



Synergistic Cytotoxic Effect of Honey Bee Venom and Cisplatin on Tongue Squamous Cell Carcinoma Cell Line

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Abstract

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BACKGROUND: Tongue cancer is one of the most common head and neck cancers in the world. Nowadays, natural compounds are important resources of many anti-cancer drugs. Venom from honey bees possesses potent anti-cancer activities. Cisplatin is a chemotherapeutic drug that has been used for decades to treat cancer cells. Recently, combination therapy has been a popular treatment choice for cancer patients.

AIM: This study was conducted to evaluate the synergistic cytotoxic effect of honey bee venom (BV) and cisplatin on tongue squamous cell carcinoma 25 (SCC-25) cell lines.

METHODS: The cytotoxic effect was determined using methyl thiazol tetrazolium assay, microscopic examination, real-time polymerase chain reaction (RT-PCR), and statistical analysis.

RESULTS: The findings revealed that the cytotoxic potential of the tested drugs on SCC-25 cells was dose-dependent. Microscopic examination showed that BV and cisplatin alone and in combination mainly produced apoptotic cell death. Regarding RT-PCR results, P53 and caspase-3 expression levels were significantly increased in SCC-25-treated cells ($p = 0.0001$).

CONCLUSION: The combined use of BV and cisplatin induced a marked synergistic cytotoxic effect on SCC-25 cell line.

Introduction

Cancer is one of the leading causes of mortality and morbidity around the globe. The World Health Organization estimated 19.3 million cancer cases and 10.0 premature deaths worldwide in 2020 [1].

Tongue squamous cell carcinoma (TSCC) is the most common head and neck cancer [2]. Tobacco and alcohol consumption are the two major risk factors of TSCC [3].

Head and neck cancer is treated with different methods that include surgery, radiotherapy, and chemotherapy. Chemotherapy remains the most often used treatment. Sometimes, it may have a variety of side effects [4].

Oncological researchers are putting too much effort into finding new and efficient therapies which can alleviate adverse side effects caused by conventional treatments. Scientists became popular in the natural biological medicines as a source of anti-cancer drugs to improve therapy efficacy and reduce undesired side effects [5].

Apis mellifera is the most prevalent honey bee species in the Middle East. Honey bee venom (BV)

is a mixture of peptides, proteins, and enzymes with antibacterial, antioxidant, and anti-cancerous activities [6].

BV has long been utilized in traditional medicine to treat inflammatory conditions such as rheumatoid arthritis and multiple sclerosis. BV is now widely used to treat a wide range of malignancies [7].

Melittin is an essential component of BV. Melittin has a wide range of pharmacological and toxicological effects [8]. Phospholipase A2 is also the most frequent enzyme present in BV. It splits phospholipids in the sn-2 membrane and releases fatty acids such as arachidonic acid and lysophospholipids [9].

Apitherapy is a type of alternative medicine in which honey bee products, specifically BV, are employed in treating a wide range of human diseases [10].

Cisplatin is the first line of treatment for several cancers such as head and neck cancer, lung cancer, ovarian cancer, testicular cancer, and sarcomas [11].

The mechanism of action of cisplatin has attributed to its capacity to crosslink with purine bases on DNA, interrupting DNA repair pathways, inducing DNA damage, and eventually cancer cell death [12].

Cisplatin induces several toxic side effects. The most common side effects are nephrotoxicity, ototoxicity, hepatotoxicity, and gastrointestinal toxicity [13].

The use of cisplatin in combination with other drugs is common in treating a variety of human malignancies. The combination of cisplatin with natural products such as BV is a novel therapeutic method for treating numerous human malignancies to overcome cisplatin resistance and reduce its undesirable side effects [14].

P53 is known as the guardian of the genome because it activates other genes that cause cell cycle arrest and DNA repair. P53 controls cisplatin-induced apoptosis through several pathways such as activation of pro-apoptotic genes and interactions with members of the Bcl-2 family in the mitochondria and cytoplasm [15].

Caspase family plays a crucial part in the apoptotic mechanism. Caspase-3 antibodies serve as excellent biomarkers for detecting induction of apoptosis. Caspases-3 activation is the first step in both intrinsic and extrinsic apoptotic pathways [16].

Apoptosis is a type of programmed cell death that helps damaged cells to be efficiently removed following DNA damage or during development. Apoptosis is a natural process that prevents malignant cells from proliferating and surviving [17].

