DOI: 10.2478/10004-1254-60-2009-1936

Scientific Paper

POTENTIATION OF BLEOMYCIN LETHALITY IN HeLa AND V79 CELLS BY BEE VENOM

Nada ORŠOLIĆ

Department of Animal Physiology, Faculty of Science, University of Zagreb, Croatia

Received in January 2009
Accepted in March 2009

This study investigated possible growth-inhibiting effects of bee venom applied alone or in combination with a cytotoxic drug bleomycin on HeLa and V79 cells *in vitro* based on clone formation, cell counting, and apoptosis. Melittin, the key component of bee venom, is a potent inhibitor of calmodulin activity, and also a potent inhibitor cell growth and clonogenicity. Intracellular accumulation of melittin correlates with the cytotoxicity of antitumour agents. Previous studies indicated that some calcium antagonists and calmodulin inhibitors enhanced intracellular levels of antitumor agents by inhibiting their outward transport. In this study, treatment of exponentially growing HeLa and V79 cells with bleomycin caused a dose-dependent decrease in cell survival due to DNA damage. This lethal effect was potentiated by adding a non-lethal dose of the bee venom. By preventing repair of damaged DNA, bee venom inhibited recovery from potentially lethal damage induced by bleomycin in V79 and HeLa cells. Apoptosis, necrosis, and lysis were presumed as possible mechanisms by which bee venom inhibited growth and clonogenicity of V79 cells. HeLa cells, on the other hand, showed greater resistance to bee venom. Our findings suggest that bee venom might find a therapeutic use in enhancing cytotoxicity of antitumour agent bleomycin.

KEY WORDS: *apoptosis, cell line, cytotoxic drug, growth inhibition, melittin*

Many studies have reported about natural products inhibiting tumour cell growth and metastasis, as well as inducing apoptosis, bringing hope of improved treatment of human tumours (1-5). Bee venom has traditionally been used to relieve pain and to treat chronic inflammatory diseases such as rheumatoid arthritis. It has also been used by oriental traditional medicine (6), and in recent times in the treatment of tumours (1-4, 7).

Bee venom plays a major role in the defence of bee colonies. It is produced in the venom gland of the bee, located in the abdominal cavity. It is a very complex mixture of active peptides, enzymes, and amines that contains histamine, catecholamines, polyamines, melittin, and phospholipase A_2 (7-10). The key component of bee venom is melittin, which makes 50 % of its dry weight. It is a strong basic polypeptide whose sequence consists of 26 amino acids (molecular weight 2850 Da) (7-10). It has strong haemolytic and cardiotoxic properties (7, 11, 12) and has been shown to revert the phenotype of H-ras-transformed cells (13). Hait et al. demonstrated that melittin was one of the most potent inhibitors of calmodulin activity and a more potent inhibitor of cell growth and clonogenicity than phenothiazines. (14-17). Drugs that inhibit calmodulin activity have been shown to inhibit DNA synthesis in the glioblastoma cell line (18), block the movement of chromosomes during metaphase (19), inhibit the growth of Chinese hamster ovary cells (20), and enhance the cytotoxicity of vincristine, doxorubicin, and bleomycin (21-23). Evidence also suggests that calmodulin inhibitors are cytotoxic to malignant cells both *in vitro* (14, 24) and *in vivo* (1, 2, 25, 26). In addition, bee venom induces single- and double-strand DNA breaks in human lymphocytes (27).

In this study we investigated the effects of bee venom on the growth and clonogenicity HeLa and V79 cells. We also considered possible use of bee venom in enhancing the cytotoxicity of antitumour agents.

MATERIALS AND METHODS

Bee venom

Lyophilised whole bee venom was purchased from Medex (Ljubljana, Slovenia). It was reconstituted in 0.5 mL of distilled water and centrifuged at 12,000 rpm for 10 min to remove insoluble materials. The resulting solutions were administered to cell cultures in concentrations 0.7125 μg mL^{-1}, 1.425 μg mL^{-1}, 2.85 μg mL^{-1}, 7.125 μg mL^{-1} or 14.25 μg mL^{-1} according to design described below.

Bleomycin

Bleomycin formulated for clinical use was obtained from Heinrich Mack Nachf (Illertissen, Germany). It was used at doses of 25 μg mL^{-1}, 50 μg mL^{-1} or 100 μg mL^{-1}.

Unless specified otherwise, the rest of the chemicals and reagents used were purchased form Sigma Chemical Co (St. Louis, USA).

Cell lines

The experiments were performed on human cervical carcinoma cells (HeLa) and Chinese hamster lung fibroblasts (V79). The average doubling time in log phase was about 20 h for HeLa cells and 12 h for V79 cells. Cells were grown in monolayer cultures in plastic disposable Petri dishes (Falcon) in minimal essential medium supplemented with 10 % foetal calf serum. Cell cultures were incubated at 37 °C in humid atmosphere containing 5 % CO_2 in air.

Cell counting

Cells were counted using Coulter Counter (Model B, Coulter Electronics, Dunstable, England). Petri dishes with different concentrations of the studied compounds were incubated for 72 h. Growth medium was then removed, and cells rinsed with phosphate-buffered saline (PBS), trypsinised, diluted, and counted in triplicate.

