NORTH-WESTERN JOURNAL OF ZOOLOGY 12 (2): 286-291
Article No.: e161501

http://biozoojournals.ro/nwjz/index.html

Cytotoxic potential of Wagner's Viper, *Montivipera wagneri*, venom

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Received: 15. June 2015 / Accepted: 10. November 2015 / Available online: 05. January 2016 / Printed: December 2016

Abstract. Snake venoms contain a variety of biologically active proteins, which have therapeutic potential. *Montivipera wagneri* is an endemic mountain viper species of Turkey. In this study, the cytotoxic activity of *M. wagneri* crude venom was investigated against A549, HeLa, CaCo-2, U-87MG, PC3 and MCF-7 cancerous cells and non-cancerous cells Vero and HEK293 by MTT assay. The IC_{50} values of *M. wagneri* crude venom on cultured cells varied from 1.02±0.02 to 19.76±0.42 µg/ml, with the most potent activities against A549 and CaCo-2 cells. The present work documents the venom of *M. wagneri* for the first time, showing promising results as a potential source for cytotoxic proteins or peptides.

Key words: snake venom, *Montivipera wagneri,* cytotoxicity.

Introduction

Snake venom is a cocktail of proteins and peptides with distinct biological activities. Major protein families found in viper venoms are serine protease, Zn^{2+} metalloprotease, group II phospholipase A_2 (PLA_2), L-amino acid oxidase (LAAO) and hyaluronidase as enzymes; disintegrin, C-type lectin (CLP), vascular endothelial growth factor (VEGF), nerve growth factor (NGF), cysteine-rich secretory protein (CRISP), kunitz-type protease inhibitor and bradykinin-potentiating peptides as non-enzyme proteins/peptides (Mackessy 2010, Igci & Demiralp 2012).

The therapeutic potential of various snake venom proteins has been shown against diseases such as cancer, infections, cardiovascular disorders and arthritis (Lewis & Garcia 2003, Fox & Serrano 2007, Samy et al. 2013). Previous studies on snake venoms resulted in the identification and characterization of novel snake venom proteins (e.g. metalloprotease, disintegrin, LAAO, CLP, PLA_2) possessing *in vitro* and *in vivo* anticancer activities through different cellular pathways (Calderon et al. 2014, Göçmen et al. 2015). Particularly a non-toxic dose of snake venom has been shown to reduce the solid tumor size and to block angiogenesis (Havey 2014, Jamunaa et al. 2012, Koh et al. 2006).

Montivipera wagneri Nilson & Andrén, 1984 is an endemic mountain viper species of Turkey. It is distributed across eastern Turkey with well-known localities in Kars and Mus provinces (Göçmen et al. 2014).

As part of our ongoing studies about the biological activity of the venom from Turkey's endemic species, this study presents the results of an investigation of *M. wagneri* venom on various cancer cells to evaluate its potential cytotoxicity and its use in medicine as a therapeutic agent.

Material and Methods

Snake venom: All tests were performed using pooled venom extracted from three adult *M. wagneri* collected in the wild, in Karakurt/Kars (Turkey), during field trips in 2013. A quantity of 50–100 µl venom was extracted from each individual. After extraction, venom samples were lyophilized by freeze-drying. The lyophilized venom samples were diluted (1 mg/ml) in physiological saline, centrifuged for 5 min at 600 × g and then filtered through a 0.22 µm cellulose acetate syringe filter before being used in the tests. Venoms were extracted following the appropriate procedures for venom sampling. NI and BG have ethical permission (approval no. 2010-43) from Ege University Animal Experiments Ethics Committee for venom collection from vipers. They also have permission from the Republic of Turkey Ministry of Forest and Water Affairs to collect vipers in the wild for venom research.

Determination of protein concentration: Protein concentration was assayed in duplicate according the method by Bradford (1976) at 595 nm using an ultraviolet (UV)-visible spectrophotometer (Thermo, Bremen, Germany) for diluted venom samples in saline. Bovine serum albumin was used as a standard.

Cell Culture and *In Vitro* Cytotoxicity Assay: Cells of human lung adenocarcinoma (A549), human cervix adenocarcinoma (HeLa), human colorectal adenocarcinoma (CaCo-2), human glioblastoma–astrocytoma (U-87MG),

human prostate adenocarcinoma (PC-3), human pancreas adenocarcinoma (Mpanc96) and human breast adenocarcinoma (MCF-7) cells were used as cancer cell lines. Human embryonic kidney (HEK293) and kidney epithelial cells from an African green monkey (Vero) were used as noncancerous cell lines. Cell lines were purchased from ATCC (Manassas, VA, USA). All the cells were maintained in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum, 2 mM Lglutamine, 100 U/ml of penicillin and 100 µg/ml of streptomycin (Biochrom, Berlin, Germany). The cells were incubated at 37°C in a humidified atmosphere of 5% carbon dioxide.

