Combined antitumor effects of bee venom and cisplatin on human cervical and laryngeal carcinoma cells and their drug resistant sublines

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ABSTRACT: In the present study, we investigated the possible combined anticancer ability of bee venom (BV) and cisplatin towards two pairs of tumour cell lines: parental cervical carcinoma HeLa cells and their cisplatin-resistant HeLa CK subline, as well as laryngeal carcinoma HEp-2 cells and their cisplatin-resistant CK2 subline. Additionally, we identified several peptides of BV in the BV sample used in the course of the study and determined the exact concentration of MEL. BV applied alone in concentrations of 30 to 60 μg m l $^{-1}$ displayed dose-dependent cytotoxicity against all cell lines tested. Cisplatin-resistant cervical carcinoma cells were more sensitive to BV than their parental cell lines (IC_{50} values were 52.50 μg m l $^{-1}$ for HeLa vs. 47.64 μg m l $^{-1}$ for HeLa CK cells), whereas opposite results were obtained for cisplatin-resistant laryngeal carcinoma cells (IC_{50} values were 51.98 μg m l $^{-1}$ for HEp-2 vs. $>$ 60.00 μg m l $^{-1}$ for CK2 cells). Treatment with BV alone induced a necrotic type of cell death, as shown by characteristic morphological features, fast staining with ethidium-bromide and a lack of cleavage of apoptotic marker poly (ADP-ribose) polymerase (PARP) on Western blot. Combined treatment of BV and cisplatin induced an additive and/or weak synergistic effect towards tested cell lines, suggesting that BV could enhance the killing effect of selected cells when combined with cisplatin. Therefore, a greater anticancer effect could be triggered if BV was used in the course of chemotherapy. Our results suggest that combined treatment with BV could be useful from the point of minimizing the cisplatin concentration during chemotherapy, consequently reducing and/or postponing the development of cisplatin resistance.

Keywords: bee venom; melittin; cytotoxicity; tumour cells; drug resistance; cisplatin; apoptosis; necrosis

Introduction

Regardless of major scientific and technological advancements in combinatorial chemistry, drugs derived from natural products still make an enormous contribution to drug discovery today (Cragg and Newman, 2000; Gordaliza, 2007; da Rocha *et al*., 2001). A large number of studies in the past few years have reported on the anticancer ability of a wide spectra of natural products derived from plants and animals (Mehta and Pezzuto, 2002; Nobili *et al*., 2009), and the majority of those studies are especially interested in the effects of venoms from snakes, spiders, scorpions and in particular from honeybees (Heinen and da Veiga, 2011; Oršolić, 2012; Son *et al*., 2007) towards different types of tumour cell lines. Venom from *Apis mellifera* has been used for centuries in traditional medicine as a source of drugs to cure different ailments (Cherniack, 2010; Gajski and Garaj-Vrhovac, 2011; Garaj-Vrhovac and Gajski, 2009; Son *et al*., 2007).

Several beneficial roles of BV are also known today, such as radioprotective (Gajski and Garaj-Vrhovac, 2009), antimutagenic (Varanda *et al*., 1999), antinociceptive (Baek *et al*., 2006) and in recent times anticancer effects (Oršolić, 2012; Son *et al*., 2007). Recent reports indicate several mechanisms of BV cytotoxicity on different types of cancer cells such as cell cycle alterations, effects on proliferation and/or growth inhibition, as well as induction of apoptosis or necrosis (Gajski *et al*., 2011; Hu *et al*., 2006; Ip *et al*., 2008a, b, 2012; Jang *et al*., 2003; Jo *et al*., 2012; Lee *et al*., 2007; Liu *et al*., 2002; Moon *et al*., 2006; Oršolić, 2009; Park *et al*., 2011). Therefore, BV has generated a great deal of interest as a possible therapeutic modality.

Venom itself is a very complex mixture of a variety of different active peptides: melittin (MEL), apamin, adolapin and mast cell degranulating (MCD) peptide (Habermann, 1972; Hider, 1988). In addition, it also contains enzymes, biologically active amines and non-peptide components including lipids, carbohydrates and free amino acids all with many cellular activities (Lariviere and Melzack, 1996). Two major components of BV are MEL and phospholipase A_2 (PLA_2) (Habermann, 1972; Stuhlmeier, 2007). MEL is a small protein containing 26 amino acids and it is the

principal toxin in the BV, constituting around 50% of the whole BV according to the literature (Oršolić, 2012; Son *et al*., 2007). MEL has a broad range of actions towards different types of cells through its interactions with the plasma membrane and the enzyme system, and its lytic activity is probably caused by its ability to insert into phospholipids layers (Dempsey, 1990; Raghuraman and Chattopadhyay, 2007). PLA_2, which makes around 10% of the whole BV, is a member of the group of enzymes that catalyze the hydrolysis of the sn-2 fatty acyl-ester bond of membrane glycero-3-phospholipids resulting in diverse biological effects (Kwon *et al*., 2002; Six and Dennis, 2000; Valentin and Lambeau, 2000). Hydrolysis of these compounds generates lysophospholipids. Interestingly, lysolipids have therefore been proposed for anticancer therapies because of their antiproliferative effects and cytotoxicity (Ashagbley *et al*., 1996; Putz *et al*., 2006; Samadder *et al*., 2004).