Colony formation

Exponentially growing HeLa and V79 cells were treated with various concentrations of bee venom for 24 h. After treatment, the bee venom was removed, and the cultures washed twice with PBS. Cells were trypsinised, and their survival determined by colony formation as described elsewhere (20). Cells were immediately plated on culture dishes in triplicate and propagated in culture for 8 to 10 days to evaluate the ability of the treated cells to form colonies. Colonies were stained with 10 % Giemsa and counted. A cell was considered to have retained reproductive capacity (survival) if it gave rise to a colony of 50 or more cells. The results were expressed as percent of colonies of untreated cells. The plating efficiency of the untreated control culture was 65 %.

Survival studies for simultaneous treatment with bleomycin and bee venom

Exponentially growing HeLa and V79 cells were treated for 1 h with various concentrations of bleomycin alone (25 μg $mL^{-1}$to 100 μg mL^{-1}) dissolved in fresh medium or with bleomycin in combination with bee venom. After treatment, both compounds were removed, and cultures washed twice with PBS. Cells were trypsinised, and survival determined by colony formation (20). Cells were then counted and seeded into Petri dishes (100 cells per dish). After 10 days, the medium was removed and cells stained with 10 % Giemsa. Colonies containing 50 cells or more were scored and results expressed as the survival index. The index was calculated by dividing the number of colonies formed per plate by the total number of singe cells initially seeded per plate.

Effect of bee venom on recovery from bleomycin-induced cell damage

Exponentially growing cells were treated with bleomycin (100 μg mL^{-1}) for 1 h. The drug was then removed and cultures washed twice with PBS. One group of cells was immediately processed for colony formation, with no time allowed for recovery. Replicate plates were treated with fresh medium alone or fresh medium containing bee venom in various concentrations. The cultures were then incubated at 37 °C and allowed to recover for 3 h. At appropriate times, replicate plates were removed and prepared for colony formation. Cell survival was determined as described above.

Apoptosis analysis

Apoptosis was determined using bivariant flow cytometry (28) on cells that were grown in the

presence or absence of bee venom for 3 h or 15 h , respectively. The cells were washed in cold PBS twice and resuspended in a small volume of 1x binding buffer (HEPES containing 2.5 mmol L^{-1} $CaCl_2$). Fluorescein-labelled annexin V and propidium iodide (PI) were added to the cells, which were then analysed using flow cytometry.

Staining with PI allowed for determination of DNA content in the tested cells. For that purpose, the cells were incubated in 100 µL of fixing solution at 4 °C for 15 min, washed in PBS, resuspended in permeabilising solution in the presence of 10 µL of PI, and incubated at 4 °C for 15 min. The cells were then washed with PBS and immediately analysed using flow cytometry.

Statistics

Results are expressed as means ± SE obtained from two or three experiments in triplicate. Statistical significance was evaluated using the Student's *t*-test.

RESULTS

Bee venom cytotoxicity

Growth inhibitory effect of bee venom administered in various concentrations to HeLa and V79 cells was estimated by counting their number after 72 h of treatment. Figure 1 shows the influence of bee venom on the growth of the tested cells.

Growth inhibition by bee venom was dose-dependent in both cell lines. IC_{50} for HeLa cells was 3 µg mL^{-1}. The inhibitory effect on V79 cells was more pronounced, and the same dose of bee venom killed about 90 % of V79 cells.

As for colony-forming, V79 cells treated in exponentially growing phase were more sensitive to the adverse effects of bee venom administered for 24 h than HeLa cells (Figure 2).

Potentiation of bleomycin lethality by bee venom

Treatment of exponentially growing cells with the DNA damaging agent bleomycin caused a dose-dependent decrease in cell survival. This lethal effect was potentiated by a nonlethal dose (2.85 µg mL^{-1}) of bee venom (Figure 3). Bee venom enhanced toxic effects of bleomycin (25 µg mL^{-1} to 100 µg mL^{-1}) on HeLa cells from 27 % to 58 %. In presence of bee venom, 55 % of the V79 cells were killed at bleomycin dose of 25 µg mL^{-1}, and increasing the dose of bleomycin to 100 µg mL^{-1} resulted in a further 79 % decrease in cell survival.