The cytotoxicity of crude venom was determined by the general procedure based on cell viability using a modified 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay (Mosmann 1983), which measures colorimetrically the mitochondrial reductase activity of viable cells. The optical density (OD) was measured at 570 nm (reference: 690 nm) by a UV-visible spectrophotometer (Thermo, Bremen, Germany) in duplicate. All cell lines were cultivated for 24 h in 96-well microplates with an initial concentration of 1×10^5 cells/ml. Then, the cultured cells were treated with different concentrations of venom (0 – 35 µg/ml) and incubated for 48 h at 37°C. Doses were selected based on previous studies on the cytotoxic effects of snake venom. The percentages of surviving cells in each culture after treatment with venom was determined.

Morphological Studies: the cells were compared to the control group 48 h after treatment using an inverted microscope (Olympus, Japan).

Determination of IC_{50} and Statistics: The half-maximal inhibitory concentration (IC_{50}), which is the concentration of venom causing 50% inhibition of cell growth compared to untreated controls, was calculated using OD values. Cytotoxicity was expressed as a decrease in the mean percentage of cell viability relative to the unexposed control ± standard deviation (SD). Control values were set at 0% cytotoxicity. The percentage viability was determined as formulated below:

%Viable cells = (Absorbance of treated cells) - Absorbance of blank) / (Absorbance of control) - (Absorbance of blank) × 100

IC_{50} was calculated by fitting the data to a sigmoidal curve, using a four-parameter logistic model, and presented as an average of three independent measurements. The IC_{50} values were reported with 95% confidence intervals and calculations were performed using GraphPad Prism 5 software (San Diego, CA, USA). The values of the blank wells were subtracted from each well of treated and control cells, and IC_{50} was calculated in comparison with untreated controls.

Results

The protein concentration of *M. wagneri* crude venom (1 mg/ml) estimated by the Bradford assay was 735 µg/ml. The effect of *M. wagneri* crude venom on cancer cells A549, HeLa, CaCo-2, U-87MG, MCF-7, mPanc-96, PC3 and non-cancerous cells Vero and HEK293 was evaluated via MTT assay following treatment with different concentrations of crude venom for 48 h. The MTT assay results showed that *M. wagneri* crude venom inhibited cell proliferation in a concentration-dependent manner with the IC_{50} values varying between 1.02±0.02 and 19.76±0.42 µg/ml (Table 1 and Fig. 1). The crude venom exhibited the most potent activity against CaCo-2 and A549 cells with the IC_{50} values of 1.02±0.02 and 1.44±0.06 µg/ml, respectively. Subsequently, MCF-7, U87MG and HEK293 cells were the most affected cells following CaCo-2 and A549 cells with their IC_{50} values between 2.17±0.01-2.42±0.05 µg/ml.

Table 1. IC_{50} values for *M. wagneri* venom for cell lines (µg/ml).

Cell lines	Crude venom	Parthenolide*
HeLa	7.40±0.62	3.32±0.24
CaCo-2	1.02±0.02	5.81±0.20
MCF-7	2.78±0.01	1.65±0.03
U87MG	2.17±0.01	1.93±0.08
A549	1.44±0.06	1.66±0.07
mPanc-96	6.11±0.33	0.58±0.03
PC3	19.76±0.42	1.28±0.08
HEK293	2.42±0.05	0.63±0.03
Vero	3.52±0.45	2.58±0.13

* Cytotoxic agent as positive control

Additionally, the results revealed that PC3 cells (IC_{50} =19.76±0.42 µg/ml) were the most resistant cells among the tested cell lines. However, the crude venom exhibited potent activity against mPanc96 (IC_{50} =6.11±0.33), Vero (IC_{50} =3.52±0.45) and HeLa (IC_{50} =7.40±0.62) cells, as well as the other cell lines. The crude venom of *M. wagneri* also decreased the viability (Fig. 1) of cells through concentration-dependent effects similar to IC_{50} results. The effect of venom on the morphology of the cells observed with light microscopy after 48 h venom exposure is demonstrated in Figs 2a and 2b.