The major obstacle for successful treatment of tumour patients with the standard chemotherapy is the development of drug resistance during the course of chemotherapy. Cisplatin is today one of the most widely used anticancer drugs for the treatment of a number of solid tumours (Boulikas and Vougiouka, 2003). It is generally accepted that cisplatin is a DNA damaging agent, because its cytotoxic effect is based upon the formation of platinum–DNA adducts. As the consequence of DNA platination, the cell cycle is arrested in order to allow the cell to repair the damage. If repair fails, apoptosis is induced by activation of various pathways (Fink & Howell, 2000; Fuertes *et al*., 2003). However, recently it has been found that cisplatin can also induce non-DNA damage owing to formation of reactive oxygen species (ROS) (Brozović *et al*., 2010). The therapeutic outcome of cisplatin-based chemotherapy can be impaired by intrinsic or acquired resistance. Cisplatin resistance is the consequence of multifactor events. Several molecular mechanisms of resistance may occur in the same cell population. They include decreased accumulation and increased detoxification of cisplatin, more efficient removal of platinum–DNA adducts, an enhanced capacity to replicate past adducts, inhibition of apoptosis and a recently recognized mechanism, cell adhesion-mediated cisplatin resistance (Brozović *et al*., 2008, 2010; Köberle *et al*., 2010; Stewart, 2007; Zisowsky *et al*., 2007).

The aim of the present study was to investigate the possible combined anticancer ability of BV and cisplatin towards tumour cells *in vitro*. As the content of BV can be influenced by many factors, first we identified several peptides of BV in the BV sample used in the course of the study and determined the exact concentration of MEL. Next, we examined the cytotoxic effect of BV alone on parental cervical carcinoma HeLa cells and laryngeal carcinoma HEp-2 cells, as well as their cisplatin-resistant HeLa CK and CK2 sublines, respectively. Because sublethal concentrations of BV can stimulate cell growth, we used higher BV concentrations, all a bit lower than those which induce the killing of a large part of the treated cell population. According to our previous data the doses used were between 30 and 60 $\mu g\ ml^{-1}$ of BV (Gajski *et al*., 2011). Additionally, we examined morphological changes and the type of cell death that was induced by BV. Finally, we investigated the combined treatment of cisplatin and BV on parental and cisplatin-resistant cells lines.

Materials and Methods

Chemicals and Cell Media

BV, MEL, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), antibiotics (penicillin and streptomycin) and cisplatin (cDDP) were purchased from Sigma (St Louis, MO, USA); acridine-orange (AO), Coomassie Brilliant Blue G-250 and ethidium-bromide (EtBr) were from Serva (Heidelberg, Germany); bovine serum albumin (BSA) and Dulbecco's modified Eagle's medium (DMEM) were from Gibco (Gaithersburg, MD, USA). All other chemicals not specifically cited here were purchased from Kemika (Zagreb, Croatia).

Analysis of BV by Mass Spectrometry

Mass spectrometry (MS) and tandem MS experiments were performed on a Bruker amaZon ETD ion trap system (Bruker Daltonik GmbH, Bremen, Germany) which incorporates a negative chemical ionization source capable of providing reagent anions for both electron transfer dissociation (ETD) and proton transfer reaction (PTR). Solutions of BV and MEL were prepared by dissolving dried samples in ethanol/water (50:50, 0.1% formic acid). All samples were introduced into the electrospray ionization (ESI) source by direct infusion at a flow rate of $65\ \mu l\ h^{-1}$. The temperature and flow rate of the drying gas were set at 210 °C and $5\ l\ min^{-1}$, respectively. ETD, PTR and collision induce dissociation (CID) conditions were optimized in order to maximize the dissociation and the appearance of the product ion spectra. All spectra were acquired in a positive mode using a scan range from 200 to 3000 m/z. DataAnalysis software 4.0 (Bruker Daltonik GmbH) was used for analysis (charge deconvolution and data reduction) and extraction of the MS and tandem MS data. Peptide sequence assignment was done using BioTools 3.2 (Bruker Daltonik GmbH).

The concentration of MEL in the BV sample was determined by direct infusion ESI-MS, without chromatographic separation. A calibration curve was obtained by plotting the peak height of the MEL-extracted ion chromatogram (all available charge states) as a function of the concentration.

Cell Cultures

Human cervical carcinoma HeLa cells and their drug-resistant subline HeLa CK as well as human laryngeal carcinoma HEp-2 cells and their drug-resistant CK2 subline were used in the present study. Cisplatin-resistant HeLa CK cells were derived from the human cervical carcinoma HeLa cell line (Osmak and Eljuga, 1993), whereas cisplatin-resistant CK2 cells were derived from HEp-2 cells as described previously (Osmak, 1992; Osmak *et al*., 1993). All cell lines were maintained as a monolayer culture in DMEM (Gibco) supplemented with 10% BSA (Gibco) and antibiotics (penicillin and streptomycin) in a humified atmosphere at 37 °C with 5% CO_2 and were sub-cultured every 3–4 days.

Cell Treatment with BV

Cells were seeded and after overnight incubation, they were treated with BV. Just before the beginning of the treatment, BV was dissolved in sterile redistilled water at 25 °C as $1\ mg\ ml^{-1}$ stock solution and then dissolved in growth medium to a broad range of concentrations. At a certain time point after the treatment, cells were collected for further experiments. Each experiment was repeated two or three times.

Cytotoxicity Assay

Cytotoxicity of the whole BV towards cervical HeLa and laryngeal Hep-2 carcinoma parental cells and their drug-resistant HeLa CK and CK2 sublines was determined by the modified colorimetric MTT assay (Mickisch *et al*., 1990). Additionally, we used the same test to evaluate the cell response to cisplatin treatment as well as

to combined treatment with BV and cisplatin. MTT stains cells that have active mitochondria, i.e. live viable cells. Briefly, 2.5×10^3 cells were seeded into 96-well microtiter tissue culture plates. On the following day, the medium was aspirated and replaced with fresh growth medium in which appropriate concentrations of whole BV diluted in medium was added. Each concentration was tested in triplicate. The cells were continuously treated with BV for 72 h at 37 °C. For the combined treatment with BV and cisplatin, cells were pretreated with concentrations of BV for 1 h and then different concentrations of cisplatin were added to the cultures. After 72 h the medium was aspirated and 20 μg of MTT dye per 0.04 ml medium was added to each well. After 4 h of incubation at 37 °C, formazan crystals were dissolved in dimethyl sulfoxide (DMSO; 0.17 ml per well), the plates were mechanically agitated for 5 min and the absorbance at 545 nm was determined on a microtiter plate reader (Awareness Technology Inc., Palm City, FL, USA).