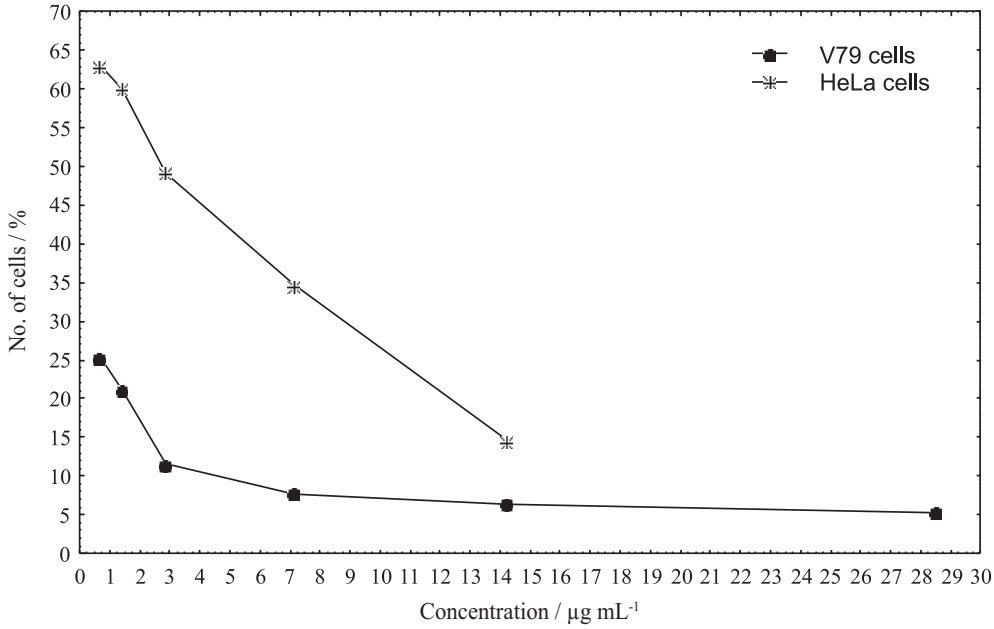

Figure 1 *Growth curves of V79 and HeLa cells treated with bee venom. Cells were seeded (1´10^5 per Petri dish) and incubated for 24 h before bee venom was added. Treatment with bee venom lasted for the next 72 h and then the cells were counted. The results are expressed in percentages with respect to untreated cells. Each point represents the mean number of cells from triplicate cultures from a representative of three experiments. There was less than 10 % variation in cell counts at each point.*

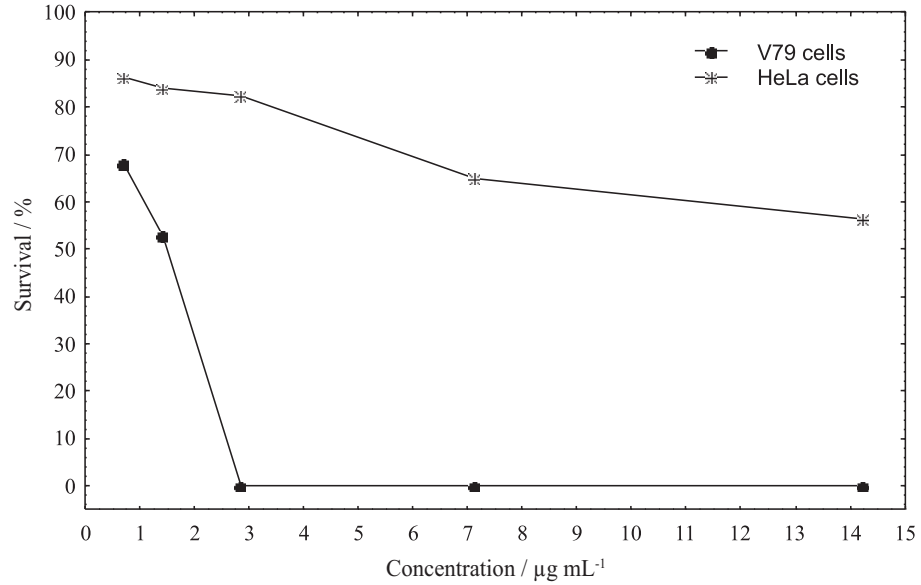

Figure 2 *Survival of proliferating V79 and HeLa cells exposed to increasing concentrations of bee venom for 24 h . Cells were seeded for colonies and treated with bee venom. Colonies were stained with 10 % Giemsa. Colonies containing 50 or more cells were counted and counts were expressed as percent of colonies of untreated cells. Each point represents the mean number of colonies from triplicate cultures from a representative of three experiments. There was less than 10 % variation in colony counts at each point.*

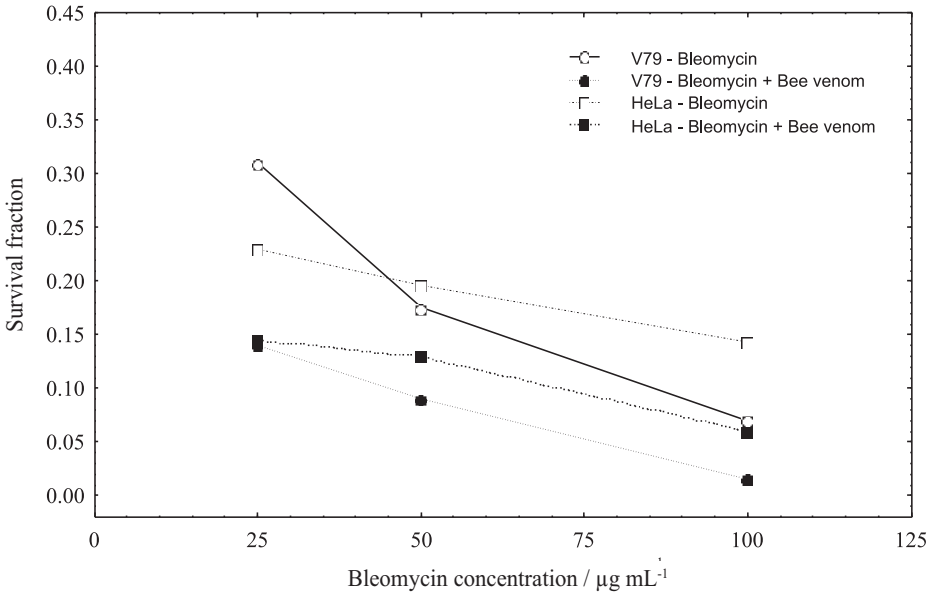

Figure 3 *Dose-response curves of exponentially growing V79 and HeLa cells treated with various concentrations of bleomycin (25 µg mL-1 to 100 µg mL-1) or bleomycin and bee venom (2.85 µg mL-1) for 1 h. After treatment, their survival was determined by colony formation. Colonies were stained with 10 % Giemsa and counted. A cell was considered to have retained reproductive capacity (survival) if it gave rise to a colony of 50 or more cells. The survival index was derived by dividing the number of colonies formed per plate by the total number of single cells initially seeded per plate.*

