

## *Naja ashei* venom induces mitochondria-mediated apoptosis in human colorectal cancer cells

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In the present study, we investigated the antiproliferative activity of *Naja ashei* full venom (NAV) on human colorectal cancer cells. The NAV-induced antiproliferative effect was associated with cell cycle arrest in S phase and increased number of cells with sub G0/G1 DNA content, which is considered a marker of apoptosis. Apoptosis has also been confirmed with annexin V/PI staining. Furthermore, flow cytometric analysis revealed loss of mitochondrial membrane potential with concomitant increase in cytochrome c and Smac/DIABLO protein content. These effects were associated with the activation of caspase-9 and caspase-3, as well as with PARP cleavage. Moreover, phosphorylation of antiapoptotic Bcl-2 protein in NAV-treated HCT116 was observed. In conclusion, our study for the first time documented antiproliferative/pro-apoptotic effect of NAV in colorectal cancer cells. Our results strongly suggest the involvement of mitochondria in NAV induced apoptosis of cancer cells. Future studies are needed to further examine the potential of NAV in the treatment of colon cancer.

**Key words:** *Naja ashei* venom, colorectal cancer cells, antiproliferative effect, apoptosis

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**Abbreviations:** CRC, colorectal cancer; BrdU, 5-bromo-2'-deoxyuridine; MMP, mitochondrial membrane potential; MTS, methyl tetrazolium salt; NAV, *Naja ashei* venom; PARP, poly (ADP-ribose) polymerase; PI, propidium iodide; PS, phosphatidylserine

### INTRODUCTION

Despite improvements in treatment and diagnosis, cancer is one of the most common causes of death in the world. It is estimated that in 2018, there were over 18 million new cancer cases and 9.6 million deaths from cancer (Bray *et al.*, 2018). Among all cancers, colorectal cancer (CRC) ranks second in terms of mortality, accounting for about 1 in 10 cancer deaths in 2018 (Bray *et al.*, 2018). In the current treatment of CRC, chemotherapy play an important role, and anti-cancer drugs such as platinum derivatives, antimetabolites or topoisomerase inhibitors are most

commonly used (Rejhova *et al.*, 2018). On-going progress in the treatment of CRC significantly improves the prognosis of patients, however, the side effects of antineoplastic drugs and their low specificity continue to be responsible for a reduced quality of life (Coppola *et al.*, 2018). Therefore, the new compounds for CRC treatment are needed. The largest source of bioactive chemicals is nature. Numerous articles have documented antiproliferative/anticancer effects of plant-derived compounds known as phytochemicals (Dutt *et al.*, 2018; Einbond *et al.*, 2017; Iqbal *et al.*, 2018). In our laboratory we have also documented antiproliferative effect of several flavonoids, chalcones or indole phytoalexins (Kello *et al.*, 2014; Kello *et al.*, 2017; Takac *et al.*, 2018; Tischlerova *et al.*, 2017). However, the antiproliferative effect of agents from animal sources was studied only marginally, as compared to plant-derived compounds. Among those, the snake venoms attracted attention for possible use as anticancer agents.

The anticancer activity of snake venom was first reported by Calmette and co-workers in 1933 (Calmette *et al.*, 1933), who observed the antitumor effect of cobra venom in adenocarcinoma cells. Since then, several *in vivo* and *in vitro* experiments have shown that snake venoms can inhibit the growth and proliferation of various types of cancer cells (Liu *et al.*, 2014).

Although the precise mechanism of anti-cancer effect of snake venoms remains to be elucidated, the existing results indicate the multitarget action including apoptosis induction (Park *et al.*, 2009), modulation of signalling pathways associated with cell survival or death (Song *et al.*, 2012), reactive oxygen species (ROS) production (Park *et al.*, 2012) and mitochondrial disruption (Fakhri *et al.*, 2017). Moreover, the ability of snake venoms to inhibit angiogenesis or metastasis has also been documented (Calderon *et al.*, 2014; Kerkkamp *et al.*, 2018).

In the present study, the effect of *Naja ashei* venom (NAV) on colon cancer cells was investigated. *Naja ashei* is an African spitting cobra species which has been classified as a separate species in 2007 (Wuster & Broadley, 2007).

According to our knowledge, no study focused on the pro-apoptotic effect of NAV has been published so far. This study is a novel investigation of the effect of NAV on apoptosis in human colorectal cancer cells.

## MATERIAL AND METHODS

**Snake venom collection.** The full *Naja ashei* venom samples were obtained from two adult snakes, which were captured and officially imported from Kenya. Venom was extracted in the Pata breeding garden near Hlohovec (Slovakia), under the veterinary certificate No. CHEZ-TT-01. After extraction, the venom was stored at  $-20^{\circ}\text{C}$  (transport temperature) and then moved to  $-80^{\circ}\text{C}$  for deep freezing.

