL	BV	Dose	IC ₅₀	Biological effects
Conjugates						
Breast cancer	BEL-7402	MEL		0.5, 1, 2, 4, 8, 16 and 32 µg/mL (<i>in vitro</i>); 80 µg/kg (<i>in vivo</i>) [62]; 1, 5 and 10 µg/mL (<i>in vitro</i>); 50 and 100 µg/kg (<i>in vivo</i>) [57]. low dose melittin (40 µg/kg), moderate dose melittin (60 µg/kg) and high dose melittin (80 µg/kg) [67].	–	Inhibited metastasis, motility and migration [62]. Induced apoptosis [57, 101]. Tumor tissue necrosis and angiogenesis inhibition [70].
		Ad-rAFP-MEL		–	–	Inhibited cellular proliferation and induced apoptosis [102, 103]. Inhibited AFP-producing cells <i>in vitro</i> and <i>in vivo</i> ; Induced significant antineoplastic effect [78, 79, 100].
	Hep3B	MEL		1, 5 and 10 µg/mL (<i>in vitro</i>); 50, 100 µg/kg (<i>in vivo</i>) [57].	–	Inhibited metastasis, motility and migration [62]. Induced apoptosis; Activated CaMKII-TAK1-MKK-JNK/p38 pathway; Inhibited TAK1-mediated activation of IKK-NFκB pathway; Synergized with TRAIL in activation of TAK1-JNK/p38 and inhibited TRAIL-induced activation of IKK-NFκB [57].
		Ad-QG511-HA-MEL		A series of MOIs (MOI = 0, 0.1, 0.5, 1, 2, 5, 10, 20, 50, and 100).	–	Cell proliferation inhibition on AFP-positive HepG2 cells [37].
	MHCC97-H	MEL		0.5, 1, 2, 4, 8, 16 and 32 µg/mL (<i>in vitro</i>); 80 µg/kg (<i>in vivo</i>).	4.06 µg/mL	Tumor cell metastasis inhibition; Cell motility and migration inhibition [62].
		VEGF165-MEL		0.5 µM	–	Cellular and tumor growth inhibition [77].
	MHCC97-L	MEL		0.5, 1, 2, 4, 8, 16 and 32 µg/mL (<i>in vitro</i>); 80 µg/kg (<i>in vivo</i>).	9.24 µg/mL	Tumor cell metastasis inhibition; Cell motility and migration inhibition [62].
	HuH7			–	–	
	PLC/RPE/5			–	–	
	NIS1	MEL		–	–	Cell necrosis [67].
	McA-RH7777			–	–	
	MDA-MB-231	MEL		0.5, 1, 2 and 4 µg/mL	–	Inhibited cell motility, migration and invasion [39].
		BV		0.5, 1, 2 and 4 µg/mL	–	Inhibited EGF-induced cell migration and motility; Reduced EGF-stimulated F-actin reorganization; Inhibited EGF-induced activation of MMP-9 and FAK [39].
		DLM		0.041, 0.082, 0.164, 0.328, 0.656, 1.313, and 2.626 µM.	130 nM	Cell necrosis [74].
		MEL-MIL-2		50, 100, 150 and 200 µg/mL (<i>in vitro</i>); 200 µM (<i>in vivo</i>).	–	Cell death induction directly or indirectly by inducing T cell and NK-cell cytotoxicity; Inhibited the growth of transplanted human tumors; Decreased the immunosuppressive cells causing reduced lung metastasis of breast cancer [76].
	MEL-PS67-b-PAA27 Polymer		10–1000 nM	40 nM	Inhibited estrogen-negative performance of the MDA-MB-231 cells [95].	

Cancers	Cell lines	MEL	BV	Dose	IC ₅₀	Biological effects
Conjugates						
	MDA-MB-435	MEL-PFC-NP		2.5 mg/kg <i>in vivo</i>	–	Synergistic delivery of significant MEL payloads to target and kill xenograft MDA-MB-435 cell lines [72].