Effect of bee venom on recovery from bleomycin-induced damage

Figure 4 shows how bee venom affected recovery from bleomycin-induced damage based on increased survival observed when cells were exposed to various concentrations of bee venom after a single exposure to a bleomycin. HeLa and V79 cells were recovering in a fresh medium, and the addition of bee venom to the medium at concentrations of 0.7125 µg mL^{-1}, 1.425 µg mL^{-1}, or 2.85 µg mL^{-1} not only inhibited recovery, but also resulted in a significant increase

($p<0.05$, $p<0.01$, $p<0.001$) in the number of killed HeLa cells within the first three hours. Similar effects were observed in experiments with V79 cells (Figure 4).

Apoptosis and necrosis assay

The effect of bee venom (1.425 μg mL^{-1} or 2.25 μg mL^{-1}) on HeLa cells that were treated for 3 h or 15 h was evaluated using flow cytometry (Figure 5). Early stage of apoptosis started three hours after the treatment of HeLa cells with bee venom. In control HeLa cells early stage apoptosis was recorded for 2.15 % of cells, whereas in the culture treated with 1.425 μg mL^{-1} of bee venom for three hours it was recorded for 6.18 % of cells. Apoptosis in HeLa cells treated with bee venom for 15 h was 7.79 % versus 4.09 % in the control line. We also observed a slight increase in the number of necrotic cells both after 3 h and 15 h of HeLa cell incubation with bee venom. Early stage apoptosis and necrosis of V79 cells was not determined. Instead, we determined the percentage of cells in A_0, which is an indicator of late apoptosis (Figure 6). Apoptosis in V79 cells treated with 1.425 μg mL^{-1} of bee venom for 3 h was 13.14 % versus 5.6 % in the control line, while apoptosis in V79 cells treated for 15 h was lower than control (Figure 6).

DISCUSSION

The results of our study provide an additional support to previous reports on antitumour activity of melittin *in vitro* (1, 4, 15-17). Melittin was a more potent inhibitor of calmodulin activity and growth of L1210 cells, C6 astrocytoma, and human and murine leukaemic cells than trifluoperazine or chlorpromazine. Weiss et al. (29, 30) demonstrated that phenothiazines and structurally similar drugs were binding to and inhibiting calmodulin activity.

Our study shows that bee venom inhibits cell growth, cell proliferation, and clonogenicity of V79 and HeLa cells, possibly through inhibition of calmodulin (Figures 1 and 2). Our results suggest that V79 cells are more sensitive to calmodulin inhibitors and the lytic effect of bee venom than HeLa cells. This effect may be owed to differences in average doubling time in the log phase between V79 and HeLa cells (12 h for V79 cells and about 20 h for HeLa cells), differences in membrane topography, and differences

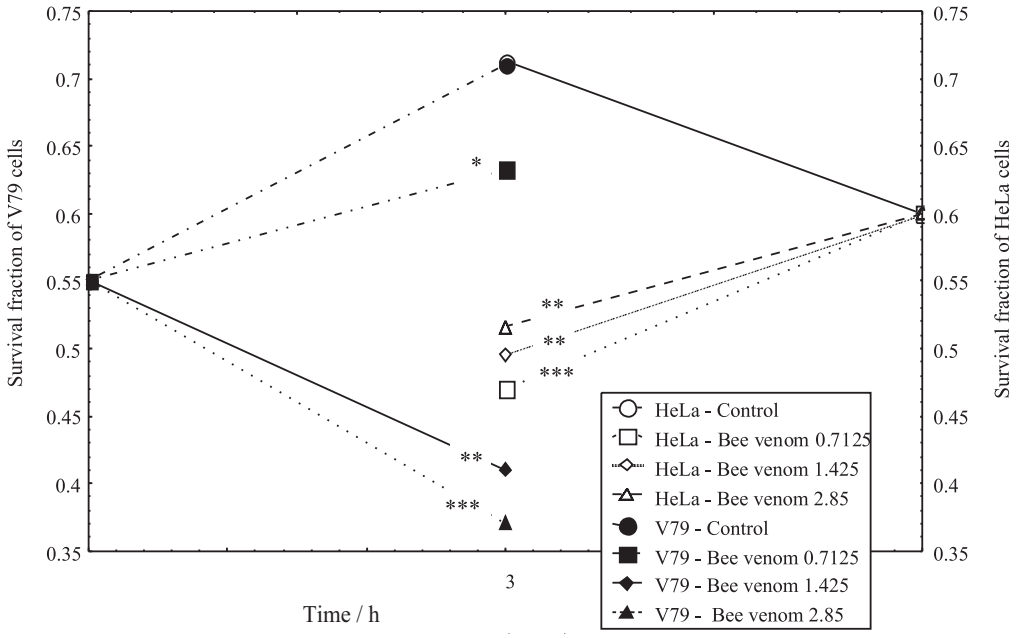

Figure 4 *Effect of bee venom on recovery from bleomycin-induced damage. Exponentially growing V79 and HeLa cells were treated with bleomycin (100 μg mL^{-1}) for 1 h. After treatment, one group of cells was immediately processed for colony formation, with no time allowed for recovery. Other cells were treated with bee venom in various concentrations. Cultures were then incubated at 37 °C and allowed to recover for 3 h. At appropriate times, replicate plates were removed and prepared for colony formation. Cell survival was determined as described in methods.*