The breeding garden serves as a quarantine station for imported animals and is an official importer of exotic animals from around the world, having the permission of the State Nature Protection of the Slovak Republic under the No. 03418/06, the trade with endangered species of wild fauna and flora and on amendments to certain laws under Law no. 237/2002 Z.z.

**Cell culture.** The human colorectal cancer cell line (HCT116) was cultured in RPMI 1640 medium and the human dermal fibroblast cell line (HDF; non-cancer) was cultured in DMEM with sodium pyruvate (Biosera, Kansas City, MO, United States). The growth media were supplemented with 10% fetal bovine serum, penicillin (100 IU/mL) and streptomycin (100  $\mu\text{g}/\text{mL}$ ) (all Invitrogen, Carlsbad, CA, United States). Cells were cultured in 5%  $\text{CO}_2$  in humidified air at  $37^{\circ}\text{C}$ . Cell viability, estimated with trypan exclusion assay before each experiment, was greater than 95%.

**MTS cell proliferation/viability assay.** MTS assay was used to determine the effects of NAV (5.7–17.1  $\mu\text{g}/\text{ml}$ ) on the metabolic activity of both HCT116 and HDF cells. After 72 hours of incubation, 10  $\mu\text{L}$  of MTS (Promega, Madison, WI, USA) was added to each well according to the CellTiter 96<sup>®</sup> Aqueous One Solution Cell Proliferation Assay protocol. After minimum 1-hour incubation, the absorbance was measured at 490 nm using the automated Cytation<sup>™</sup> 3 Cell Imaging Multi-Mode Reader (Biotek, Winooski, VT, USA). The absorbance of the control wells was taken as 1.0 (100%) and the results were expressed as a percent of the control. All experiments were performed in triplicate.

**BrdU cell proliferation assay.** Cell proliferation was confirmed using the 5-bromo-2'-deoxyuridine (BrdU) Cell Proliferation Assay Kit (Roche Diagnostics, Man-

nheim, Germany). Cells were seeded at a density of  $10 \times 10^3$  cells/well in 96-well polystyrene microplates (SARSTEDT, Nümbrecht, Germany). Twenty-four hours after cell seeding, different concentration (5.7–17.1  $\mu\text{g}/\text{ml}$ ) of NAV were added. After 48 h of treatment, cells were incubated with BrdU labelling solution (10 mM final concentration) for another 24 h at  $37^{\circ}\text{C}$  followed by fixation and incubation with anti-BrdU peroxidase conjugate for an additional 1.5 h at room temperature. Finally, after substrate reaction, the "stop solution" was added (25 ml 1 M  $\text{H}_2\text{SO}_4$ ), and color intensity was measured with an automated Cytation<sup>™</sup> 3 Cell Imaging Multi-Mode Reader (Biotek). Absorbance of control wells was taken as 100%, and the results were expressed as a percentage of the untreated control.

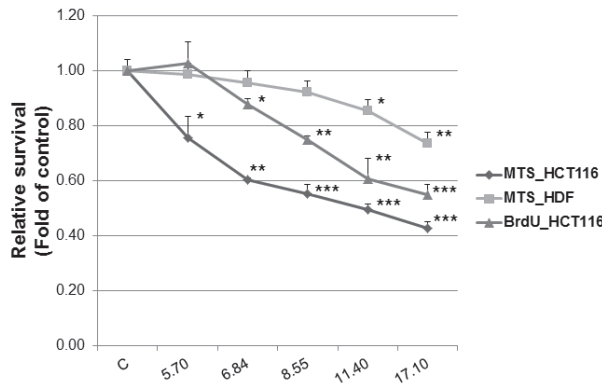
**Cell cycle analysis.** The HCT116 cells, both floating and adherent, were harvested ( $1 \times 10^6$  cells) 24, 48 and 72 hours after adding NAV (6.84  $\mu\text{g}/\text{ml}$ ), washed in PBS, fixed in cold 70% ethanol and kept at  $+4^{\circ}\text{C}$  overnight. Prior to analysis, the cells were washed in PBS, resuspended in staining solution (0.2% Triton X-100, 0.5 mg/ml ribonuclease A and 0.025 mg/ml propidium iodide in 500  $\mu\text{l}$  PBS; all Sigma Aldrich, St. Louis, MO, USA) and incubated for 30 minutes at room temperature in the dark. The DNA content of the stained cells was analyzed using a BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). For each sample, a minimum of  $1 \times 10^4$  cells were evaluated and the sample flow rate during analysis did not exceed 200–300 cells per second.