Apoptosis is the main effector mechanism in many anti-cancer drugs. The best way to treat cancer is to kill it. The elimination of apoptotic cells ensures a minimal risk of inflammation [18].

The morphological hallmarks of apoptosis are plasma membrane blebbing, cell shrinkage, peripheral chromatin condensation along the nuclear membrane forming a crescent-like structure, nuclear fragmentation, cytoplasmic vacuolization, and cell lysis [19].

This study aimed to evaluate the synergistic cytotoxic effect of BV and cisplatin on tongue squamous cell carcinoma 25 (SCC-25) cell lines.

Materials and Methods

Reagents

The Egyptian *apis mellifera* BV was obtained from the cell culture department-VACSERA-EGYPT. The dried venom sample was dissolved in normal saline (0.9% NaCl).

Cisplatin has a chemical formula of $\text{PtCl}_2(\text{NH}_3)_2$ and a molecular weight of 300.05 g/mol. Cisplatin was dissolved in dimethyl sulfoxide (DMSO) (Sigma, USA) using an orbital shaker to yield a clear solution.

Cell line

SCC-25 cells were supplied from the cell culture department-VACSERA-EGYPT in the form of a

frozen vial from the American Type Culture Collection with the reference number "CRL-1628."

Cell culture protocol

SCC-25 cultures were grown in cultured flasks (Griner-Germany) in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin in a 37°C humidified with 5% CO₂ incubator.

SCC-25 cells were divided into four groups: Control SCC-25 group, BV-treated SCC-25 group, cisplatin-treated SCC-25 group, and BV/cisplatin mixture-treated SCC-25 group.

It was confirmed that cell cultures were not contaminated using standard light microscopy, gram stain, and polymerase chain reaction (PCR).

Methyl thiazol tetrazolium (MTT) assay protocol

1.2–1.8 × 10³ SCC-25 cells were pre-cultured in 96 microtiter plates (5 × 10⁴ cells/mL). The cells were filled with 10L of MTT (0.5 mg/ml stock) solution and incubated at 37°C for 24 h. The medium was removed, and the purple Formosan crystals were dissolved in 100 L of DMSO.

The Dynatech MR5000 spectrophotometer (Dynatech Laboratories, Inc., Chantilly, VA) was used to measure the absorbance at 570 nm, which was proportional to the number of viable cells. The viability percentage was calculated as follows: Viability Percentage = (Mean OD of Test Dilution/Mean OD of Negative Control) × 100.

Microscopic examination

The microscopic fields were photomicrographed using a digital video camera (C5060, Olympus, Japan) mounted on a light microscope at a magnification of ×1000 oil immersion (BX60, Olympus, Japan). The photomicrographs assessed the existence of morphological apoptotic criteria.

Evaluation of the expression levels of P53 and caspase-3 genes by real-time PCR (RT-PCR)

Total RNA was extracted from the control and SCC-25 treated cells using the GeneJET RNA purification kit (Fermentus-UK) according to the manufacturer's instructions. The quantity and integrity of RNA were spectrophotometrically evaluated at a 260/280 nm ratio. First-strand cDNA was produced with 1 g of total RNA using a QuantiTect Reverse Transcription kit (Qiagen, Germany) according to the manufacturer's instructions.

The samples were kept at -80°C until used to evaluate the expression levels of P53 and caspase-3

genes. Glyceraldehyde-3-phosphate dehydrogenase was the endogenous expression standard. Quantitative RT-PCR was performed with QuantiTect SYBR Green PCR kits and forward and reverse primers for each gene on a Rotor-Gene Q cyclo (Qiagen, Germany). The nucleic acid sequences of the primers were as shown in Table 1:

Table 1: The nucleic acid sequences of the primers

P53 F	5'-ATGTGTGTGGAGACCGTCAA-3'
P53 R	5'-GCCGTACAGTTCACAAAGG-3'
Casp-3	5'-TGTTTGTGTGCTTCTGAGCC-3'
Casp-3	5'-CACGCCATGTCAATCAAC-3'
GAPDH F	5'-AATGCATCCTGCACCACCA-3'
GAPDH R	5'-GATGCCATATTCATTGTGATA-3'

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

Statistical analysis

Experimental data were collected, tabulated, and statistically analyzed by Statistical Package for the Social Sciences version 16.0 window software. Data were expressed as mean \pm standard deviation to determine the significance of differences between groups using one-way analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