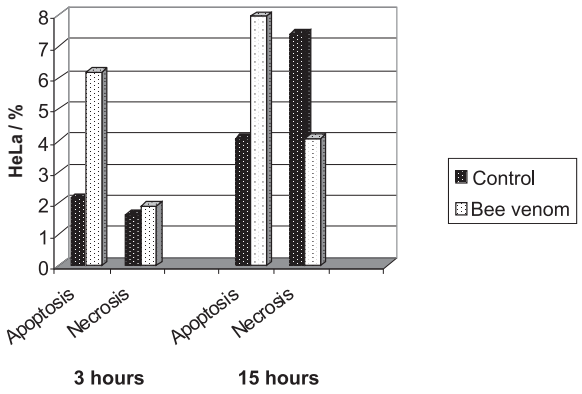

Figure 5 *Induction of apoptosis and necrosis in HeLa cells by bee venom. Cells were cultured in the absence or presence of bee venom (1.425 μg mL^{-1}) for 3 h or 15 h, washed and stained with fluorescein-labelled annexin V and propidium iodide and then analysed by flow cytometry.*

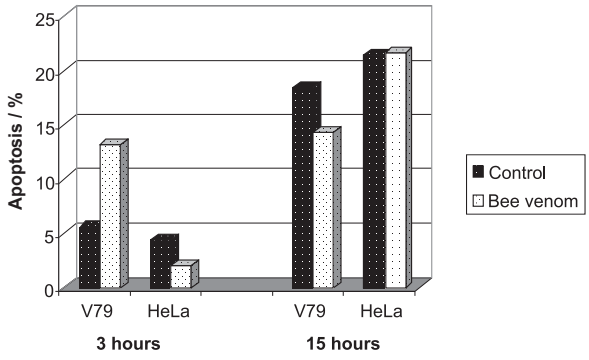

Figure 6 *Induction of apoptosis in V79 and HeLa cells by bee venom. Cells were cultured in the absence or presence of bee venom (1.425 μg mL^{-1}) for 3 h or 15 h, washed, fixed, permeabilised, and stained with propidium iodide and then analysed by flow cytometry.*

in cell calcium and magnesium levels. In addition, V79 cells are rich in amino-group/carbohydrate binding sites, which also bind melittin (31).

We also observed differences in the growth curves of HeLa and V79 cells treated with bee venom. Since the number of cells in a culture is a measure of both cell proliferation and death, growth inhibition by calmodulin inhibitors could be due to the inhibition of proliferation rather than to cytotoxicity, which is in line with our results. Balk et al. (32) strongly suggest that both calcium and magnesium play significant roles in triggering cell replication. However, other evidence indicates that extracellular calcium is necessary for DNA synthesis and cell proliferation in normal cells, but not in cancer cells (33).

The mechanism by which bee venom exerts anticalmodulin activity and inhibits cell growth is unknown. One possible mechanism might be the inhibition of a calmodulin-sensitive enzyme such as cyclic nucleotide phosphodiesterase. Inhibition appears to be mediated by the formation of a calcium-dependent high-affinity complex of calmodulin and melittin (34-37). Although phosphodiesterase was the first enzyme demonstrated to be activated by calmodulin, it is now known that numerous enzymes and structural proteins depend on this pervasive calcium-binding protein (38, 39). Calmodulin is essential for many processes in normal cell function, including the assembly and disassembly of microtubules, calcium extrusion from cells by a calcium-magnesium ATPase, and activation of numerous intracellular enzymes such as protein kinases, phosphatases, and cyclic nucleotide phosphodiesterase (38, 39). Interference with any of these known functions of calmodulin could be toxic to cells. Other cell functions affected by calmodulin inhibitors may or may not be mediated exclusively by calmodulin inhibition. These effects include depletion of intracellular ATP (40), destabilisation of the membranes (1, 10, 13, 14), and inhibition of protein kinase C.

Our data indicate that bee venom inhibits DNA repair and that this may be the mechanism by which it increases bleomycin lethality and inhibits recovery from bleomycin-induced damage (Figures 3 and 4). Several mechanisms may explain our findings. Firstly, efflux of antitumor agents from cells may be controlled by calcium and calmodulin (calcium-calmodulin complex), and calmodulin inhibitors could directly inhibit the calcium-calmodulin complex. Secondly, permeability of cell membranes might be perturbed by bee venom or by changes in calcium environment. Thirdly, the affinity of bleomycin for intracellular targets may be altered by calcium antagonist-induced changes in calcium environment or by calmodulin inhibitors.

In this study, we have shown that bee venom might result in apoptosis and/or necrosis of HeLa and V79 cells. Bee venom caused dose- and time-dependent changes in V79 cells, but not in HeLa cells (Figures 5 and 6). The absence of apoptosis or necrosis in HeLa cells remains unexplained, and calls for a long-term study with bee venom. It is also possible that melittin as a Ca^{2+} antagonist also blocks the Ca^{2+} channel, and offers complete protection against cell death. In

certain cell lines it was demonstrated that Ca^{2+} channel blockers stop apoptosis or necrosis and thus reduce DNA damage (41). Studies with well-known Ca^{2+} channel blockers and specific genes such as p53, c-myc, bcl_2 and bcl_x, may help to clarify this question.