**Apoptosis detection via externalized phosphatidylserine (PS).** For apoptosis detection, the floating and adherent HCT116 cells ( $1 \times 10^6$ ) were harvested 24, 48 and 72 hours after treatment with NAV (6.84  $\mu\text{g}/\text{ml}$ ), washed in PBS and stained using Annexin-V-Alexa Fluor 647 conjugated antibody (Invitrogen, Carlsbad, CA, USA) for 15 minutes at room temperature in the dark. Prior to analysis the cells were stained with PI and were next observed using a BD FACSCalibur flow cytometer.

**Flow cytometric analysis.** HCT116 cells ( $1 \times 10^6$ ) were treated with NAV at concentration 6.84  $\mu\text{g}/\text{ml}$  for 24, 48 and 72 hours. Floating and adherent cells were harvested, washed in PBS, split for particular analysis and stained prior to analysis (Table 1). Fluorescence was detected after 15 minutes incubation at room tempera-

**Table 1. Flow cytometry cell staining.**

Analysis	Staining solution	Manufacturer
Caspase activation	Cleaved Caspase-3-PE	Cell Signaling Technology, Danvers, MA, USA
	Cleaved Caspase-9-FITC	BIOSS Antibodies, Woburn, MA, USA
Cytochrome c release	Cytochrome c antibody (6H2) FITC conjugate	Invitrogen, Carlsbad, CA, USA
Cleavage of PARP	Cleaved-PARP (Asp214) XP <sup>®</sup> rabbit mAb (PE Conjugate)	Cell Signaling Technology, Danvers, MA, USA
Mitochondrial membrane potential	TMRE (tetramethylrhodamine ethyl ester perchlorate), final concentration 0.1 $\mu\text{M}$	Sigma-Aldrich, St. Louis, Missouri, USA
	Bcl-2 mouse mAb (PE conjugate)	
Protein analysis	Phospho-Bcl-2 (Ser70) rabbit mAb Alexa Fluor <sup>®</sup> 488 conjugate	Cell Signaling Technology <sup>®</sup> , Danvers, MA, USA
	Smac/Diablo (D5S3R) rabbit mAb	
	Goat anti-rabbit IgG (H+L) secondary antibody conjugated with Alexa Fluor 488	Thermo Scientific, Rockford, IL, USA



**Figure 1.** The effect of NAV (5.7 to 17.1 µg/ml) on the metabolic activity and cell proliferation in HCT116 (as assessed with MTS and BrdU incorporation assays) and in HDF cells (as assessed with MTS assay).

Data were obtained from three independent experiments. Statistical significance of comparison with control cells (untreated) is marked as \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; and for the comparison between the cell lines is marked as \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

ture in the dark using a BD FACSCalibur flow cytometer. A minimum of  $1 \times 10^4$  events was used per analysis.

**Statistical analysis.** The results are expressed as mean  $\pm$  S.D. The statistical analysis of the data was performed using standard procedures, with one-way ANOVA followed by the Bonferroni multiple comparisons test. Values of  $p < 0.05$  were considered statistically significant.

**RESULTS**

**MTS cell proliferation/viability assay and BrdU cell proliferation assay**

The metabolic activity and cell proliferation on the human cancer cell line HCT116 showed that NAV inhibits cell proliferation in a dose-dependent manner. Incubation of HCT116 cells with NAV in all concentrations used, significantly decreased metabolic activity ( $p < 0.05 - p < 0.001$ ). In comparison with HCT116, the effect of NAV on non-cancer HDF cells was significantly weaker (Fig. 1). To confirm antiproliferative effect of NAV, we used BrdU cell proliferation assay which detected 5-bromo-2'-deoxyuridine (BrdU) incorporated into cellular DNA during cell proliferation. With the exception of the lowest concentration (5.7 µg/ml), NAV significantly decreased incorporation of BrdU into DNA, i.e. inhibited cell proliferation ( $p < 0.05 - p < 0.001$ ).