Morphological Changes and the Type of Cell Death

Next, 2.5×10^3 cells per well of cervical carcinoma HeLa cells or their drug-resistant subline HeLa CK cells were plated in tissue culture plates. Cells were then incubated with the whole BV at different concentrations for 1 h. As a positive control cells were treated for 24 h with 20 μM cisplatin which is known to induce apoptotic cell death (Eastman, 1990). The type of cell death was determined by staining cells with DNA-intercalators AO and EtBr, which give green fluorescence of the nuclei in live cells and red fluorescence of the nuclei in dead cells, respectively. In brief, adherent and floating cells were collected by centrifugation and resuspended in a small volume of culture medium, after which in 10 μl of cell suspension, 2 μl of AO [15 μg ml^{-1} in phosphate-buffered saline (PBS)] and 2 μl of EtBr (50 μg ml^{-1} in PBS) were added. Samples were viewed under the epifluorescence microscope Axiovert 35 (Opton, Germany). Fluorescence was detected through the BP 450-490, FT 510, LP 520 filter. Images were taken with the camera Pixera Pro150ES (San Jose, CA, USA).

Western Blot Analysis

Next, 4×10^5 cells were seeded and on the next day treated with the whole BV in different concentrations. As a positive control, cells were treated for 24 h with 20 μM cisplatin which is known to induce apoptotic cell death (Eastman, 1990). Total cellular extracts were obtained by lysing the cells in lysis buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100, 10 mM $MgCl_2$, 1 mM EDTA, 2% glycerol, 1 mM PMSF) on ice. The protein concentration was determined by Bradford (1976) and equal amounts of protein were separated by SDS–PAGE (12.5%) and blotted onto nitrocellulose membrane (Schleicher & Schüll, Dassel, Germany). After overnight incubation at 4 °C in blocking buffer [5% milk (w/v) in TBS buffer with 0.1% Tween20 (v/v)], the membranes were probed with monoclonal antibody against poly(ADP-ribose) polymerase (PARP; Pharmingen, San Diego, CA, USA). Primary antibodies were detected with corresponding horseradish-peroxidase conjugated secondary antibodies (GE Healthcare, Piscataway, NJ, USA), followed by Western Blot Chemiluminescence Reagent Plus detection according to the protocol provided by the manufacturer (PerkinElmer Life Science, Boston, MA, USA). As an internal protein loading control, ERK2 protein expression was determined by re-probing the membranes with ERK2 specific rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Statistical Analysis

The isobologram analysis method was used to determine the extent of synergism on combining two agents for their possible therapeutic effect. The synergy index (q) was calculated according to the formula $q = E(A + B)/(EA + EB - EA \times EB)$ (Jin, 1980). This method was used to elucidate the possible additive and/or synergistic effect of BV and cisplatin. E(A + B) represent the inhibition rate of the combination group, and EA and EB represent the individual group. If the value of q ranges from 0.85 to 1.15, the role of combination is just the simple addictive effect. While from 1.15 to 2.0, the role of combination will be a synergistic effect. Greater than 2.0, it represents that there is a significant synergistic effect between the two agents. Statistical differences between the mean values were calculated using Student's t-test. Differences were considered significant when $P < 0.05$.

Results

Analysis of BV Content

MS and tandem MS experiments were performed in order to examine the content of BV and to determine the exact concentration of MEL in BV that was used in the present study. Peptides MEL, apamin, MCD peptide and tertiapin were identified by tandem MS as components of BV. Charge deconvoluted low-energy CID/ETD spectra are shown on Fig. 1. Identified peptides are in agreement with previous findings regarding peptide components of BV (Oršolić, 2012; Son *et al*., 2007). Additionally, the concentration of MEL in BV was determined (Fig. 2). MEL mass fraction in the BV sample was estimated at 0.19.

Cytotoxicity of BV *in vitro*

A cytotoxic effect of whole BV was evaluated on human cervical carcinoma HeLa cells and human laryngeal carcinoma HEp-2 cells and their drug-resistant HeLa CK and CK2 sublines, respectively. The results of the MTT assay are presented in Fig. 3. BV was cytotoxic for all examined cell lines, and the toxicity was dose dependent. The cytotoxicity was also cell type dependent. Although parental HeLa and HEp-2 cells exhibited almost the same sensitivity with IC_{50} values of 52.50 and 51.98 μg ml^{-1}, respectively, we observed a different pattern in the sensitivity of their drug-resistant sublines. HeLa CK cells were more sensitive towards BV than the parental cells with the IC_{50} value of 47.64 μg ml^{-1}, compared with CK2 cells that displayed the highest resistance towards BV with a IC_{50} above 60.00 μg ml^{-1}.