Most examples of apoptosis are accompanied by significant cytosolic and nuclear Ca^{2+}accumulation and Ca^{2+}/Mg^{2+} endonuclease activation. However, there is a growing evidence showing no increase in intracellular Ca^{2+} (42) and no early appearance of internucleosomal DNA fragments characteristic of Ca^{2+}/Mg^{2+} endonuclease activation (43-45). Although these reports argue that Ca^{2+}/Mg^{2+}endonuclease fragmentation of DNA may not always be involved in apoptosis, and that, when present, it may be a relatively late molecular event, they did not dismiss other forms of DNA damage, such as detachment of duplex DNA loops from the nuclear scaffold and the cleavage of DNA into large kilobase and megabase fragments, as important early events in the stereotype changes in DNA morphology. Although the role of Ca^{2+} in early chromatin disruption is ambiguous, DNA damage remains the leading candidate for initiating apoptotic cell death.

The underlying mechanisms of apoptosis are largely unknown. A recent study by Tu et al. (46) demonstrated anticancer effects of bee venom in human melanoma A2058 cells and its mode of action at the cellular and molecular levels. The authors found that honeybee venom induced calcium-dependent but caspase-independent apoptotic cell death in human melanoma A2058 cells, but not in normal skin fibroblast Detroit 551 cells. They suggested that treatment of A2058 cells with melittin, the key component of bee venom, resulted in similar elevation of calcium levels and cell death (47). Observations by Ip et al. (48) indicated that bee venom induced cell cycle arrest and apoptosis in human cervical epidermoid carcinoma Ca Ski cells via a Fas receptor pathway, involving mitochondrial-dependent pathways, and that it is closely related to the level of cytoplasmic Ca^{2+} in Ca Ski cells. Flow cytometry in their study showed that bee venom induced reactive oxygen species, increased the level of cytoplasmic Ca^{2+}, reduced mitochondrial membrane potential which led to cytochrome c release, and promoted the activation of caspase-3 which then led to apoptosis. Bee venom also induced an increase in the levels of Fas, p53, p21 and Bax, but a decrease in the level of Bcl-2. The activities of both caspase-8 and caspase-9 were enhanced by bee venom, promoting caspase-3 activation and leading to DNA fragmentation. Apart from the caspase-dependent pathway, these authors also suggested a caspase-independent pathway through expression of a*poptosis*-inducing factor (AIF) and endonuclease G (Endo G) in the Ca Ski cells. A similar mechanism of bee venom was confirmed in human breast cancer MCF7 cells (49), U937 cells (50), and in human MG63 osteosarcoma cells (47). Data by Wang et al. (51) suggest that melittin can synergize with TRAIL in the induction of human hepatocellular carcinoma cell (HCC) apoptosis, and that the combination of melittin with tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) may be a promising therapeutic approach in the treatment of TRAIL-resistant human cancer.

In summary, this study suggests that proliferation and clonogenicity of V79 and HeLa cells in culture with the bee venom is not entirely due to the inhibition of calmodulin, and that the cytotoxic effect of bee venom could partially be related to its apoptotic and necrotic activities. This study also points to possible new ways to use bee venom to improve the cytotoxic effects of antitumour, calmodulin-inhibiting agents.

REFERENCES

1. Oršolić N, Šver L, Verstovšek S, Terzić S, Bašić I. Inhibition of mammary carcinoma cell proliferation *in vitro* and tumor growth *in vivo* by bee venom. Toxicon 2003;41:861-70.
2. Oršolić N, Sacases F, Percie du Sert P, Bašić I. Antimetastatic ability of honey bee products. Period Biol 2007;109:173-80.
3. Liu S, Yu M, He Y, Xiao L, Wang F, Song C, Sun S, Ling C, Xu Z. Melittin prevents liver cancer cell metastasis through inhibition of the Rac1-dependent pathway. Hepatology 2008;47:1964-73.
4. Liu X, Chen DW, Xie LP, Rongqing Z. Effect of honey bee venom on proliferation of K1735M2 mouse melanoma cells in-vitro and growth of murine B 16 melanomas in-vivo. J Pharm Pharmacol 2002;54:1083-9.
5. Yusuf N, Irby C, Katiyar SK, Elmets CA. Photoprotective effects of green tea polyphenols. Photodermatol Photoimmunol Photomed 2007;23:48-56.
6. Kwon YB, Lee JD, Lee HJ, Han HJ, Mar WC, Kang SK, Beitz, AJ, Le E JH. Bee venom injection into an acupuncture point reduces arthritis associated edema and nociceptive responses. Pain 2001;90:271-80.
7. Raghuraman H, Chattopadhyay A. Melittin: a membrane-active peptide with diverse functions. Biosci Rep 2007;27:189-223.
8. Habermann E, Jentsch J. Sequential analysis of melittin from triptic and peptic fragments. Biochem J 1967;348:37-42.
9. Gauldie J, Hanson JM, Shipolini RA, Vernon CA. The structures of some peptides from bee venom. Eur J Biochem 1978;83:405-10.

