L-amino acid oxidase from snake venom: Biotransformation and induction of apoptosis in human colon cancer cells

Danijela D. Nikodijević[1*], Jovana V. Jovankić[1], Danijela M. Cvetković[2], Marko Z. Anđelković[3], Aleksandra G. Nikezić[1], Milena G. Milutinović[1]

[1] University of Kragujevac, Faculty of Science, Department of Biology and Ecology, Radoja Domanovića 12, 34000 Kragujevac, Serbia

[2] University of Kragujevac, Institute for Information Technologies Kragujevac, Department of Natural Sciences, Jovana Cvijića bb, 34000 Kragujevac, Serbia

[3] University of Belgrade, Institute for Biological Research "Siniša Stanković", National Institute of Republic of Serbia, Department of Evolutionarl Biology, Bulevar Despota Stefana 142, 11060 Belgrade, Serbia

*Corresponding author: Danijela Nikodijević, MSci, University of Kragujevac, Faculty of Science, Department of Biology and Ecology, Radoja Domanovića 12, 34000 Kragujevac, Serbia; E-mail address: danijela.nikodijevic@pmf.kg.ac.rs

Abstract

This study evaluated the potential of antitumor activity of snake venom from *Vipera ammodytes* and L-amino acid oxidase from *Crotalus adamanteus* on different colorectal cancer cell lines through determination of cytotoxic activity by MTT assay, pro-apoptotic activity by acridine orange/ethidium bromide staining, and concentrations of redox status parameters (superoxide, reduced glutathione, lipid peroxidation) by colorimetric methods. The expression of genes involved in the biotransformation process and metabolite efflux was determined by qPCR method, while protein expression of glutathione synthetase and P-glycoprotein were analysed by immunocytochemistry. The analysis of cell death shows that snake venom dominantly leads cells to necrosis. Induction of apoptosis by L-amino acid oxidase was in correlation with oxidative disbalance in cancer cells. Gene expression profile of membrane transporters and *CYP* genes were different in each cell line and in correlation with their sensitivity of treatment. Our results show that L-amino acid oxidase from snake venom is a potent cytotoxic substance with pronounced pro-apoptotic activity. The inhibition of P-glycoprotein suggests that L-amino acid oxidase is a good substance for furter research of antitumor effect, with unexpressed potential for occurrence of drug resistance *in vitro*.

Keywords

Anticancer, ABC transporters, drug resistance, *Vipera ammodytes*

1. Introduction

There is an increased incidence of colorectal cancer globally, taking the third position in frequency ranking among the most common cancers according to the data of World Health Organization (Sung et al., 2020). The conventional treatment of this disease is demanding, and in addition to standard procedure requires the examination of new therapeutic agents. Recently, due to their numerous favourable pharmacological effects, additional therapies with substances of natural origin have proved to be excellent. Among them, animal products mainly from animal venoms have been increasingly investigated (Roy and Bharadvaja, 2020).

Snake venom is a mixture of many substances with numerios side effects on humans; however, some of them can be useful. Nose-horned viper (*Vipera ammodytes*) is the most venomous snake in Europe (Latinović et al., 2016). It belongs to the subfamily of Viperinae, characterized by venoms, composed of a combination of neuro and chemo toxins (Latinović et al., 2016). It contains many active compounds mostly of protein structure such as proteinases, lipases, disintegrins, L-amino acid oxidases, and other molecules (Georgieva et al., 2008). L-amino acid oxidase is a flavoprotein, the enzyme that catalyzes oxidation of L-amino acids. On antitumor investigation, L-amino acid oxidases are only reported to induce apoptosis and disbalance of redox status in cancer cells by producing hydrogen peroxide (Izidoro et al., 2014). Thus, they are a good starting point in preclinical testing of anticancer properties of snake venom, regardless of the fact that they are not a predominant component of the venom, with destructive effects on human cells (Du and Clemetson, 2002).

In anticancer treatment, one of the main mechanism of action of natural products is their ability to induce apoptosis (Wang et al., 2018; Milutinović et al., 2019; Nikodijević et al., 2019). Cancer cells avoid apoptosis, which may be associated with the development of resistance to potential anticancer agents. Numerous intracellular mechanisms can lead to resistance such as avoiding