BV-Induced Morphological Changes and the Type of Cell Death

Light and fluorescent microscopy was used for the determination of morphological changes induced in the pair of cervical carcinoma cells: parental HeLa cells and their cisplatin-resistant HeLa CK subline. On both cell lines we saw similar morphological alterations that were induced rapidly after the addition of whole BV: rounded and granulated cells, shrinkage, and separation from neighbouring cells and eventual detachment from the culture plates (Fig. 4). Very few such features were observed in the control cells. One hour after the treatment we also determined the type of cell death using fluorescent microscopy. As shown in Fig. 4, BV induced a necrotic type of cell death in both HeLa and HeLa CK cells as opposed to treatment with cisplatin where apoptosis was observed. Detection of cells dying by a necrotic

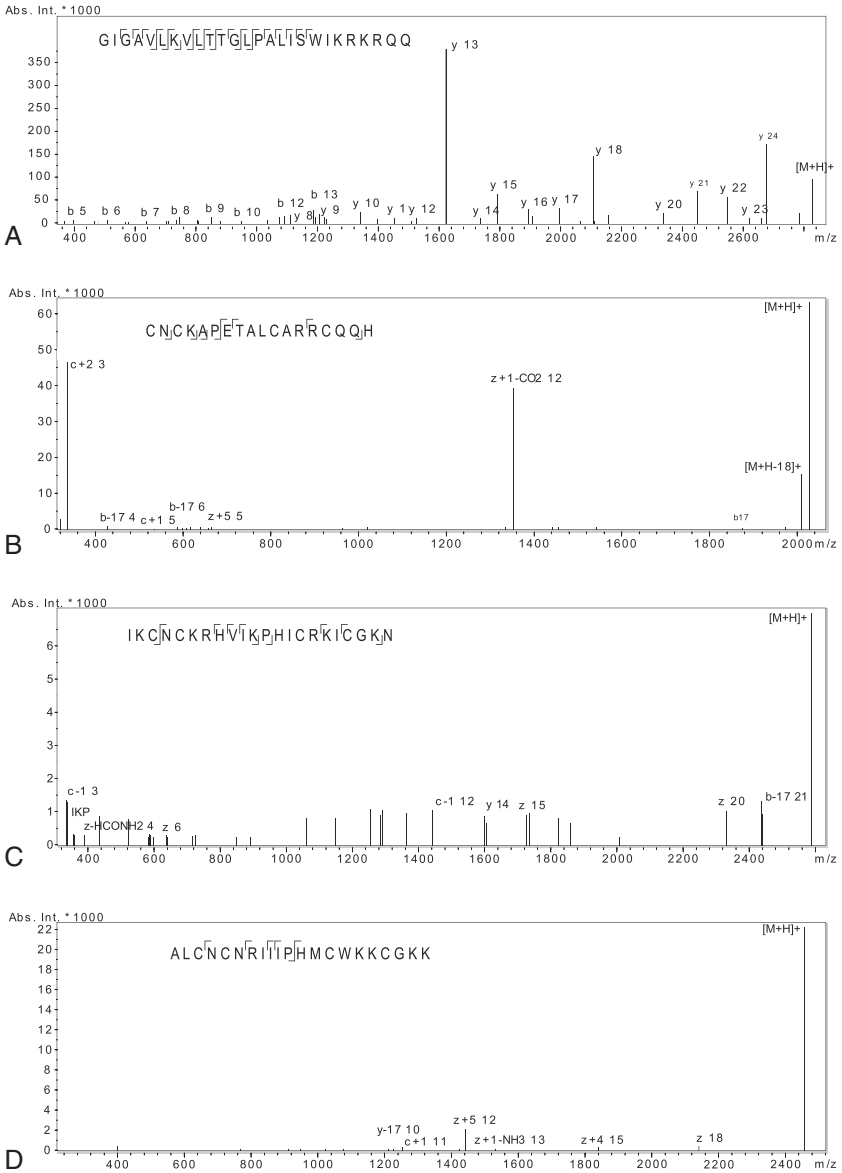

Figure 1. Charge deconvoluted low-energy collision induce dissociation/electron transfer dissociation (CID/ETD) spectrum of (A) melittin (GIGAVLKVLTTGLPALISWIKRKRQQ), (B) apamin (CNCKAPETALCARRCQQH – amidated C-terminus, crosslink SS 1-11 and SS 3-15), (C) mast cell degranulating peptide (IKCNCKRHVIKPHICRKICGKN – amidated C-terminus, crosslink SS 3-15 and SS 5-19) and (D) tertiapin (ALCNCNRIIIPHMCWKKCGKK – amidated C-terminus, crosslink SS 3-14 and SS 5-18).

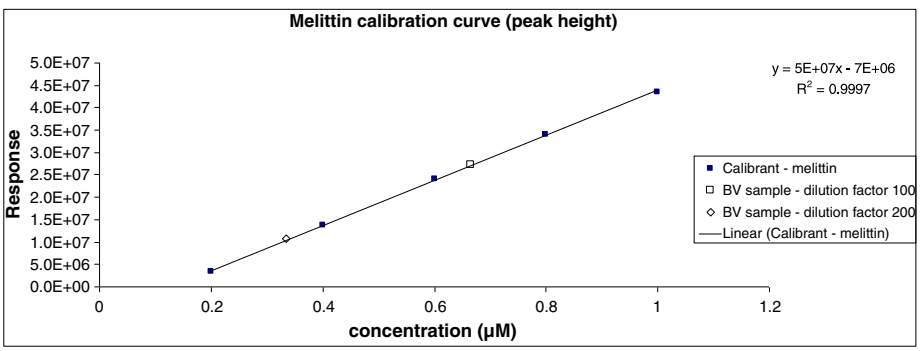

Figure 2. Melittin (MEL) calibration curve obtained by direct infusion electrospray ionization-mass spectrometry (ESI-MS) assay.