Results

MTT cytotoxicity assay

In the present study, the cytotoxic effect of BV, cisplatin, and BV/cisplatin mix on SCC-25 cells was assessed for 24 h incubation. Data obtained revealed that the cytotoxicity was dose-dependent. The mean viability percentage of the treated cells decreased as the drug concentrations increased from 0.4 $\mu\text{g/ml}$ to 100 $\mu\text{g/ml}$. The half-maximal inhibitory concentration (IC_{50}) values were 1.56 $\mu\text{g/ml}$, 12.56 $\mu\text{g/ml}$, and 0.44 $\mu\text{g/ml}$ for BV-treated cells, cisplatin-treated cells, and BV/cisplatin mix-treated cells as shown in Figure 1.

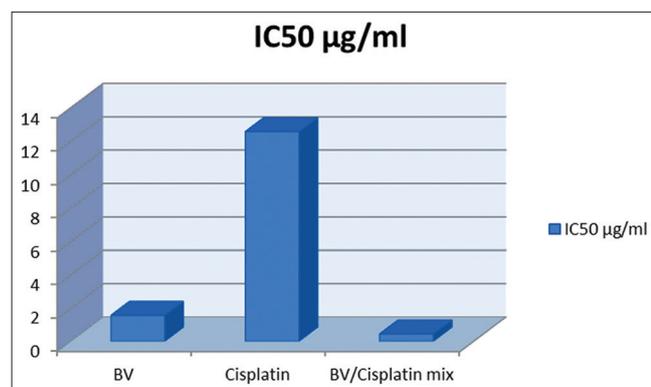


Figure 1: IC_{50} values of SCC-25 cells treated with BV, cisplatin, and BV/cisplatin mix for 24 h incubation. SCC-25: Squamous cell carcinoma 25, BV: Honey bee venom, IC_{50} : inhibitory concentration 50

Microscopic examination

Microscopic findings showed that control SCC-25 cells were almost rounded and showed criteria of malignancy such as hyperchromatism, nuclear pleomorphism, and increased nuclear/cytoplasmic ratio.

On the other hand, SCC-25-treated cells exhibited morphological apoptotic characteristics. These criteria were obvious in BV-treated SCC-25 cells and cisplatin-treated SCC-25 cells, but they became more apparent in BV/cisplatin mix-treated group. The apoptotic criteria included membrane blebbing, peripheral condensation of chromatin, nuclear shrinkage, and nuclear fragmentation.

In addition to the apoptotic criteria, some cells revealed nuclear alterations that resembled the morphological hallmarks of necrosis particularly with cisplatin-treated SCC-25 cells such as nuclear and cellular swelling, increased eosinophilia of the cytoplasm, and rupture of cell membrane.

In cisplatin-treated SCC-25 cells and BV/cisplatin mix-treated SCC-25 cells, the presence of secondarily necrotic cells with both apoptotic and necrotic characteristics such as nuclear fragmentation and cytoplasmic swelling, is shown in Figure 2.

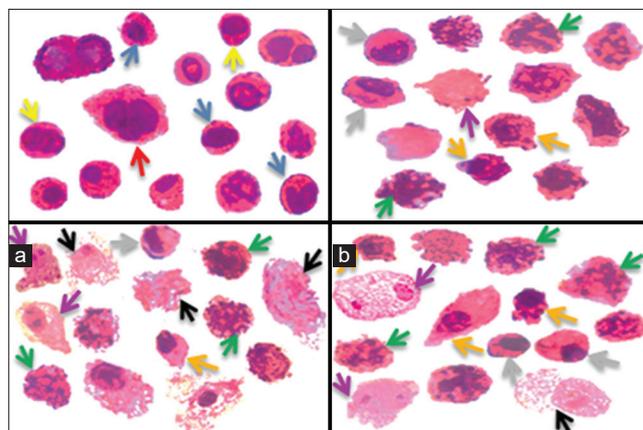


Figure 2: Photomicrographs of (a) control cells, (b) BV-treated cells, (c) cisplatin-treated cells, and (d) BV/cisplatin mix-treated cells showing: Hyperchromatic nuclei (blue arrows), abnormal mitotic figure (red arrow), increased nuclear-cytoplasmic ratio (yellow arrows), membrane blebbing (orange arrows), peripheral chromatin condensation (grey arrows), necrotic cells (black arrows), nuclear fragmentation (green arrows), and secondary necrotic cells (purple arrows) (H & E $\times 1000$ oil immersion). BV: Honey bee venom

Evaluation of the expression levels of P53 and caspase-3 genes by RT-PCR

Regarding the apoptotic profiles of the tested drugs, RT-PCR analysis demonstrated that BV, cisplatin, and BV/cisplatin mix significantly increased the expression levels of P53 and caspases-3 in treated SCC-25 cells when compared with control SCC-25 cells, as shown in Tables 2 and 3.