	MCF-7	MEL		0.5, 1, 2 and 4 µg/mL	–	Inhibited cell motility, migration and invasion via suppressing of EGF-induced tumor invasion; Inhibition of MMP-9 expression and FAK phosphorylation; PI3K/Akt/mTOR phosphorylation and mTOR signaling pathway inhibition [39].
		BV		0.5, 1, 2, 4 µg/mL	–	Suppressed PMA-induced MMP-9 expression and activity [54]. Inhibited EGF-induced cell migration and cell motility; Inhibition of EGF-induced activation of MMP-9 and FAK [39].
		DLM		0.041, 0.082, 0.164, 0.328, 0.656, 1.313, and 2.626 µM.	130 nM	Cell necrosis [74].
		MEL-PLGA-NP		1.25, 2.5, 5, 10, 20 and 40 µg/mL	–	Enhanced growth inhibitory effects [97].
		FAP-proMEL		45 mg/kg <i>in vivo</i>	–	Significant lysis and growth inhibition of cancer cells with minimal toxicity to the host animal [89].
		MEL-PS67-b-PAA27 Polymer		10–1000 nM	80 nM	Inhibited estrogen-positive performance of the MCF-7 cells [95].
		MEL-DSNS-NP		0.1–10 µM	–	Cellular death without any hemolytic effect [73].
	BT-549	AbDR-MEL		0.1–0.5 mM	–	Decreased cell viability and cell growth inhibition combined with dwindling in HER2 expression [98].
	SK-BR-3	DLM		0.041, 0.082, 0.164, 0.328, 0.656, 1.313, and 2.626 µM.	130 nM	Cell necrosis [74].
Lung cancer		MEL		1, 2 and 3 µg/mL	–	Suppressed PMA-induced invasion and migration [66]
		AbDR-MEL		0.1–0.5 mM	–	Inhibited cell viability and cell growth in highly HER2-positive overexpression [98].
	JIMT-1	AbDR-MEL		0.1–0.5 mM	–	Cell viability and cell growth inhibition with down-regulation of HER2-positive overexpression [98].
	MCa	BV		<i>In vitro</i> (0.7125, 1.425, 2.85 µg/mL) and <i>in vivo</i> (150, 300, 600 µg/kg Mouse.	1.43 µg/mL (48 h) and 2.15 µg/mL (72 h)	Inhibited proliferation and metastasis <i>in vitro</i> and <i>in vivo</i> [52].
	A549	BV		1, 2 and 5 µg/mL [43]. 1, 2, 3 and 4 µg/mL [52]	2.91 µg/mL [43] or 3.6 µg/mL [52] 48 h	Inhibited cancer growth through activation of DR-induced apoptotic pathway and inactivation of NF-κB [44, 50].
		MEL-MIL-2		50, 100, 150 and 200 µg/mL (<i>in vitro</i>); 200 µM (<i>in vivo</i>).	–	Cellular death directly or indirectly by inducing T cell and NK-cell cytotoxicity; Inhibition of transplanted human tumor growth via increasing of IFN-γ production in PBMCs and decreasing the immunosuppressive cells causing reduced lung metastasis of breast cancer [76].

Cancers	Cell lines	MEL	BV	Dose	IC ₅₀	Biological effects
		Conjugates				
Leukemia		ASGPR-MEL		1.5 or 0.15 µg/mL	–	Cellular death [83].
	NCI-H69	CA-MEL		1, 10 and 100 µg/mL	32.2 µM	Hybrid peptide showed greater antitumor activity than MEL does alone [104].
	NCI-H128				28.6 µM	
	NCI-H146				28.6 µM	
	NCI-H1299	BV		1 and 10 µg/mL	–	Induced apoptosis [49].
	NCI-H460	BV		1, 2 and 5 µg/mL [43], 1, 2, 3 and 4 µg/mL [52]	3.14 µg/mL [43] or 3.3 µg/mL [52] 48 h	Inhibited cancer growth through activation of DR-induced apoptotic pathway and inactivation of NF-κB [44, 50].
	LLC	MEL		0.5 and 5 mg/kg <i>in vivo</i>	–	Suppressed VEGF-A-induced tumor growth by blocking VEGFR-2 and the COX-2-mediated MAPK signaling pathway [68].