Discussion

Most venoms contain a vast variety of lethal proteins / peptides targeting normal biological processes in snake prey such as blood clotting or nerve-cell signaling. The high reaction efficiency of snake venoms on many biochemical processes has led researchers to investigate the toxins for

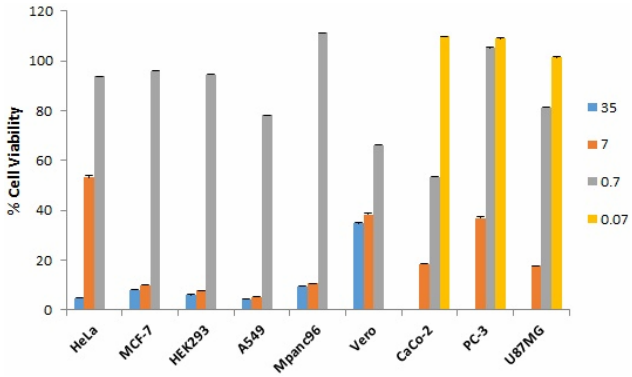

Figure 1. Cytotoxic effect of *M. wagneri* crude venom on cancerous and non-cancerous cells following 48-h exposure to different venom concentrations. Cell viability was determined by MTT assay, control was exposed to vehicle only which was taken as 100 % viability. Data are expressed as mean±SD.

their suitability for drug development. Nevertheless, earlier investigations on the potential of toxin-related compounds showed that the drug developers needed to modify the toxins to remove their damaging effects and render them safe for drug use (Harvey 2014). For example, the successful antihypertensive drug Captopril was developed from a compound of a neotropical pitviper (*Bothrops jararaca*) venom by modifying its chemical structure. This drug is an inhibitor of angiotensin converting enzyme (ACE), and is extremely efficient in lowering blood pressure (Cushman et al. 1977, Opie & Kowolik 1995). Other successful examples are anti-platelet agents Tirofiban (Aggrastat®), a non-peptide tyrosine derivative developed by Merck, and eptifibatide (Integrilin®), a circular heptapeptide. Tirofiban and Eptifibatide were developed based on disintegrins barbourin isolated from Southeastern Pygmy Rattlesnake (*Sistrurus miliaris barbouri*) and echistatin from African Saw-scaled Viper (*Echis carinatus)* venom, respectively. These drugs were designed to mimic the RGD and KGD sequences that is the recognition motif for binding to GPIIb/IIIa integrin receptors (Hashemzadeh et al. 2008). Ziconotide, the synthetic version of the venom peptide MVIIA from the sea snail *Conus magus* that selectively blocks N-type calcium ion channels (Cav2.2), was approved by the FDA in 2004 for treating patients with intractable pain. Thus, researchers revealed that there might be many harmless versions of venom toxins of medical importance waiting discovery throughout new drug development. Indeed, recent studies on snake venoms have shown them to be promising sources of new drug leads (Essack et al. 2012, Harvey 2014, Pope & Deer 2013, Vetter & Lewis 2012, Vyas et al. 2013). In this context, we examined the cytotoxic effects on cancer and non-cancerous cells of *M. wagneri* crude venom to evaluate its potential for further bioactivity-guided study.

As cancer is one of the most important health problems among the diagnosed diseases and due to issues of increasing drug resistance, the need for new chemotherapeutic agents has become essential. Based on research of natural products, it is vital to perform general screening and biological characterization of the crude substance at the beginning of the discovery phase to seek out the possible targets.

Mechanisms for the cytotoxicity of snake venoms are quite complex and multi-faceted. These include necrosis, induction of apoptosis, regulation of cell cycle proteins, and worsening of cell membrane integrity. Apoptosis-inducing cytotoxic activities of crude venoms or purified proteins such as metalloproteinases and LAAO from different viper species have been reported on various cancer and non-cancerous cells (Park et al. 2009, Shebl et al. 2012, Samel et al. 2013).