Table 2: P53 gene fold change in control and treated SCC-25 cells using RT-PCR

Sample		P53			Test cells				FLD
Ser	Code	Control cells			GAPDH	P53	Δ CTE	$\Delta\Delta$ CT	$2^{\Delta\Delta$ CT
		GAPDH	P53	Δ CTC					
		HC	TC	TC-HC					
		HE	TE	TE-HE	Δ CTE- Δ CTC	Eamp = 1.854			
1	Control SCC-25	24.49	34.06	9.57	24.49	34.06	9.57	0	1
2	BV/SCC-25	24.49	34.06	9.57	23.79	31.77	7.98	-1.59	2.66867
3	Cis/SCC-25	24.49	34.06	9.57	23.88	30.46	6.58	-2.99	6.33356
4	BV-cis/SCC-25	24.49	34.06	9.57	24.05	29.91	5.86	-3.71	9.87841

SCC-25: Squamous cell carcinoma-25, RT-PCR: Real-time polymerase chain reaction, GAPDH: Glyceraldehyde-3-phosphate dehydrogenase, BV: Honey bee venom

Statistical analysis

ANOVA test demonstrated a high statistically significant difference between control SCC-25 cells and SCC-25 cells treated with different concentrations of BV, cisplatin, and BV/cisplatin mix for 24 h ($p = 0.0001$) as shown in Table 4.

Discussion

Cancer is a leading cause of death in both developing and developed countries. Hence, finding a better therapeutic strategy is crucial [20]. Oral cancer is the most common oral malignancy. It accounts for 80–90% of all oral malignant neoplasms [21].

The most significant challenges of chemotherapy treatment are the development of drug resistance and undesired toxicity. Cancer therapy aims to maximize the effect on cancer cells while minimizing the influence on healthy cells [22].

BV is one of the most commonly encountered animal venoms. BV is a complex mixture of several active peptides, proteins, enzymes, carbohydrates, minerals, and water [10].

For thousands of years, BV was used in medical applications as a non-steroidal anti-inflammatory drug for pain relief and in the modern days it is used in cancer treatment [23].

The discovery of novel BV activities is a promising strategy for discovering natural cancer treatments. The natural extracts of BV showed anti-cancer properties in numerous investigations [23].

BV has a powerful pharmacological effect, but at the same time, it has adverse effects depending on the individual. The possible side effects include allergic reactions and anaphylaxis in severe cases. The severity of BV depends on venom concentration and the frequency of venom administration [24].

Cisplatin is one of the most often used anticancer drugs. Cisplatin kills cancer cells by disrupting nuclear and mitochondrial DNA, blocking DNA replication, and triggering apoptosis. Cisplatin caused P53 activation, which resulted in cell death [25].

Combination therapy of cisplatin with other drugs is common in the treatment of various human cancers. Combination therapy is used to improve therapeutic response and reduce cisplatin resistance [26].

In this study, we evaluated the synergistic cytotoxic effect of honey BV and cisplatin alone and in combination on TSCC cell line (SCC-25).

There were four groups of SCC-25 cells: Control group, BV-treated SCC-25 group, cisplatin-treated SCC-25 group, and BV/cisplatin mix-treated SCC-25 group.

MTT cytotoxicity assay was used to examine the influences of BV, cisplatin, and BV/cisplatin mix on the cultivated SCC-25 cells, as well as to determine the IC_{50} values of the tested drugs after 24 h incubation.

The IC_{50} value is the dose of a drug that kills 50% of a cell population after specified test duration. Determination of the IC_{50} is essential for understanding the pharmacological and biological characteristics of chemotherapeutic agents [27].

MTT assay showed that BV and cisplatin successfully suppressed SCC-25 proliferation in a dose-dependent manner. The results revealed a remarkable *in vitro* cytotoxic effect of the tested drugs on SCC-25 cells after 24 h.

Regarding the cytotoxic profiles of the tested compounds, the IC_{50} value of the BV/cisplatin mix was less than those of BV and cisplatin alone. BV/cisplatin mix had a higher effect on malignant cells with a small concentration of the drug.