	K562	sTRAIL-MEL		0.5, 1 and 2 µM	–	Cellular apoptosis [84].
	Jurkat	MEL		1, 5 and 10 µg/mL (<i>in vitro</i>), 50 and 100 µg/kg (<i>in vivo</i>)	–	Induced apoptosis; Activated CaMKII-TAK1-MKK-JNK/p38 pathway; Inhibited TAK1-mediated activation of IKK-NFκB pathway; Synergized with TRAIL in activation of TAK1-JNK/p38 and inhibited TRAIL-induced activation of IKK-NFκB [57].
	U937	MEL		0.5, 1, 2 and 3 µg/mL [56], 1–70 µM [103]	–	Induced apoptosis [56]. <i>in vitro</i> cell lysis through activation of cellular PLD that might hydrolyze membrane phospholipids leading to pore formation [105].
Ovarian cancer		BV		0.5, 1, 2 and 3 µg/mL	–	Induced apoptosis via Bcl-2 and caspase-3 key regulators through down-regulation of ERK and Akt pathway [45].
	LI210	MEL		0.25, 0.5, 1, 2, 4, 8 µM [106]	<1 µM [107]	Caused cellular lysis [106]. Modulated the cell proliferation through calmodulin inhibition [107].
	L5178Y HL-60	MEL		1–100 µM		Modulated cell proliferation through calmodulin inhibition [107].
	SKOV3	MEL		0.5, 1 and 2 µg/mL	1.5 µg/mL (24 h)	Induced apoptotic cell death via enhancement of DR3, and DR6 expressions and inhibition of STAT3 pathway [41].
		MEL-N		0.1, 0.25, 0.5, 1, 2.5, and 5 µM	–	Demonstrated cytotoxic activity [55].
		BV		1, 2, 5 µg/mL	3.8 µg/mL (24 h)	Induced apoptotic cell death via activation of DR3, and DR6 expressions and inhibition of STAT3 pathway [41].
		MEL-MIL-2		50, 100, 150 and 200 µg/mL (<i>in vitro</i>); 200 µM (<i>in vivo</i>)	–	Killed cancer cells directly <i>in vitro</i> or indirectly by inducing T cell and NK-cell cytotoxicity. In addition, inhibited the growth of transplanted human tumors <i>in vivo</i> via increasing of IFN-γ production in PBMCs decreasing the immunosuppressive cells causing reduced lung metastasis of breast cancer [76].
		MEL-DSNS-NP		0.1–10 µM	–	Significant cell death without any hemolytic effect [73].

Cancers	Cell lines	MEL	BV	Dose	IC ₅₀	Biological effects
Conjugates						
Melanoma	PA-1	rhupa1-43-MEL		20, 40 and 80 µg/mL	–	Induced cell cycle arrest and apoptosis [90].
		MEL-MhIL-2		0.125, 0.5, 2 and 8 µM <i>in vitro</i> ; 8 µM <i>in vivo</i>	–	Inhibited the growth and decreased tumor xenograft growth [75].
		DLM		0.041, 0.082, 0.164, 0.328, 0.656, 1.313, and 2.626 µM	130 nM	Cell necrosis [74].
		MEL-Avidin		–	–	<i>In vitro</i> , showed strong cytolytic activity with high MMP2 activity; <i>in vivo</i> , the size of tumors injected with the conjugate was significantly smaller as compared to untreated tumors [92].
		MEL-IL-2((88)A, (125)A)		1, 2, 3, 4 and 5 µM	–	Inhibited the cellular growth <i>in vitro</i> [108].
	A2780	rATF-MEL		7.5, 15, 30, 60 and 120 µg/mL	–	Inhibited growth of cancer cells with no cytotoxicity on normal cells [91].
		MEL		0.5, 1 and 2 µg/mL	1.2 µg/mL (24 h)	Induced apoptotic cell death via enhancement of DR3, and DR6 expressions and inhibition of STAT3 pathway [41].
		MEL-N		0.1, 0.25, 0.5, 1, 2.5, and 5 µM	–	MEL showed stronger cytotoxic activities than MEL-N against ovarian cancer cell lines [55].