apoptosis, biotransformation of drug, reducing accumulation of an antitumor drug in cells which is a direct consequence of their active ejection from the cell, etc. (Komarova and Wodarz, 2005; Torigoe, 2005; Hall et al., 2008; Podolski-Renić et al., 2013). The first phase of biotransformation process in a cancer cell involves hydrolysis, reduction, and oxidation of anticancer drug-mediated by CYP family of enzymes (proteins), which results in its increased solubility (Guengerich, 2007). The second phase involves reactions of glucuronidation, sulfonation, acetylation, methylation, and conjugation with glutathione by GST, Glutathione S-Transferase. At the end of the biotransformation process, the elimination of xenobiotics from the cell is performed by ABC transporters (ATP Binding Cassette) on the cell membrane. Multidrug resistance-associated proteins (MRPs) are a transporter protein family that export drugs previously conjugated to glutathione (Leslie et al., 2005). The MRP1 and MRP2 transporters are members of this family, expressed in both colon cancer tissue and normal intestinal tissue (Kerb et al., 2001; Sandusky et al., 2002; Hoffman et al., 2004). However, the most common transporter, associated with the occurrence of resistance in antitumor therapy is P-glycoprotein (Multidrug resistance protein 1, PGP1). It ejects a large spectrum of xenobiotics and does not require conjugation of substrates with glutathione (Haber et al., 2006).

In this study we evaluated the effects of snake venom (SV) from *Vipera ammodytes* and L-amino acid oxidase (LAAO) from *Crotalus adamanteus* against colorectal cancer cell lines (HCT-116, SW-480, and HT-29). A potential cytotoxic effect of SV and LAAO was also observed on normal human keratinocytes (HaCaT). Furthermore, LAAO was examined regarding its impact on redox status parameters ($O_2^{\cdot -}$, GSH, and lipid peroxidation) and expression of genes (*CYP1A1*, *CYP1B1*, *GSTP1*, *MRP-1*, *MRP-2*, *PGP1*) and proteins (GSS - glutathione synthetase, and PGP1) related to biotransformation process and cancer cell resistance.

2. Materials and methods

2.1. Chemicals and reagents

Primary antibody GSS, Trichloroacetic acid, Thiobarbituric and Hydrochloric acids were obtained from Sigma Aldrich. Dulbecco's Modified Eagle Medium (DMEM) and Ethidium bromide (EB), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), NBT (Nitro Blue Tetrazolium), absolute ethanol, and chloroform were obtained from SERVA, Germany. Acridine Orange (AO) was from Acros organics, New Jersey, USA. Reverse Transcription Kit and qPCR MasterMix, secondary antibody conjugated with Cy3, Diamidino-2-phenylindole (DAPI), and primary antibody PGP1 were from Thermo Fisher Scientific, US. All solvents and chemicals were of analytical grade.

2.2. Snake venom collection

Snake venom (SV) was collected from *Vipera ammodytes* females in laboratory conditions at Institute for Biological Research "Siniša Stanković" in Belgrade, Serbia in 2018 (permission for animal capture by Serbian Ministry for Energy, Development, and Environmental Protection No: 353-01-212/2018-04; permission by Ethical Committee for the use the animals for experimental purposes No: 02-09/18). Snake venom was dried (powder was stored at -20 °C), and dissolved in distilled water before use. Dilutions were made in DMEM. L-amino acid oxidase from snake venom (powder) was purchased, stored at -20 °C, and dissolved in the same way. L-amino acid oxidase from *Crotalus adamanteus* (dry powder), was commercially produced by Sigma Aldrich Co. LLC (https://www.sigmaaldrich.com/catalog/product/sigma/a9253?lang=en&region=SX).

2.3. Investigated cell lines

Human cancer cells - HCT-116 colorectal carcinoma cell line, SW-480 and HT-29 colorectal adenocarcinoma cell lines were obtained from American Type Culture Collection, USA. Normal

human keratinocytes - HaCaT cell line was obtained from Cell Lines Service, Eppelheim, Germany. All cell lines were cultivated in DMEM, under laboratory conditions described previously (Milutinović et al., 2019).

2.4. MTT assay

The effects of SV and LAAO on colorectal carcinoma HCT-116, SW-480, HT-29 cells, and normal HaCaT keratinocytes viability were examined by MTT assay (Mosmann, 1983). The cells were seeded in a 96-well plate (10^4 cells/well) and after a pre-incubation period of 24 h were treated with 100 µl of SV (concentration range of 0.01-10 µg/ml) and LAAO (concentration range of 1-50 µg/ml). Normal cells were treated with concentration range of 1-200 µg/ml by SV and 1-250 µg/ml by LAAO. Yellow MTT was reduced in live cells to purple formazan and dissolved in DMSO. Untreated cells served as a control. At the end of the incubation with MTT, 150 µl of DMSO was added to each well and the absorbances were read on an ELISA reader at 550 nm. Percentages of cell viability were obtained as the ratio between the absorbance of treated and untreated cells, multiplied by 100. The IC_{50} values were performed from the dose response curves by CalcuSyn program. The assay was performed 24 and 72 h after the applied treatments.