Those findings were consistent with Gajski *et al.* 2016, who found that BV and cisplatin had a cytotoxic influence and inhibited human glioblastoma A1235 cells in a dose-dependent manner [14].

Table 3: Caspase-3 gene fold change in control and treated SCC-25 cells using RT-PCR

Sample		Caspase-3			Test cells				FLD
Ser	Code	Control cells			GAPDH	P53	Δ CTE	$\Delta\Delta$ CT	$2^{\Delta\Delta$ CT
		GAPDH	P53	Δ CTC					
		HC	TC	TC-HC					
		HE	TE	TE-HE	Δ CTE- Δ CTC	Eamp = 1.854			
1	Control SCC-25	24.49	33.86	9.37	24.49	33.86	9.37	0	1
2	BV/SCC-25	24.49	33.86	9.37	23.79	31.44	7.65	-1.72	2.89167
3	Cis/SCC-25	24.49	33.86	9.37	23.88	31.18	7.3	-2.07	3.58911
4	BV-cis/SCC-25	24.49	33.86	9.37	24.05	30.74	6.69	-2.68	5.23039

SCC-25: Squamous cell carcinoma-25, RT-PCR: Real-time polymerase chain reaction, GAPDH: Glyceraldehyde-3-phosphate dehydrogenase, BV: Honey bee venom

Table 4: ANOVA test for the mean values±standard deviation of the different groups

Group	Number	Mean ± Standard deviation	Standard error	P-value
Control SCC-25 cells	100	586.2 ± 88.3	8.834	0.0001
BV-treated SCC-25 cells	100	501.1 ± 121.9	12.188	
Cisplatin-treated SCC-25 cells	100	267.1 ± 78.6	7.859	
BV/cis mix- treated SCC-25 cells	100	163.1 ± 76.9	7.693	

ANOVA: One-way analysis of variance, SCC-25: Squamous cell carcinoma-25, BV: Honey bee venom

Gajski *et al.* discovered that combining BV with cisplatin improved cytotoxicity, which could be beneficial in decreasing cisplatin concentration throughout treatment. Melittin produces holes in the cell membrane bilayer, which increases cisplatin uptake and accumulation, resulting in a synergistic increase in the cytotoxic effect of cisplatin [14].

The findings showed that BV/cisplatin mix suppressed cell viability more effectively than BV and cisplatin alone. MTT assay findings supported the cytological analysis, which revealed that when drug concentrations increased, morphological apoptotic criteria increased as well.

At the microscopic and cellular level, SCC-25 cells demonstrated a considerable increase in apoptotic cells after 24 h of BV, cisplatin, and BV/cisplatin mix therapy. Inhibiting cancer cell proliferation through the apoptotic mechanism becomes an apparent mode of action of the numerous anticancer compounds [28].

There were also necrotic and secondary necrotic cells. Secondary necrosis is beneficial in cancer therapy because it indicates the death of malignant cells.

To investigate the molecular mechanism of BV, cisplatin, and BV/cisplatin mix-induced apoptosis in SCC-25 cells, the expression levels of P53 and caspase-3 in both the control and experimental SCC-25 cells were evaluated by RT-PCR.

The research results showed that P53 and caspase-3 genes were significantly up-regulated in treated SCC-25 cells compared with the control group. Furthermore, both drugs were more effective when used together than when used separately.

Caspase-3 is considered to be a key mediator of apoptosis. Caspase-3 is activated in the apoptotic cells by both extrinsic and intrinsic pathways. Caspase-3 is a central regulator of chromatin condensation and DNA fragmentation during apoptosis [29].

P53 gene plays a crucial role in cancer prevention. P53 gene encodes proteins that bind to DNA and regulate gene expression to avoid genome mutations. P53 can trigger cell death through activation of the apoptotic effector proteins BAK and BAX in the outer mitochondrial membrane, compromising its integrity, and releasing cytochrome c from mitochondria [30].

That agreed with Jo *et al.* 2016 results, who observed the expression levels of P53, P21, caspase-3, caspase-7, and caspase-9 increased in the oral squamous cell carcinoma cell line after cisplatin treatment [31].

Conclusion

Both BV and cisplatin exhibited anti-cancer activities on SCC-25 cells. Using BV and cisplatin in a mixed formulation induced a significant synergistic cytotoxic effect on SCC-25 cells.

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