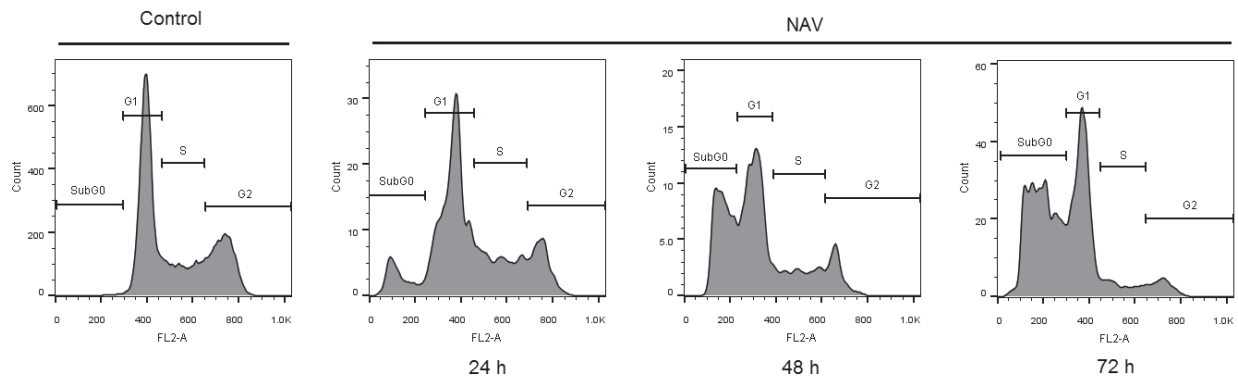
Based on of MTS and BrdU assays, we selected a concentration of 6,84 µg/ml for all other experiments.

**Cell cycle analysis.** Cell cycle phases distribution was determined using flow cytometric analysis of HCT116 cells after NAV treatment for 24, 48 and 72 hours. The results showed a significant increase in the number of cells with sub-G<sub>0</sub>/G<sub>1</sub> DNA content after 24 h treatment, which increased after 48 and 72 hours. At the same time, an increase in the population of cells in S phase was observed after 24 and 48 treatment, which suggested cell cycle arrest (Table 2, Fig. 2). Accordingly, a significant concomitant reduction of populations in G<sub>1</sub>

**Table 2.** Percentage analysis of the cell cycle phases in the NAV-treated (6.84 µg/ml) HCT116 cells, determined with the use of flow cytometry.

Treatment	Time (h)	sub-G <sub>0</sub> /G <sub>1</sub>	G <sub>1</sub>	S	G <sub>2</sub> /M
Control	24	0.92 $\pm$ 0.17	42.10 $\pm$ 0.86	27.53 $\pm$ 1.80	29.43 $\pm$ 2.35
	48	0.97 $\pm$ 0.35	51.40 $\pm$ 2.61	26.50 $\pm$ 2.08	21.13 $\pm$ 2.82
	72	1.82 $\pm$ 0.44	56.90 $\pm$ 2.24	20.13 $\pm$ 1.79	21.13 $\pm$ 3.34
NAV	24	15.9 $\pm$ 3.2 <sup>b</sup>	33.35 $\pm$ 3.27 <sup>a</sup>	38.50 $\pm$ 1.22 <sup>b</sup>	12.20 $\pm$ 1.91 <sup>b</sup>
	48	24.0 $\pm$ 1.8 <sup>c</sup>	33.80 $\pm$ 1.38 <sup>b</sup>	33.35 $\pm$ 1.74 <sup>a</sup>	8.87 $\pm$ 1.65 <sup>b</sup>
	72	39.9 $\pm$ 3.4 <sup>c</sup>	43.45 $\pm$ 2.23 <sup>b</sup>	9.42 $\pm$ 2.61 <sup>a</sup>	7.25 $\pm$ 1.18 <sup>b</sup>

The results from three independent experiments are presented as a mean  $\pm$  SD. Statistical significance is marked as <sup>a</sup>when  $p < 0.05$ , <sup>b</sup> $p < 0.01$ , <sup>c</sup> $p < 0.001$  in comparison with the untreated cells (control). The sub- G<sub>0</sub>/G<sub>1</sub> fraction of cells is identified as the apoptotic population.

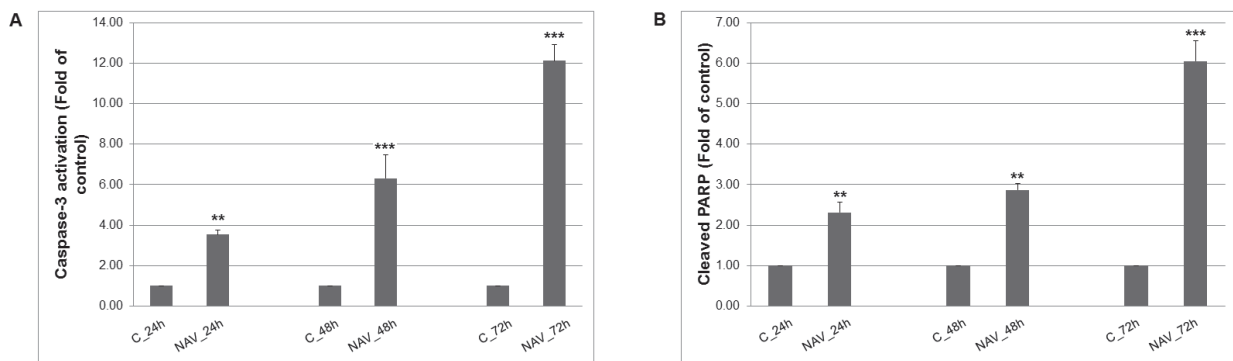


**Figure 2.** Cell cycle phases distribution in HCT116 cells treated with 6,84 µg/ml NAV for 24, 48 and 72 h.

**Table 3. Annexin V/PI flow cytometry analysis of apoptosis occurrence in HCT116 cells after NAV treatment (6.84 µg/ml).**