		BV		1, 2 and 5 µg/mL	2.6 µg/mL (24 h)	Induced apoptotic cell death via activation of DR3, and DR6 expressions and inhibition of STAT3 pathway [41].
		MEL		0.4, 0.15, 0.1, 0.35, 0.6, 0.85 and 1.1 µg/mL	6.8 µg/mL (24 h)	Reduced levels of amino acids in the proline/glutamine/arginine pathway; Decreased levels of carnitines, polyamines, ATP and NAD ⁺ ; affected the lipid composition of the cancer cells [109].
C32	NCI/ADR-RES B16F10	BV		4 and 8 µg/mL	8 µg/mL (24 h)	Induced apoptosis [53].
		MEL-DSNS-NP		0.1–10 µM	–	Cellular death without any hemolytic effect [73].
		p5RHH/siRNA-NP		10–200 nM	–	Inhibited cell proliferation and decreased viability <i>in vitro</i> , and inhibited tumor growth and prevent angiogenesis <i>in vivo</i> [99].
		α-MEL-NPs		5, 10 and 20 µM (<i>in vitro</i>); 20 mg/kg (<i>in vivo</i>)	11.26 µM	Blocked the growth of cells <i>in vivo</i> through intravenous administration with minimal hemolysis [85].
		MEL-PFC-NP		8.5 mg/kg <i>in vivo</i>	–	Incorporation of MEL onto nanoemulsions inhibited cancer growth producing a 7-fold protection from hemolysis [72].
	C32	MEL-MMP2-LAP		100 µL <i>in vivo</i>	–	70% decrease in tumor volume <i>in vivo</i> as compared to control mice with no significant systemic toxicity in the treated mice [86].
		PA-MEL-PFC-NP		10, 20, 30, 40, 50 and 60 µM (<i>in vitro</i>); 1 mg/kg (<i>in vivo</i>)	8.8 µM	Inhibited cell viability <i>in vitro</i> and decreased tumor growth rate <i>in vivo</i> via intravenous administration without hemolytic activity [96].
		MEL-PFC-NP		13.5 mg/kg <i>in vivo</i>	–	A 5-fold reduction in IC ₅₀ after specific targeting of MEL-loaded NP to melanoma cells. Therefore, a 5-fold protection

Cancers	Cell lines	Conjugates			Dose	IC ₅₀	Biological effects
		MEL	BV				
Gastric cancer	A2058	MEL		0.5, 1, 2, 4 and 6 µg/mL	–		from hemolysis were added to MEL by nano conjugate in melanoma growth inhibition [72]. Induced apoptosis via elevation of calcium and caspase-independent pathway [43].
		BV		0.5, 1, 2 and 4 µg/mL	–		Raised calcium and regulated caspase-independent pathway inducing apoptosis. Incubation of cells with BV increased JNK and ERK rapidly, while BV <i>in vivo</i> administration inhibited p38 and AKT [43].
	M2R	FI-MEL		0.1–100 µM	–		Inhibited cell proliferation suppressing MSH receptor function, prostaglandin E1-, GTP gamma S, and forskolin-stimulated AC activity in M2R cell lines [110].
	K1735M2	BV		2.8, 11, 14.2 µg/mL	10 µg/mL (24 h)		Inhibited cell proliferation <i>in vitro</i> [48].
	BGC-823	5-Fu + MEL DDP + MEL TXT + MEL		–	–		Inhibited cells growth through down-regulating chemotherapeutic agent-associated genes TS, ERCC1, BRCA1, TUBB3, and MAPT [71].
Prostate cancer	AGS	MEL		0.25, 0.5, 1, 2, 4 and 8 µg/mL	–		Induced necrosis, apoptosis and inhibited the proliferation of the cancer cells [66].
	SGC-7901	MEL		1, 2 and 4 µg/mL	–		Induced cell apoptosis through mitochondria pathways that was confirmed by typical morphological changes [58].
	PC-3	MEL		0.5, 1 and 2.5 µg/mL	1.8 µg/mL (72 h)		Induced apoptotic cell death [42, 111] through activation of caspase pathway via inactivation of NF-κB [42].