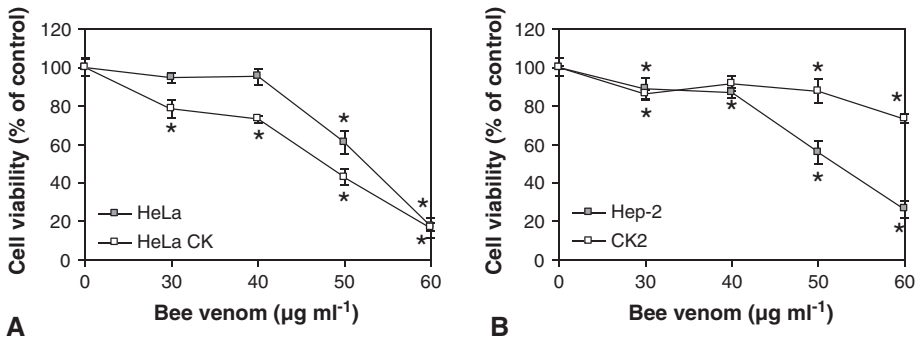

Figure 3. Cytotoxic effect of bee venom (BV) on human cervical carcinoma HeLa cells (A) and human laryngeal carcinoma HEp-2 cells (B) and their drug-resistant HeLa CK and CK2 sublines, respectively. Cells were seeded in 96-well tissue culture plates and 24 h later BV was added. Cells were incubated for 72 h with indicated concentrations of BV and percentage of cell viability was calculated in relation to the untreated control. The viability was determined with the modified colorimetric MTT assay. *Statistically significant compared with the corresponding control ($P < 0.05$).

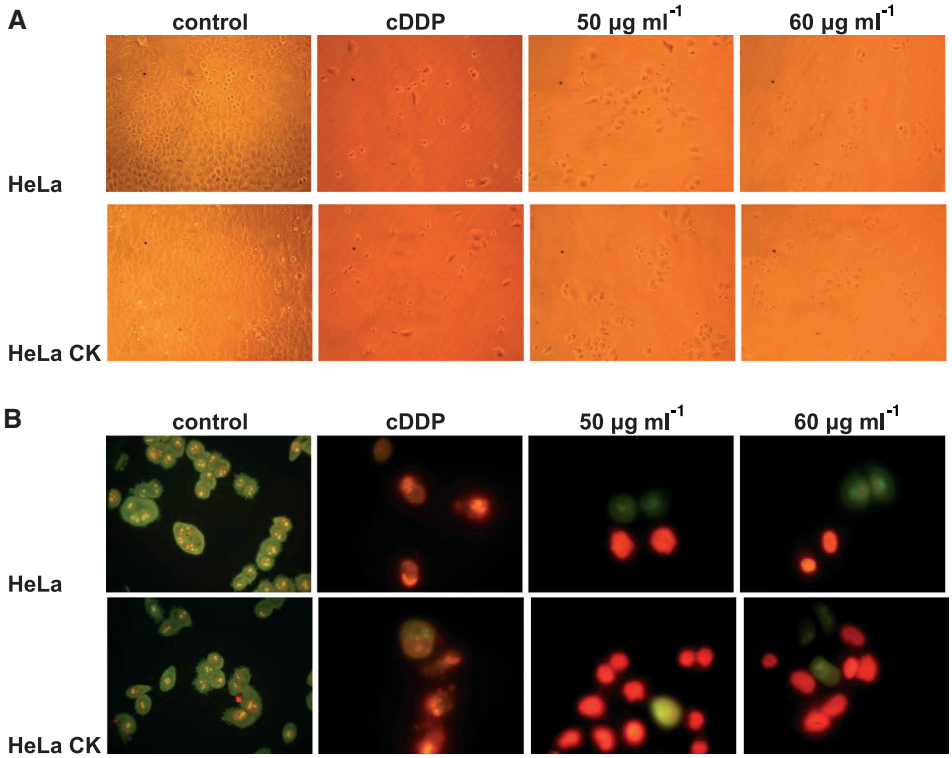

Figure 4. Microphotographs of human cervical carcinoma HeLa cells and their drug-resistant HeLa CK subline after bee venom (BV) treatment (μg ml^{-1}). Cells were incubated for 1 h with indicated concentrations of BV and viewed and photographed under the light microscope to determined morphological changes induced by the treatment with the whole BV. Pictures were taken at magnification 400× (A). For determination of apoptotic and/or necrotic nuclear morphology cells were also incubated for 1 h with indicated concentrations of BV, stained with DNA-intercalators acridine-orange (AO) and ethidium-bromide (EtBr), and viewed and photographed under the epifluorescent microscope. Pictures were taken at magnification 1000× (B). As a positive control cells were treated for 24 h with cisplatin (cDDP, 20 μM) which is known to induce apoptotic cell death.

type of cell death employs the fact that, in contrast to apoptosis, the cell membrane becomes permeable very early, but the nucleus disintegrates late. So, pyknotic and fragmented nuclei are characteristic for apoptosis, but round and intact nuclei indicate a necrotic type of cell death. It is important to note that cells in the late stages of apoptosis are also membrane permeable owing to secondary necrosis.

Induction of PARP Cleavage by BV

Using morphological alteration and staining with fluorescent dyes, we show in this study that BV induces necrosis in both HeLa and HeLa CK cells. However, to confirm this observation, we additionally explored whether BV can induce apoptosis. For this purpose we used HeLa cells and examined whether treatment with BV induces cleavage of PARP, which is a regulatory molecule whose cleavage is known to indicate apoptotic cell death (Kaufmann *et al.*, 1993). As expected, Western blot analysis did not show any cleavage of PARP as shown in Fig. 5.