Treatment	Time (h)	An-/PI-	An+/PI-	An+/PI+	An-/PI+
Control	24	92.30 ± 1.17	0.76 ± 0.47	3.09 ± 0.30	3.85 ± 1.01
	48	92.83 ± 2.25	1.14 ± 0.49	2.85 ± 1.14	3.17 ± 0.96
	72	94.60 ± 1.41	0.49 ± 0.14	1.86 ± 0.50	3.03 ± 1.00
NAV	24	82.67 ± 0.64 <sup>a</sup>	10.40 ± 1.66 <sup>a</sup>	1.61 ± 0.52	5.32 ± 1.29
	48	51.13 ± 2.78 <sup>c</sup>	24.94 ± 2.82 <sup>b</sup>	10.27 ± 1.42 <sup>a</sup>	13.64 ± 1.50 <sup>a</sup>
	72	33.67 ± 3.42 <sup>c</sup>	40.23 ± 1.65 <sup>c</sup>	12.46 ± 2.1 <sup>a</sup>	13.61 ± 2.18 <sup>a</sup>

The results from 3 independent experiments are presented as a percentage of An-/PI- events (living cells), An+/PI- events (cells in a stage of early apoptosis), An+/PI+ events (late apoptotic cells) and An-/PI+ events (necrotic/dead cells). Statistical significance of comparison with untreated cells (control) is described as: a  $p < 0.05$ ; b  $p < 0.01$ ; c  $p < 0.001$ .

**Figure 3. Effect of NAV treatment (6.84 µg/ml) on caspase activity and PARP cleavage.**

(A) Activation of caspase-3; (B) Cleavage of PARP based on flow cytometric analysis at 24, 48 and 72 h after treatment. Graph shows control – normalized mean  $\pm$  S.D. for 3 independent experiments. Statistical significance of treated vs. untreated cells is shown with \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

and G2/M phase after 24, 48 and 72 h after treatment was also observed. These findings suggest significant changes in cell cycle progression and induction of apoptosis in HCT116 cells treated with NAV.

**Apoptosis detection, caspase-3 activation and PARP cleavage.** Because the increase in the number of cells with sub-G0/G1 DNA content is a marker of apoptosis, we performed several complementary assays to confirm the apoptosis-inducing effect of NAV.

One of the earliest features of apoptosis is the translocation of phospholipid phosphatidylserine from the inner to the outer plasma membrane. Due to the high affinity of annexin V for phosphatidylserine, staining with annexin V-FITC conjugate is used to detect early apoptosis. A co-staining with propidium iodide (PI) is used to distinguish between apoptotic and necrotic cells (Crowley *et al.*, 2016). Our results showed that NAV induced a significant increase in the number of annexin V – positive cells with a concomitant increase in the number of PI positive cells that was time dependent (Table 3). Therefore, we suggest that apoptosis plays an important role in the death mechanisms of HCT116 cells treated with NAV.

Another characteristic feature of apoptosis is the proteolytic cleavage of poly (ADP-ribose) polymerase (PARP). This enzyme is involved in DNA repair and stability, and cleavage of PARP by caspases is considered to be a marker of apoptosis.