According to the cytotoxicity results of this study, the crude venom of *M. wagneri* exhibited concentration-dependent cytotoxic effects at several levels on selected cell lines as proven by previous studies which demonstrated cytotoxic effects of different crude snake venoms or purified venomic proteins/peptides on various cancer and non-cancerous cells (Bustillo et al. 2009, Jamunaa et al. 2012, Yalcin et al. 2014, Ozen et al. 2015, Suzergoz et al. 2016). The results suggested that the *M. wagneri* crude venom has a significant potent cytotoxic effect against CaCo-2, A549 MCF-7, U87MG and HEK293 cells with the IC_{50} values in a range between 1.02±0.02 and 2.42±0.05 µg/ml. The results also seem to be more compelling than positive control drug agent parthenolide. Comparing

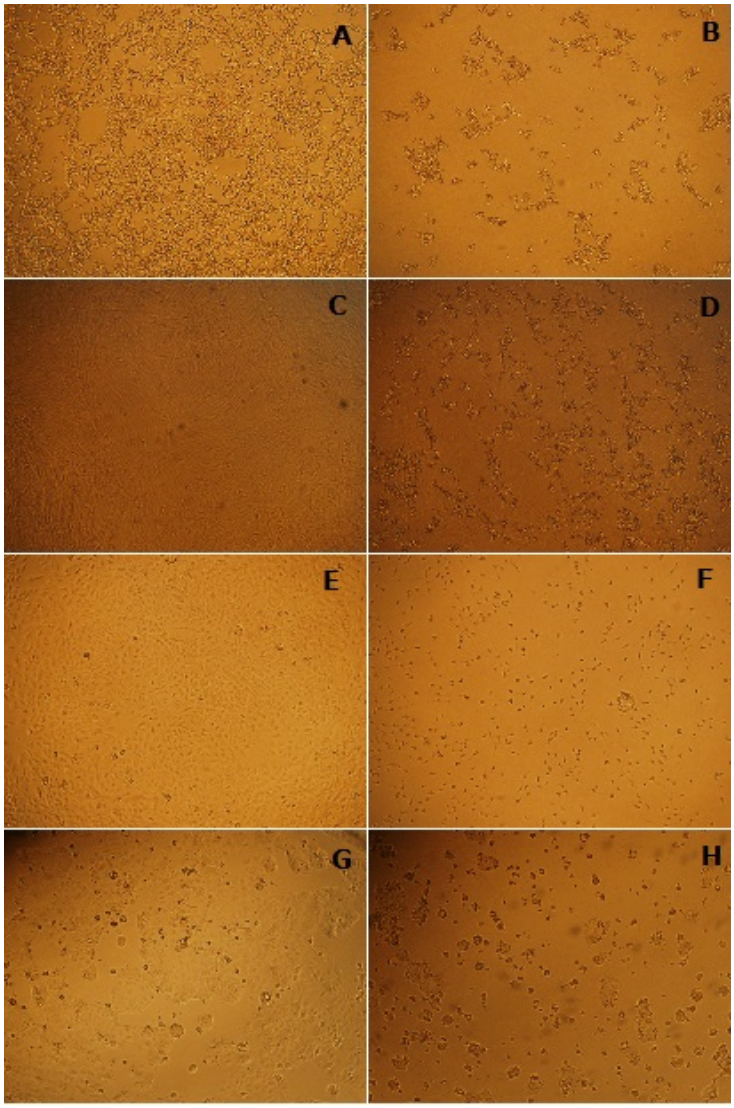

Figure 2a. Dose-dependent venom induced morphologic changes of cancerous and non-cancerous cells viewed by inverted microscope (10x). Cells were treated with different concentrations of crude venom for 48 h at 37°C. A. HEK293 untreated B. HEK293, 7 µg/ml C. Vero untreated D. Vero, 7 µg/ml E. A549 untreated F. A549, 7 µg/ml G. CaCo-2 untreated H. CaCo-2, 0.7 µg/ml.

the parthenolide activity with the activity of crude venom, higher cytotoxicity was assessed against CaCo-2 and A549 cells whereas U87MG IC_{50} results were similar. Snake venoms show specific cytotoxic effect between different cell lines attributable to the unique properties of distinct venom components from different species. Although cytotoxicity was also observed against non-cancerous HEK293 and Vero cells, IC_{50} values of the most sensitive cancer cells (CaCo-2, A549 and U87MG) were lower than healthy cells. In order to elucidate the mechanism of action and selectivity of the venom, further studies should be carried out using purified proteins and peptides.

Microphotography (Figs 2a and 2b) following incubation of the cells with different venom concentrations exhibited similar results in comparison with the MTT assay. The morphological changes observed throughout the 48 h period of venom exposure varied depending on the origin of the cell lines. Increasing venom concentrations resulted in increased rounding up and detachment of cells, and relatively multicellular aggregate formation (Figs 2a and 2b). In addition, cell disorganization and large areas without cells were detected with increasing concentrations of venom.