Effect of Combined Treatment with BV and Cisplatin

To test the impact of the combined treatment of BV and cisplatin, human cervical carcinoma HeLa cells and human laryngeal

carcinoma HEp-2 cells, as well as their cisplatin-resistant HeLa CK and CK2 sublines, were pre-treated with BV in different concentrations for 1 h, after which cisplatin was added. Cytotoxicity of such a combined treatment against human cervical carcinoma HeLa cells and their cisplatin-resistant HeLa CK subline is shown in Fig. 6, whereas cytotoxicity on human laryngeal carcinoma HEp-2 cells and their cisplatin-resistant CK2 subline was shown in Fig. 7. When combined treatment with cisplatin and BV was given to HeLa cells, cell sensitization was observed for a higher dose of cisplatin, and the highest dose of BV and a higher dose of cisplatin. In contrast, all three given doses of BV sensitized HeLa CK cells to both doses of cisplatin (Fig. 6). The combination of BV and cisplatin was again less cytotoxic to parental laryngeal carcinoma cells, while all three selected doses of BV increased the cell sensitivity of CK2 cells to both doses of cisplatin (Fig. 7). Thus, according to statistical analysis, both cisplatin-resistant sublines, HeLa CK and CK2 cells were more sensitive to cisplatin when pre-treated with BV compared with parental cells. The isobologram analysis method showed that the effect of BV and cisplatin, given in combination, on HeLa and HeLa CK cells was synergistic (q ranges from 1.15 to 2.0) for all the BV and cisplatin concentrations, although a significant synergistic effect was not observed. In contrast, the synergistic effect on HEp-2 cells was observed only for the highest BV concentration and for both cisplatin doses, whereas on CK2 cells only 40 μg ml^{-1} BV and both cisplatin doses showed a synergistic effect. It has to be pointed out that the synergistic effect was again non-significant since q was lower than 2.0. All the other BV and cisplatin concentrations on HEp-2 and CK2 cells only gave an additive effect.

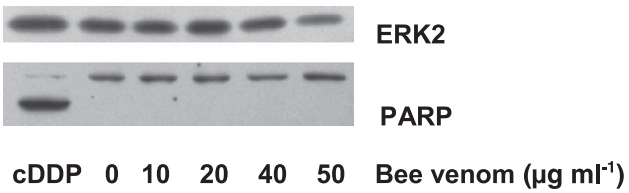

Figure 5. Determination of cell death after bee venom (BV) treatment. Human cervical carcinoma HeLa cells were incubated for 1 h with indicated concentrations of BV. As a positive control cells were also treated with cisplatin (cDDP, 20 μM) for 24 h which is known to induce apoptotic cell death. The induction of apoptosis was determined by the cleavage of characteristic apoptotic protein poly (ADP-ribose) polymerase (PARP) by Western blot analysis. As an internal protein loading control, ERK2 protein expression was determined by re-probing the membranes with ERK2-specific antibody. A representative of three independent experiments is shown.

Discussion

Today there is increasing interest in anticancer treatment strategies involving natural products such as BV and strategies that combine standard chemotherapy with novel agents that could target cancer

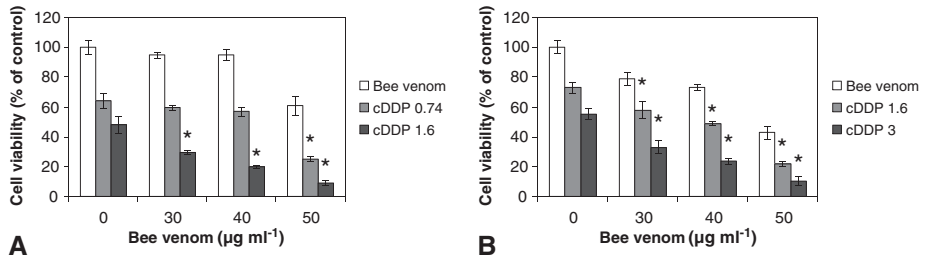

Figure 6. Sensitivity of human cervical carcinoma HeLa cells (A) and their drug-resistant HeLa CK cell subline (B) towards joint treatment with bee venom (BV) and cisplatin (cDDP). Cells were seeded in 96-well tissue culture plates and 24 h later BV was added. Cells were pre-treated for 1 h with indicated concentrations of BV (μg ml^{-1}) after treatment with indicated concentrations of cDDP (μM) and percentage of cell viability was calculated in relation to untreated control. The viability was determined with the modified colorimetric MTT assay after 72 h of treatment. White columns represent cell viability obtained with BV treatment (from 0 to 50 μg ml^{-1}), light-gray and dark-gray columns (under 0 μg ml^{-1} BV) represents viability obtained with indicated cDDP doses, whereas light-gray and dark-gray columns (under 30, 40 or 50 μg ml^{-1} BV) represent cell viability obtained by combined treatment with BV and cisplatin. *Statistically significant compared with the corresponding cDDP treatment ($P < 0.05$).

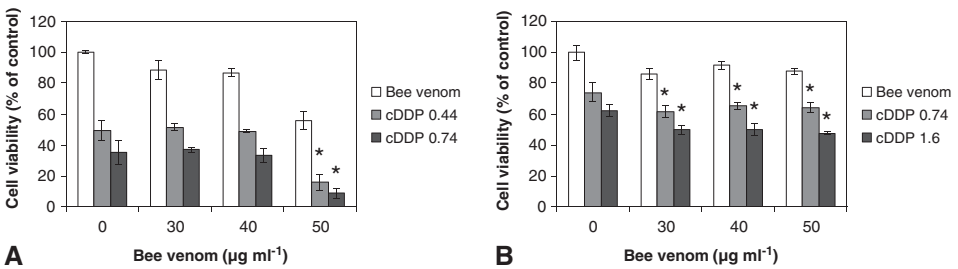

Figure 7. Sensitivity of human laryngeal carcinoma HEp-2 cells (A) and their drug-resistant CK2 cell subline (B) towards joint treatment with bee venom (BV) and cisplatin (cDDP). Cells were seeded in 96-well tissue culture plates and 24 h later BV was added. Cells were pre-treated for 1 h with indicated concentrations of BV (μg ml^{-1}) after treatment with indicated concentrations of cDDP (μM) and percentage of cell viability was calculated in relation to untreated control. The viability was determined with the modified colorimetric MTT assay after 72 h of treatment. White columns represent cell viability obtained with BV treatment (from 0 to 50 μg ml^{-1}), light-gray and dark-gray columns (under 0 μg ml^{-1} BV) represent viability obtained with indicated cDDP doses, while light-gray and dark-gray columns (under 30, 40 or 50 μg ml^{-1} BV) represent cell viability obtained by combined treatment with BV and cisplatin. *Statistically significant compared with the corresponding cDDP treatment ($P < 0.05$).

cells. New therapeutic approaches have attempted to use chemotherapeutic agents derived from venoms of animals and the most frequently used in recent years is venom derived from *A. mellifera* (Gajski *et al*., 2012; Gajski and Garaj-Vrhovac, 2013; Oršolić, 2012; Son *et al*., 2007).