10. Habermann E. Bee and wasp venoms: The biochemistry and pharmacology of their peptides and enzymes are reviewed. Science 1972;177:314-22.
11. Orlov BN, Romanova EB, Omarov SM. Immunological properties of bee venom. In: Proceedings of the XXVIIIth International Congress of Apiculture; 23-29 Oct 1981; Acapulco, Mexico. Bucharest, Romania; Apimondia Publishing House; 1981. p. 490-3.
12. Orlov BN. Theoretical basis for therapeutical use of bee venom. Apiacta 1979;14:12-5.
13. Sharma SV. Melittin-induced hyperactivation of phospholipase A2 activity and calcium influx in ras-transformed cells. Oncogene 1993;8:939-47.
14. Hait WN, Cadman E, Benz C, Cole J, Weiss B. Inhibition of cyclic nucleotide phosphodiesterase and calmodulin. Proc Am Assoc Cancer Res 1983;2:5-9.
15. Hait WN, Grais L, Benz C, Cadman EC. Inhibition of growth of leukemic cells by inhibitors of calmodulin: phenothiazines and melittin. Cancer Chemother Pharmacol 1985;14:202-5.
16. Hait WN, Lee GL. Characterization of the cytotoxic effects of calmodulin inhibitors. Clin Res 1984;32:416A.
17. Lee GL, Hait WN. Growth inhibition of C6 astrocytoma cells by inhibitions of calmodulin. Life Sci 1985;36:347-54.
18. Okumara K, Kato T, Ito J, Tanaka R. Inhibition by calmodulin antagonists of glioblast DNA synthesis and morphological changes induced by glial maturation factor. Dev Brain Res 1982;255:661-5.
19. Means AR, Chafouleas JA, Lagace L, Lai E, Stein JP. Multiple roles for calmodulin in the regulation of eukaryotic cell metabolism. In: O'Malley BW, editor. Gene regulation. New York: Academic Press; 1982. p. 307-26.
20. Chafouleas JG, Bolton WE, Hidaka H, Boyd AE, Means AR. Calmodulin and the cell cycle: involvement in regulation of cell cycle progression. Cell 1982;28:41-50.
21. Chafouleas JG, Botton WE, Means AR. Potentiation of bleomycin lethality by anti-calmodulin drugs: a role for calmodulin in DNA repair. Science 1984;224:1346-8.
22. Gamapathi R, Grabowski D. Enhancement of sensitivity to adriamyc in resistant P388 leukemia by the calmodulin inhibitor, trifluoperazine. Cancer Res 1983;43:3696-9.
23. Tsuro T, Iida H, Tsukagoshi S, Sakurai Y. Increased accumulation of vineristine and adriamycin in drug-resistant P388 tumor cells following with calcium antagonists and calmodulin inhibitors. Cancer Res 1982;42:4730-3.
24. Wei JW, Hickie RA, Klaassen DJ. Inhibition of human breast cancer colony formation by anticalmodulin agents, trifluoperazine, W-7 and W-13. Cancer Chemother Pharmacol 1983;11:86-90.
25. Ito, H, Hidaka, H. Antitumor effect of a calmodulin antagonist on the growth of solid sarcoma-180. Cancer Lett 1983;19:215-9.
26. Oršolić N, Josipović P, Bašić I. Direct antitumor activity of honey bee venom *in vivo* and *in vitro.* Egypt J Nat Tox 2009;6:1-16.
27. Gajski G, Garaj-Vrhovac V. Genotoxic potential of bee venom (Apis Mellifera) on human peripheral blood lymphocytes in vitro using single cell gel electrophoresis assay. J Environ Sci Health A Tox Hazard Subst Environ Eng 2008;43:1279-87.
28. Telford WG, King LE, Fraker PJ. Rapid quantitation of apoptosis in pure and heterogeneous cell populations using flow cytometry. J Immunol Methods 1994;172:1-16.
29. Levin RM, Weiss B. Binding of trifluoperazine to the calcium-dependent activator of cyclic nucleutide phosphodiesterase. Mol Pharmacol 1977;13:690-5.
30. Sellinger-Barnette M, Weiss B. Interaction of various peptides with calmodulin. In: Greengard P, Robinson GA, editors. Advances in cyclic nucleotide and protein phosphorylation research. New York (NY): Raven Press; 1984. p. 261-76.
31. Killion JJ, Dunn JD. Differential cytolysis of murine spleen, bone-marrow and leukemia cells by melittin reveals differences in membrane topography. Biochem Biophys Res Commun 1986;139:222-7.
32. Balk SD, Polimeni PI, Hoon BS, LeStourgeon DN, Mitchell RS. Proliferation of Rous sarcoma virus-infected, but not of normal chicken fibroblasts in a medium of reduced calcium and magnesium concentration. Proc Natl Acad Sci USA 1979;76:3913-6.
33. Whitfield JF, Boynton AL, MacManus JP, Sicorska M, Tsang BK. The regulation of cell proliferation by calcium and cyclic AMP. Mol Cell Biochem 1979;27:155-79.
34. Barnette MS, Daly R, Weiss B. Inhibition of calmodulin activity by insect venom peptides. Biochem Pharmacol 1983;32:2929-33.
35. Comte M, Maulet Y, Cox JA. Ca^{2+} dependent high affinity complex formation between calmodulin and melittin. Biochem J 1983;209:269-72.
36. Maulet Y, Cox JA. Structural changes in melittin and calmodulin upon complex formation and their modulation by calcium. Biochemistry 1983;22:5680-6.
37. Tanaka J, Takahashi S, Mitsui Y, Itoh S, Iitaka Y, Kasai H, Okuyama T. X-Ray crystallographic and chromatographic characterization of the crystals of Ca^{2+}-calmodulin complexed with bee venom melittin. J Mol Biol 1985;186:675-7.
38. Cheung WY. Calmodulin: an overview. Fed Proc 1982;41:2253-7.
39. Weiss B, Wallace TL. Mechanism and pharmacological implications of altering calmodulin activity In: Cheung WY, editor. Calcium and cell function. Vol. 1. New York (NY): Academic Press; 1980. p. 329-79.
40. Zimmerman TP, Wolberg G. Effect of calmodulin antagonists on lymphocyte-mediated cytolysis. Proc Am Assoc Cancer Res 1984;25:351-5.
41. Melino G, Knight RA, Nicotera P. How many ways to die? How many different models of cell death? Cell Death Differ 2005;12(Suppl 2):1457-62.
42. Lennon SV, Kilfeathr SA, Hallett MB, Campbell AK, Cotter, TG. Elevations in cytosolic free Ca^{2+} are not required to trigger apoptosis in human leukemia cells. Clin Exp Immunol 1992;87:465-71.
43. Oberhammer F, Fritsch G, Schmied M. Condensation of the cromation at the membrane of an apoptotic nucleus is not associated with activation an endonuclease. J Cell Sci 1993;104:317-26.
44. Oberhammer F, Wilson JW, Dive C, Morris ID, Hickman JA, Wakeling AE, Walker PR, Sikorska M. Apoptotic death in epithelial cells: cleavage of DNA to 300 and/or 50 kb fragments prior to or in the absence of internucleosomal fragmentation. EMBO J 1993;12:3679-84.
45. Zakeri ZF, Quaglino D, Latham T, Lockshin, RA. Delayed internucleosomal DNA fragmentation in programmed cell death. FASEB J 1993;7:470-8.
46. Tu WC, Wu CC, Hsieh HL, Chen CY, Hsu SL. Honeybee venom induces calcium-dependent but caspase-independent