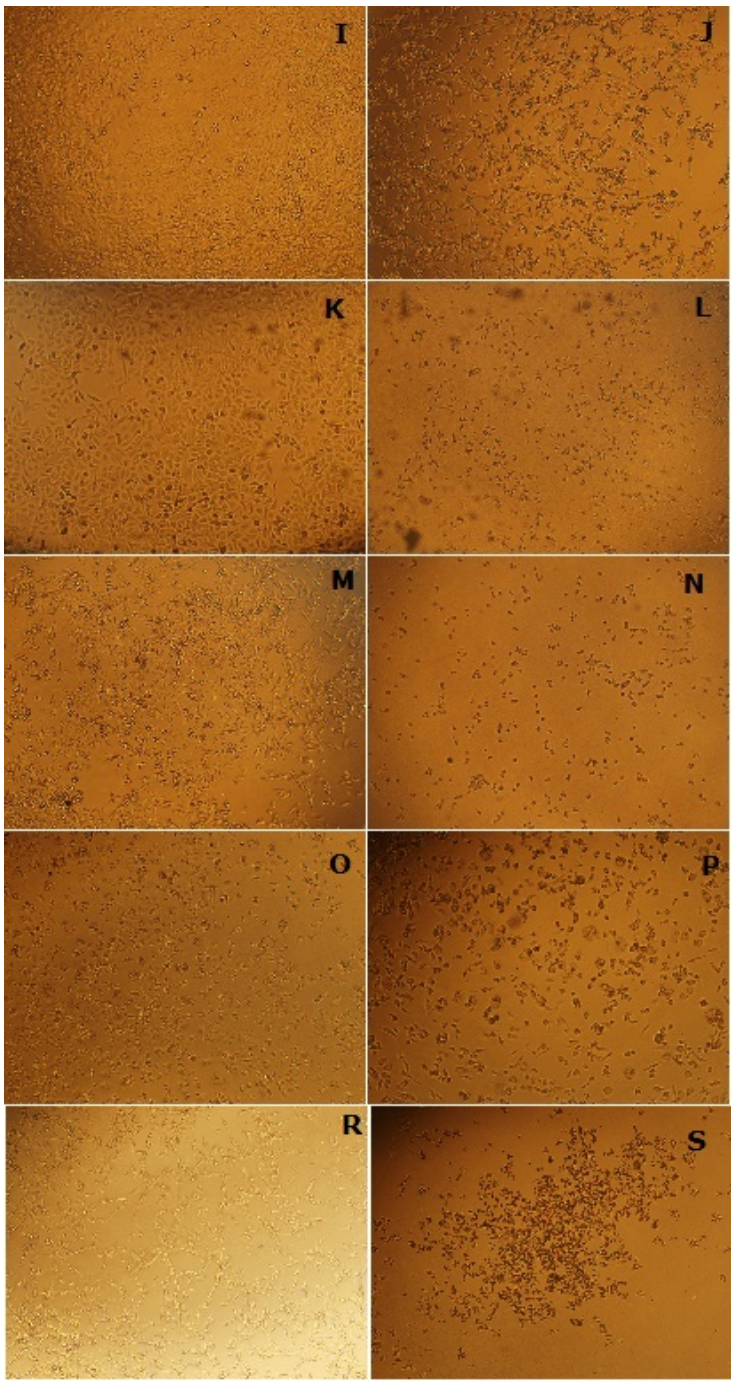

Figure 2b. I. HeLa untreated J. HeLa, 7 µg/ml K. MCF-7 untreated L. MCF-7, 7 µg/ml M. mPANC96 untreated N. mPANC96, 7 µg/ml O. PC3 untreated P. PC3, 0.7 µg/ml R. U87MG untreated S. U87MG, 7 µg/ml.

Untreated cells were homogeneously spread in wells and exhibited their normal typical morphological phenotype depending on their origins (Figs 2a and 2b).

In conclusion, cytotoxic activities of *M. wagneri* crude venom was screened for the first time indicating the potential for cytotoxicity against cancer cells. The results showed that *M. wagneri* crude

venom could be a source of novel bioactive protein prototypes with anti-cancer activities. Regarding the results, further studies are needed to characterize bioactive peptides / proteins possessing selective biological activity.

Acknowledgements. We would like to extend our thanks to the AREL (Research and Education Laboratory, Ege University School of Medicine) for their support for the laboratory facilities during this study.

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