		BV		1, 5, 10 µg/mL (<i>in vitro</i>) and 3, 6 mg/kg (<i>in vivo</i>)	6.1 µg/mL (72 h)		Induced apoptosis through upregulation of caspase pathway via NF-κB inhibition [42].
	LNCaP	MEL		0.5, 1 and 2.5 µg/mL	2.9 µg/mL (72 h)		Induced apoptotic cell death through activation of caspase pathway via inactivation of NF-κB [42].
DU-145		BV		1, 5 and 10 µg/mL (<i>in vitro</i>), 3, 6 mg/kg (<i>in vivo</i>)	14.2 µg/mL (72 h)		Significant lysis and growth inhibition of cancer cells with minimal toxicity to the host animal [89].
		FAP-proMEL		45 mg/kg <i>in vivo</i>	–		<i>In vitro</i> inhibition effects as well as inhibited tumor growth <i>in vivo</i> improving survival for treated mice [87].
		IMM (MEL-101)		–	–		Cell apoptosis through activation of caspase pathway via inactivation of NF-κB [42].
		MEL BV MEL-MMP2-LAP		0.5, 1 and 2.5 µg/mL 1, 5 and 10 µg/mL (<i>in vitro</i>), 3, 6 mg/kg (<i>in vivo</i>) 100 µL <i>in vivo</i>	1.5 µg/mL (72 h) 6.3 µg/mL (72 h) –		Decreased cell viability <i>in vitro</i> ; 70% decrease in B16 tumor volume in recombinant adenovirus-treated mice; No significant systemic toxicity [86].

Cancers	Cell lines	MEL	BV	Dose	IC ₅₀	Biological effects
Conjugates						
Cervical cancer	HeLa	MEL-Avidin		–	–	<i>In vitro</i> , showed strong cytolytic activity against cancer cells with high MMP2 activity. <i>In vivo</i> , the size of tumors injected with the MEL-Avidin conjugate was significantly smaller as compared to untreated tumors [92].
		IMM (MEL-101)		2, 4, 6 and 8 μ moles	–	<i>In vitro</i> inhibition effects as well as inhibited tumor growth <i>in vivo</i> improving survival for treated mice [87].
		MEL		1, 5 and 10 μ g/mL (<i>in vitro</i>), 50 and 100 μ g/kg (<i>in vivo</i>)	–	Induced apoptosis; activated CaMKII-TAK1-MKK-JNK/p38 pathway, inhibits TAK1-mediated activation of IKK-NF κ B pathway, synergized with TRAIL in activation of TAK1-JNK/p38 and inhibited TRAIL-induced activation of IKK-NF κ B [57].
		BV		0.7125, 1.425, 2.85, 7.125 or 14.25 μ g/mL	3 μ g/mL (72 h)	Inhibited cell proliferation, and clonogenicity might be through inhibition of calmodulin [46].
		Gel-MEL		10^{-11} – 10^{-5} M	1300 $\pm$ 480 nM (48 h)	Inhibited the growth via inhibiting of cellular protein synthesis and protein translation; Exhibited enhanced cytotoxic activity and greater cellular uptake [88].
Colon cancer		pLEGFP-C1-MEL-IL-2 ⁽⁸⁸⁾ A, 125A ^b		–	–	Inhibited cell proliferation and induced apoptosis in the tumor cells [112].
		ASGPR-MEL		1.5 or 0.15 μ g/mL	–	Cell death [83].
		PBV-SIL [hdscFv25]		–	–	Provided higher tumor cells selectivity and killed cancer cells <i>in vitro</i> with higher efficiency than non-targeted liposomes [94].
	CaSki	MEL-IL-2((88)A, (125)A1)		1, 2, 3, 4 and 5 μ M	–	Inhibited the growth cancer cells <i>in vitro</i> [108].
		MEL		0.5, 1 and 2 μ g/mL [64], 1, 2 and 3 μ g/mL [69].	–	Suppressed EGF-induced VEGF secretion and new blood vessel formation by inhibiting HIF-1 α . [64]. Repressed PMMA-induced invasion and migration via MMP-9 expression inhibition through blocking the activations of AP-1 and NF- κ B [69].