apoptotic cell death in human melanoma A2058 cells. Toxicon 2008;52:318-29.
47. Chu ST, Cheng HH, Huang CJ, Chang HC, Chi CC, Su HH, Hsu SS, Wang JL, Chen IS, Liu SI, Lu YC, Huang JK, Ho CM, Jan CR. Phospholipase A2-independent Ca2+ entry and subsequent apoptosis induced by melittin in human MG63 osteosarcoma cells. Life Sci 2007;80:364-9.
48. Ip SW, Wei HC, Lin JP, Kuo HM, Liu KC, Hsu SC, Yang JS, Mei-Dueyang Chiu TH, Han SM, Chung JG. Bee venom induced cell cycle arrest and apoptosis in human cervical epidermoid carcinoma Ca Ski cells. Anticancer Res 2008;28:833-42.
49. Ip SW, Liao SS, Lin SY, Lin JP, Yang JS, Lin mL, Chen GW, Lu HF, Lin MW, Han SM, Chung JG. The role of mitochondria in bee venom-induced apoptosis in human breast cancer MCF7 cells. In Vivo 2008;22:237-45.
50. Moon DO, Park SY, Choi YH, Kim ND, Lee C, Kim GY. Melittin induces Bcl-2 and caspase-3-dependent apoptosis through downregulation of Akt phosphorylation in human leukemic U937 cells. Toxicon 2008;51:112-20.
51. Wang C, Chen T, Zhang N, Yang M, Li B, Lu X, Cao X, Ling C. Melittin, a major component of Bee Venom, sensitizes human hepatocellular carcinoma cells to TRAIL-induced apoptosis by activating CaMKII-TAK1-JNK/p38 and inhibiting IKK-NFkappa B. J Biol Chem 2009;284:384-13.

Sažetak

POVEĆANJE LETALNOG UČINKA BLEOMICINA NA STANICE HeLa I V79 S POMOĆU PČELINJEG OTROVA

U uvjetima *in vitro* istražen je inhibitorni učinak pčelinjeg otrova, samog ili združenog s citostatikom bleomicinom, na rast stanica HeLa i V79. Rabljene su sljedeće metode: brojenje stanica, metoda klonskog rasta i apoptoza. Poznato je da neki antagonisti kalcija i kalmodulinski inhibitori povisuju unutarstaničnu razinu protutumorskih lijekova inhibirajući njihov prijenos iz stanice. Unutarstanična akumulacija melitina izravno povećava citotoksični učinak protutumorskog lijeka. Obrada stanica HeLa i V79 u eksponencijalnoj fazi rasta bleomicinom uzrokuje oštećenje DNA ovisno o dozi te smanjenje broja živih stanica. Uočeno je da se letalni učinak bleomicina može pojačati dodatkom neletalne doze pčelinjeg otrova. Pčelinji otrov pritom inhibira popravak nastalih oštećenja u stanicama HeLa i V79 te sprječava oporavak stanica tretiranih bleomicinom. Apoptoza, nekroza i liza mogući su mehanizmi kojima pčelinji otrov inhibira rast i stvaranje kolonija stanica V79, dok HeLa-stanice pokazuju pojačanu otpornost na pčelinji otrov. Istraživanje također potvrđuje mogućnost uporabe pčelinjeg otrova u povećanju citotoksičnosti bleomicina.

KLJUČNE RIJEČI: *apoptoza, citotoksičnost, inhibicija rasta, melitin, stanične linije*

CORRESPONDING AUTHOR:

Nada Oršolić
Department of Animal Physiology
Faculty of Science, University of Zagreb
Rooseveltov trg 6, 10 000 Zagreb, Croatia
E-mail: *norsolic@yahoo.com*