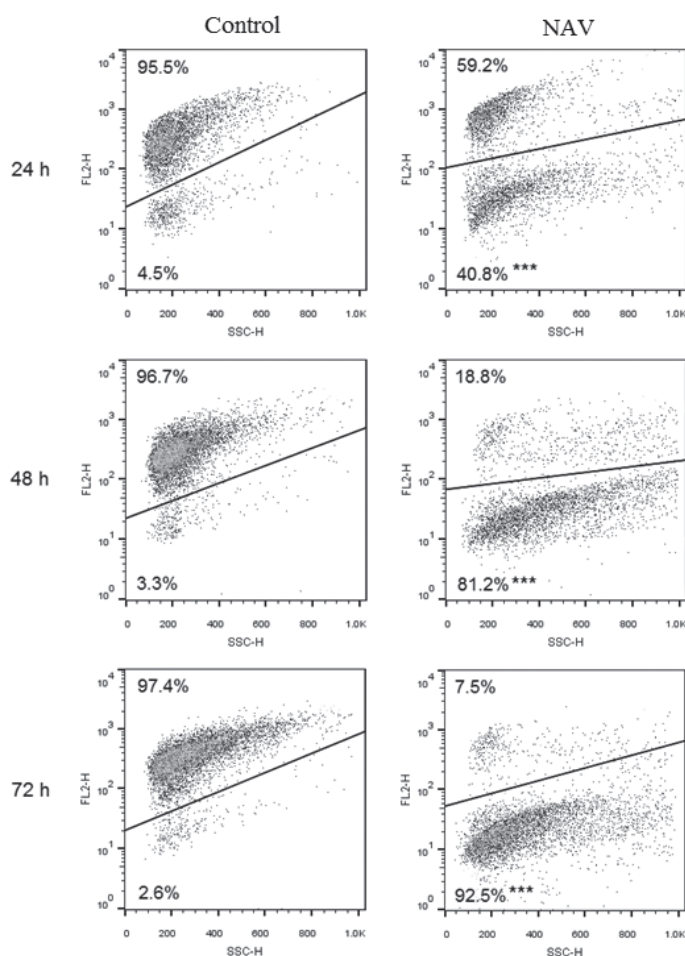
To confirm the apoptosis-inducing effect of NAV, we tested its influence on caspase-3 activity and PARP cleavage. After NAV treatment, caspase-3 activity signifi-

cantly increased in all the tested time periods ( $p < 0.01$ ,  $p < 0.001$  vs. untreated controls) (Fig. 3A). Increase in caspase-3 activity strongly correlated with PARP cleavage ( $p < 0.01$ ,  $p < 0.001$  vs. untreated controls) (Fig. 3B).

**Mitochondrial membrane potential (MMP) dissipation.** Mitochondria represent the key organelles affected by several extra-/intra-cellular stimuli. Dissipation of transmembrane potential ( $\Delta\Psi_m$ ) is the first and a very early indicator of mitochondrial dysfunction. In our experiments, significant decrease of the MMP was observed 24, 48 and 72 hours after treatment with NAV ( $p < 0.001$ ) (Fig. 4), indicating the activation of the mitochondrial apoptotic cell death pathway.

**Mitochondrial apoptotic pathway activation.** Mitochondrial changes induced by several stimuli activate intrinsic apoptotic pathway. Loss of MMP, membrane dysfunction and damage led to the activation of several proteins associated with programmed cell death, for example pro/apoptotic proteins, cytochrome *c*, apoptosis inhibitors and activators, etc.

Cytochrome *c* is an important member of the mitochondrial apoptosis cascade. During apoptosis, this protein is released from mitochondria to cytosol, where it binds to Apaf-1 protein forming the apoptosome complex and triggering activation of initiator caspase-9. Our experiments showed significant changes in the release of cytochrome *c* shortly after 24 h in the NAV-treated HCT116 cells (Fig. 5A). Similar effects were observed in caspase-9 activation in a time dependent manner (Fig. 5B). Concomitantly with caspase-9 activation, we observed a significant increase in Smac/DIABLO pro-



**Figure 4.** Representative dot-plot diagram of MMP changes in HCT116 cells after NAV treatment (6.84  $\mu\text{g/ml}$ ). Data were obtained from three independent experiments, and significant differences versus control cells (untreated) were marked as \*\*\* $p < 0.001$ .

tein levels (a second mitochondria-derived activator of caspases) 24 hours after HCT116 incubation with NAV (Fig. 5C).

To support our hypothesis about mitochondrial pathways involvement in cell apoptosis after the NAV treatment, we analysed Bcl-2 protein status and activity. We observed a significant increase of Bcl-2 protein levels in cytosol after 24 h of NAV treatment, which culminated after 48 h. Moreover, the phosphorylation of Bcl-2 significantly increased in a time-dependent manner, suggested deactivation of antiapoptotic function (Fig. 6).

## DISCUSSION

Snake venom, a complex mixture of different components such as peptides, proteins, enzymes and carbohydrates, possesses a variety of biological effects. Several proteins from snake venoms, in spite of their toxicity, are promising pharmaceutical agents in the field of cancer treatment (Tan *et al.*, 2018; Waheed *et al.*, 2017).

The present study is the first to show the pro-apoptotic effect of *Naja ashei* venom in human colorectal cancer cells. Apoptosis plays a crucial role in the elimination of unnecessary cells, including the malignant cells. However, one of the typical cancer hallmarks is the ability of cancer cells to evade apoptosis, and activation of apoptotic machinery plays an essential role in the anticancer

activity of several conventional anti-neoplastic drugs (Hanahan & Weinberg, 2000).