Malignant glioma	HCT-116	MEL-DSNS-NP		0.1–10 μ M	–	Significant cellular death without any hemolytic effect [73].
	CT26	Gel-MEL		10^{-11} – 10^{-5} M	1500 $\pm$ 350 nM (48 h)	Inhibited the growth via inhibiting of cellular protein synthesis and protein translation. Exhibited enhanced cytotoxic activity ted greater cell uptake [88].
	LS174T			10^{-11} – 10^{-5} M	3600 $\pm$ 990 nM (48 h)	
	9L	Gel-MEL		10^{-11} – 10^{-5} M	1200 $\pm$ 330 nM (48 h)	Exhibited enhanced cytotoxic activity ted greater cell uptake and inhibited the growth via inhibiting of cellular protein synthesis and protein translation [88].
	U251	MEL		1, 10, 20, 40, 80, 160 and 200 mg/L	–	Inhibited cell proliferation and induced apoptosis of cell lines <i>in vitro</i> [113].

Cancers	Cell lines	MEL	BV	Dose	IC ₅₀	Biological effects
		Conjugates				
Skin cancer	U87	MEL		1, 10, 20, 40, 80, 160 and 200 mg/L	–	
		Gel-MEL		10 ⁻¹¹ –10 ⁻⁵ M	890 ± 190 nM (48 h)	Inhibited the growth via inhibiting of cellular protein synthesis and protein translation. Exhibited enhanced cytotoxic activity ted greater cell uptake [88].
	SCC12	MEL		1–10 µM	1 µM (48 h)	Inhibited cell proliferation <i>in vivo</i> [30].
	SCC13	MEL		1–10 µg/mL	–	Inhibited cell proliferation <i>in vitro</i> via biochemical pathways of AA metabolism [114].
Osteosarcoma	SCC25	MEL		1–10 µM	2.2 µM (48 h)	Inhibited cell proliferation <i>in vivo</i> [30].
	MG63	MEL		0.5, 1 and 2 µM	–	Cell apoptosis via phospholipase A2 activation and Ca ²⁺ influx induction and Cell proliferation inhibition through activating inositol-requiring protein-1α and X-box binding protein 1-mediated apoptosis [59].
	U2 OS	MEL		16, 32 and 64 mg/L	–	Induced Cell apoptosis and inhibited cell proliferation via up-regulation of Fas expression [115].
HNSCC	CNE-2	MEL		1, 2, 3, 4 and 5 µM		<i>In vitro</i> and <i>in vivo</i> growth inhibition via cell apoptosis, and inhibition of HIF-1α and VEGF expressions that has been linked to hypoxia cell radio-resistance. The intraperitoneal injection significantly reduces the growth of tumors in CNE-2 tumor-bearing mice [65].
ESCC	KB	MEL		1, 2, 3, 4 and 5 µM		Induced cell apoptosis, and reduced HIF-1α and VEGF expressions that has been linked to hypoxia cell radio-resistance [65].
	ECA109	MEL		0.5, 0.75, 1, 2 and 5 µM	1.88 µM	Potently sensitized cells to radiation with a sensitization enhancement ratio of 1.15–1.42. In the same time, this radio-sensitization was accompanied with enhanced apoptosis and regulated by apoptosis proteins [37].
	TE13				1.64 µM	
Renal cancer	Caki-1	MEL		1, 2 and 3 µg/mL		Suppressed PMA-induced invasion and migration via MMP-9 expression inhibition through blocking the activations of AP-1 and NF-κB [69].
Bladder cancer	EJ	EV (Pre-pro-MEL)		–	–	Reduced tumorigenicity [93].
Neuroblastoma		EV (Pre-MEL)		–	–	
	LA-N-1	MEL		0.1–100 µM	–	Inhibited cell proliferation through induction of glycolysis [116].
Retinoblastoma	Y79	MEL		10–500 ng/mL	–	Induced cell apoptosis via AA pathway [117].