BV is a complex mixture of different active compounds and its toxic effects could be largely attributed to its small protein MEL that comprises around 19% of dry BV in our sample. PLA_2 whose activity is enhanced by MEL, as well as other peptide components such as apamin, MCD peptide and/or tertiapin, was identified in the present study as well. Those are the components with known cytotoxic effects towards a variety of cells and are likely to be responsible for the effects encountered in our study.

It has also been demonstrated that BV inhibits the growth of several types of cancer cell lines (Hu *et al*., 2006; Ip *et al*., 2008a, b, 2012; Jang *et al*., 2003; Jo *et al*., 2012; Lee *et al*., 2007; Moon *et al*., 2006; Park *et al*., 2011). Previously, we reported that BV is cytotoxic against different types of tumour and non-tumour cell lines *in vitro* (Gajski *et al*., 2011). Those results showed a decreased viability after BV treatment that was more prominent in tumour than in non-tumour cells suggesting that BV preferentially kills tumour cells. In addition to evaluating the cytotoxic effect of BV alone, and the morphological changes and the type of cell death induced after BV treatment, the present study aimed to investigate the possible combined anticancer ability of BV and cisplatin towards tumour cells *in vitro* in a narrower concentration range around IC_{50} values. This was done using two different cultured human cell lines: human cervical carcinoma HeLa and laryngeal carcinoma HEp-2 cells and their cisplatin-resistant sublines HeLa CK and CK2 cells, respectively.

As the properties of carcinogenic agents are growth-inhibition power and the ability to induce cell death, these properties are widely used in anticancer therapies. In the present study, we examined the cytotoxic effect of the whole BV and its impact on cisplatin cytotoxicity on two tumour cells lines and their sublines resistant to cisplatin. Using the spectrophotometric MTT assay, we first determined cell viability after treatment, whereas using light and fluorescent microscopy, in addition to Western blot analysis, we described morphological changes and the type of cell death induced. Our results showed that BV was cytotoxic to all tested cell types and that cytotoxicity was dose- and cell-type dependent. Parental HeLa and HEp-2 cells exhibited almost the same sensitivity towards BV treatment. In contrast, in their drug-resistant sublines we observed a different pattern of sensitivity. HeLa CK cells were more sensitive towards BV than the parental cells, whereas CK2 cells displayed resistance towards BV. Such differences may be as a result of the biological and genetic variations among the investigated cell types.

The cytotoxic effect of BV could be based on the effect of MEL. Specifically, this compound shares its amphipatic properties with a series of peptides that are characterized by their capacity to disturb cell membrane bilayer integrity, either by creation of defects, disruption, or through pore formation. The resulting opening in the lipid bilayer leads to the collapse of transmembrane electrochemical gradients (Bechinger and Lohner, 2006). In contrast to the normal cells with low membrane potential, the cell membranes of tumour cells maintain a large membrane potential. Therefore, many lytic peptides together with MEL selectively disrupt the tumour cell membranes rather than those of normal cells, and that could be the important mechanism of the anticancer activity of BV (Holle *et al*., 2003; Moon *et al*., 2006; Son *et al*., 2007).

The collapse of the membrane integrity was confirmed in our system by the staining of treated cells with EtBr, a dye that only enters the cells with a damaged cell membrane. In all cell lines tested we observed similar morphological alterations induced by BV. These changes occurred rapidly after cell treatment. Cell rounding and granulation, as well as detachment from the substratum, along with the staining with fluorescent dye EtBr suggests the cell membrane damage as the cause of cell death, and the necrosis as the most probable type of the cell death in the given concentrations. In the literature there are contradictory data about the type of cell death which could be induced by BV. BV caused apoptosis in lung carcinoma cells, hepatoma cells, leukemic cells, breast cancer cells, cervical epidermoid carcinoma cells, synovial fibroblasts, prostate cancer cells, bladder cancer cells and melanoma cells (Hong *et al*., 2005; Hu *et al*., 2006; Ip *et al*., 2008a, b, 2012; Jang *et al*., 2003; Moon *et al*., 2006; Park *et al*., 2011; Tu *et al*., 2008). Recently reported results demonstrate that in fibroblast-like synoviocytes, in addition to apoptotic-like cell death induced by BV, necrotic cell death was present as well whereas in human mammary carcinoma cells, cervical carcinoma cells and Chinese hamster lung fibroblasts both apoptotic and necrotic activities were encountered (Oršolić, 2009; Oršolić *et al*., 2003; Stuhlmeier, 2007). In addition, Lee *et al*. (2007) in their study on human lymphoma cells did not observe sub-G1 fractions or cleavage of caspase-9, -3 or PARP suggesting that BV induced cell death but these cellular events were not accompanied by the activation of the apoptotic machinery.