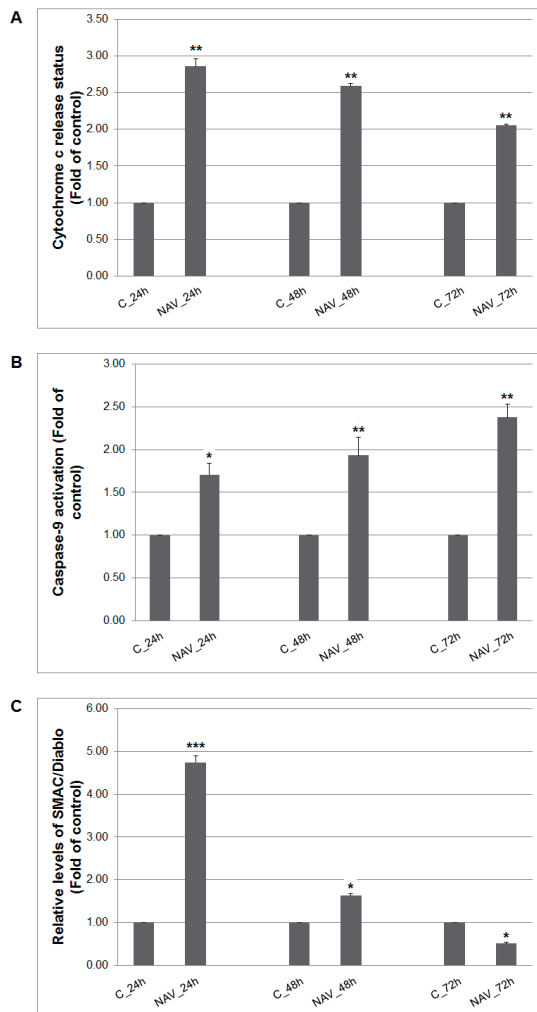
Our study was designed to reveal whether NAV influences proliferation of HCT116 colorectal cancer cells and further to examine the possible mechanism of its pro-apoptotic effect. We found that viability and proliferation of NAV-treated cancer cells significantly decreased in comparison with the untreated cells. We also showed that suppression of cancer cell growth was associated with the induction of apoptosis, as revealed by several complementary assays. In the NAV-treated cell population, we detected an increase in the fraction of cells with sub-G<sub>0</sub>/G<sub>1</sub> DNA content, which is considered a marker of apoptosis. Apoptosis was further confirmed with annexin V/PI staining and detection of cleaved PARP.

Because the mechanisms of the NAV-induced apoptosis in cancer cells have not yet been studied, in the present paper we tried to evaluate the machinery by which NAV induces apoptosis.

It is well known that mitochondria are essential for cell survival. However, there is also plentiful evidence on crucial role of mitochondria in apoptosis and several factors such as DNA damage or chemotherapeutic agents can induce mitochondria-operated apoptosis (Gillies & Kuwana, 2014; Wang & Youle, 2009).

Mitochondrial dysfunction can be associated with the loss of mitochondrial membrane potential ( $\Delta\Psi\text{m}$ ) (Cosentino & Garcia-Saez, 2014) and modulation of function of Bcl-2 proteins (Danial, 2007). The release of intermembrane space proteins such as cytochrome *c*, Smac/DIABLO or apoptosis-inducing factor into the cytosol, leads to the activation of downstream caspases to carry out the apoptosis process (Burke, 2017). We found a significant loss of MMP in the HCT116 cells after 24, 48 and 72 h of incubation with NAV. At the same time, we detected increased content of cytochrome *c* in cytosol after 24, 48 and 72 h of treatment, as well as Smac/DIABLO protein after 24 h of incubation. In the NAV-treated colorectal cancer cells, we observed, as a consequence of the release of apoptosis activators, the activation of both caspase-9 and caspase-3 with a subsequent PARP cleavage. Similar pro-apoptotic effect of cobra venom associated with the loss of MMP and caspase-3 activation have been recently documented by Ebrahim and co-workers (2016). Later, the ability of cobra venom from *Naja oxiana* to induce the loss of MMP, release of cytochrome *c* and caspase-3 activation was confirmed either in hepatocellular carcinoma (Seydi *et al.*, 2017) or in colorectal carcinoma (Fakhri *et al.*, 2017).