In the present study, the effects of BV were related to necrosis, which was confirmed by fluorescent microscopy and Western blot analysis. Although decades of research clarified the pathways that regulate apoptosis and allowed the development of novel diagnostic and therapeutic modalities in cancer treatment, only recently has the significance of necrosis become the focus of investigations (Amaravadi and Thompson, 2007). Necrosis is an irreversible inflammatory form of cell death with a possible implication for cancer therapy. Necrotic cell death is often referred to as unscheduled cell death, suggesting that within a multicellular organism it is an unregulated process. The disruption of the plasma membrane that is characteristic of necrotic cell death leads to the spillage of intracellular proteins that activates a damage response from the host immune system (Amaravadi and Thompson, 2007; Zeh and Lotze, 2005). This rapid inflammatory response and immune amplification of the damage signal is in contrast to apoptotic cells that are silently removed by tissue macrophages. Thus, necrosis was viewed as strictly a pathologic form of cell death that is not a physiologically programmed process. However, a large number of experimental data indicate that, much like apoptosis, specific genes have evolved to regulate necrotic cell death, suggesting that necrosis may be a well-regulated process activated by rather specific physiological and pathological stimuli (Amaravadi and Thompson, 2007; Festjens *et al*., 2006; Golstein and Kroemer, 2007; Proskuryakov and Gabai, 2010). Hence, necrosis is not to be excluded as a possible way of cancer cell death.

Most of the anticancer agents, including cisplatin, can induce drug resistance. In order to interact with intracellular targets, they need to penetrate through membranes. Once they have reached the cytosol many of them are deactivated and than transported out of the cell before interacting with intracellular targets (Köberle *et al*., 2010; Pérez-Tomás, 2006; Schweizer, 2009; Stewart, 2007). In order to battle such resistance and to

reduce normal cell cytotoxicity, the development of novel drugs and delivery systems with novel mode of actions and high cancer cell selectivity are crucial. In the search for new cancer agents and delivery systems, cationic amphiphilic peptides such as MEL have recently attracted attention owing to their novel mode of actions and decreased possibility of resistance development (Hoskin and Ramamoorthy, 2008; Leuschner and Hansel, 2004; Papo and Shai, 2005; Schweizer, 2009).

In our study, pre-treatment with BV enhanced cytotoxicity of cisplatin in all cell lines. We used the isobologram analysis method to determine the extent of synergism when combining BV and cisplatin. The synergy index (*q*) on HeLa and HeLa CK cell lines, for all the concentrations tested, indicated that a combination of BV and cisplatin could be synergistic although the synergistic effect was not significant. As for the HEp-2 and CK2 cells, the same was observed only for certain BV and cisplatin concentrations, whereas other BV and cisplatin concentrations gave only an additive effect. Nevertheless, not only is there a direct effect of BV on the growth of tested tumour cells, but combined BV and cisplatin treatment showed enhanced cytotoxicity which could be useful from the point of minimizing the cisplatin concentration during chemotherapy. The possible mechanism of action could be a creation of defects, disruption and pore formation in the cell membrane bilayer by MEL, enhancing cisplatin uptake and accumulation and thus causing synergistic i.e. enhanced cytotoxic effect of cisplatin. The effects of BV and MEL on cytotoxicity of different cytostatic drugs was previously noticed in leukemic L1210 cells (Lazo *et al*., 1985), HeLa cells, Chinese hamster lung fibroblasts V79 cells (Oršolić, 2009) and human ovarian cancer A2780cp cells (Alizadehnohi *et al*., 2012). Oršolić (2009) investigated the cytotoxic effects of BV applied alone or in combination with the DNA damaging drug bleomycin on HeLa and V79 cells. Bleomycin caused a dose-dependent decrease in cell survival, and when used with a non-lethal dose of BV, its lethal effect was potentiated. Oršolić (2009) concluded that BV, by preventing the repair of damaged DNA, increases bleomycin lethality and inhibits recovery from the bleomycin-induced damage. Moreover, Alizadehnohi *et al*. (2012) showed that lethal effects of cisplatin are potentiated by the addition of a non-lethal dose of BV. As DNA is the main target of cisplatin, it is very likely that BV is able to potentiate the lethal effect of cisplatin in this way. Although the detailed mechanism for the action of BV and cisplatin needs further clarification, the inhibitory effect on tumour cell lines is apparent. Because cisplatin cytotoxicity was enhanced with another drug, in this particulate case with BV, combination therapy may help overcome the challenge of tumour resistance towards standard chemotherapy.

In conclusion, BV is a mixture of different active compounds and its toxic effects could be largely attributed to its small protein MEL that comprises around 19% of dry venom in our sample, PLA_2 which activity is enhanced by MEL, in addition to other peptide components such as apamin, MCD peptide and/or tertiapin that were identified in the course of our study. BV, given in the range of 30 to 60 $\mu g\ ml^{-1}$, was cytotoxic to parental cervical HeLa and laryngeal HEp-2 carcinoma cells. Their cisplatin-resistant sublines exhibited an opposite pattern of sensitivity: HeLa CK cells were more sensitive to BV than their parental HeLa cells, whereas CK2 cells were resistant compared with HEp-2 cells. BV given alone induced very fast necrosis. The combined treatment of BV and cisplatin induced an additive and/or weak synergistic effect towards all the tested cell lines, suggesting that BV could enhance the killing effect of selected cells when combined with cisplatin, therefore, a greater anticancer effect could be triggered if BV was used in the course of chemotherapy. Minimizing the cisplatin concentration during chemotherapy by the addition of compounds that increase their cytotoxicity could be very useful for the patients, and also from the point of reducing and/or postponing the development of cisplatin resistance. Activities displayed by BV alone and by its combination with cisplatin could merit clinical investigation as a new agent in the treatment of cancer. In view of accumulating evidence on anti-proliferative and pro-cell death activity, BV could be used in the development of antitumor drugs. Nevertheless, more studies are required to show the suitability and safety of these types of drugs in anticancer therapies.

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Conflict of Interest

The Authors did not report any conflict of interest.

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