The Bcl-2 family of proteins plays an important role in apoptosis either as anti- or pro-apoptotic factors (Tsujiyama, 1998). Anti-apoptotic Bcl-2 protein prevents activation of apoptotic pathway by blocking the activation of caspases. It has been documented that its antiapop-



**Figure 5.** The release of cytochrome *c* (A), caspase-9 activation (B) and SMAC/Diablo presentation (C) in HCT116 cells 24, 48 and 72 h after NAV treatment (6.84  $\mu\text{g/ml}$ ), as assessed with flow cytometry analysis.

Data represents the control-normalized mean  $\pm$  S.D. for 3 independent experiments; \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$  vs. untreated cells (control).

otic activity depends on its phosphorylation status, and Bcl-2 phosphorylation suppresses its anti-apoptotic function (Tamura *et al.*, 2004). In our study we found significant increase of phosphorylated Bcl-2 after 24, 48 and 72 h of incubation in NAV-treated cells. Loss of Bcl-2 function can influence mitochondrial membrane integrity and cytochrome *c* release (Kale *et al.*, 2018).

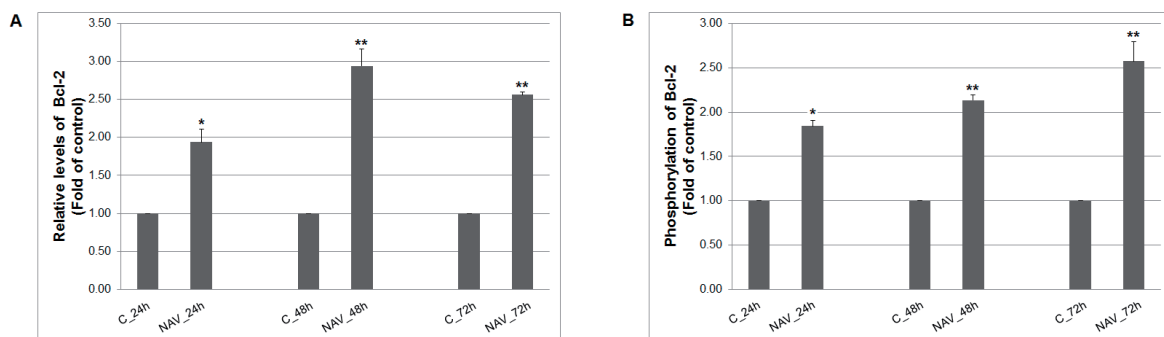
Due to the fact that there is no record of NAV effect on cancer cell lines in the literature, it is impossible to compare our result with others. On the other hand, recently Hus and co-workers analyzed protein composition of *Naja ashei* venom (Hus *et al.*, 2018) and they found that the main components of analysed venom were three-finger toxins (3FTxs) and phospholipases  $A_2$  (PLA<sub>2</sub>) accounting 70% and 27% of the total venom proteins.

3FTxs is a family of non-enzymatic polypeptides found in the venom of terrestrial or sea snakes (Kini&Doley, 2010). As documented by Hus and co-authors (2018), cytotoxin 1 (CTX1) and cytotoxin 2 (CTX2) are the most abundant proteins from 3FTxs family in the *Naja ashei* venom. It has been shown that both cytotoxins are potent apoptosis stimulators in cancer cells (Ebrahim *et al.*, 2014; Wu *et al.*, 2013). Similar to our results, CTX1 and CTX2 induced apoptosis was associated with mitochondrial dysregulation. As shown recently, the disruption of mitochondrial membranes may be the result of binding of CTX1 and CTX2 to cardiolipin (Gasnov *et al.*, 2015).

Furthermore, PLA<sub>2</sub>, the second most common component of NAV, has also been found to possess pro-apoptotic activity. Chen and co-workers (2010) and Liu and co-workers (2009) documented the apoptosis inducing effect of the cobra PLA<sub>2</sub> associated with modulation of Bcl-2 family proteins, reactive oxygen species production, loss of mitochondrial membrane potential, cytochrome *c* release or modulation of several signalling pathways.

## CONCLUSION

Taken together, our results show for the first time that NAV has potent antiproliferative effect in the colorectal carcinoma cell line. *Naja ashei* venom induces this effect *via* the induction of apoptosis through cell cycle arrest in S phase, an increase in the number of cells with sub-G0/G1 DNA content, mitochondrial damage, release of cytochrome *c* and Smac/DIABLO protein with subsequent activation of caspase-9 and caspase-3. These findings suggest that NAV-induced apoptosis was mediated by the intrinsic pathway.



**Figure 6.** Analysis of mitochondrial apoptotic pathway associated proteins in HCT116 cells treated with NAV. Relative levels (A) and activation (B) of Bcl 2 protein after NAV treatment (6.84  $\mu\text{g/ml}$ ); \* $p < 0.05$ , \*\* $p < 0.01$  vs. untreated cells (control).

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