2.5. Cell death quantification

The type of induced cell death in HCT-116, SW-480, HT-29, under the influence of SV and LAAO was performed by Acridine orange/ethidium bromide (AO/EB) double staining assay (Baskić *et al*. 2006). The cells were seeded in a 96-well plate (10^4 cells/well) and after a pre-incubation period of 24 h treated with 100 µl of SV and LAAO (2 and 5 µg/ml). Untreated cells served as controls. After the incubation of treatment, 10 µl of AO and EB dye were added to each sample, and the cells were counted immediately after staining. The dyes were pre-dissolved,

100 μg per milliliter of distilled water. The percentages of viable (V), apoptosis (early - EA, and late - LA), and necrosis (N) cells were calculated proportionally, concerning the total cell number per sample (a minimum of 300 cells was counted in each sample). The assay was performed using an inverted fluorescent microscope (Nikon Ti-Eclipse) at 400x magnification, 24 h after treatment.

2.6. Impact of treatments on redox status parameters

2.6.1. Determination of superoxide anion radical

The level of superoxide anion radical ($O_2^{\cdot -}$) was determined by NBT assay (Auclair and Voisin, 1985). The cells were seeded in a 96-well plate (10^4 cells/well) and treated with 100 μl of LAAO (1, 2, 5, and 10 μg/ml). After the end of the treatment, 10 μl of NBT solution at the concentration of 5 mg/ml was added to the each well, followed by an incubation period of 45 min at 37 ºC. At the end of the incubation, 10 μl of DMSO was added to each well and the absorbances were read on an ELISA reader at 550 nm. The absorbances were used to calculate the concentration of $O_2^{\cdot -}$, expressed in nmol/ml, according to the formula: **nmol NBT/ml = A/0.015 x Vcuv / Vex** (A - absorbance; Vcuv - total volume of solution in the well = 120 μl; Vex - cell volume with treatment = 100 μl; 0.015 - molar extinction coefficient for monoformazan (15,000 M-1 cm^{-1}). The results were firstly expressed in nmol/ml and then calculated according to the number of viable cells in each applied concentration (data obtained by MTT).

2.6.2. Determination of reduced glutathione

The concentration of reduced GSH was determined by the colorimetric method (Baker et al., 1990). The cells were seeded in a 96-well plate ($5x10^4$ cells/well) and treated with 100 μl of LAAO (1, 2, 5, and 10 μg/ml). At the end of the treatment incubation time, the plate was centrifuged at 1000 rpm for 10 min. The medium was replaced with 100 μl 2.5% sulfosalicylic

acid followed by incubation on ice for 15 min. After the incubation time, the plate was centrifuged for 15 min at 1000 rpm. 50 µl of each sample was added to new microtiter plate in triplicate. then 100 µl of reaction mixture was added to each well (reaction mixture containing 1 mM NADPH, 0.7 U GSH reductase per milliliter of reaction mixture, 1 mM DTNB, dissolved in DMSO and supplemented with PBS to the required volume). Colour reaction was measured by spectrophotometer at 405 nm. The results were firstly expressed in nmol/ml (related to standard curve constituted of known molar BSA concentrations), and then calculated concerning the number of viable cells (related to the results of MTT assay) after 24 h.

2.6.3. Determination of malondialdehyde

The concentration of malondialdehyde (MDA) as an indicator of lipid and membrane damage under conditions of oxidative stress was determined by TBARS assay (Buege and Aust, 1987). The cells were seeded in a 6-well plate (10^6 cells/well) and treated with 100 µl of LAAO (1, 2, 5, and 10 µg/ml). Absorbances were observed on a biophotometer at 405 nm (Eppendorf BioPhotometer plus), and protein concentration was determined based on the standard curve factor. The rest of the supernatant was mixed with 1 ml of the reaction mixture TCA-TBA-HCl (15% of Trichloroacetic acid; 0.375% of Thiobarbituric and 0.25 M Hydrochloric acid). Supernatant with reaction mixture was warmed at 90 ºC, 30 min, then refrigerated for 5 min on ice, and vortexed (10 min on 6600 rpm, 4 ºC). The supernatant was transferred to 96 well plate (100 µl per well) and absorbance was measured at 405 nm on Microplate Reader (ELISA RT-2100C). The concentration of MDA was calculated by the formula previously described in Buege and Aust, 1987. The results were presented in pmol/mg of protein.

2.7. Immunocytochemistry

The effects of LAAO on GSS and PGP1 protein expression in HCT-116, SW-480, and HT-29 cells treated with LAAO were detected by the immunofluorescence method (Higuchi et al., 2001). Untreated cells served as control. The cells were seeded in 6-well plate on glass coverslips (5 x 10^4 cells/well) and after a pre-incubation period of 24 h were treated with 2 ml of LAAO (2 and 5 µg/ml). At the end of the treatment time, the supernatant was aspirated, cells were washed with PBS and fixed with 4% paraformaldehyde (20 min, RT). This was followed by a series of three washings with PBS and permeabilization of the cell membrane with methanol at -20 °C, for a few seconds, and then Methanol was also washed three times with PBS, followed by blocking the cells with 1% BSA for 20 min. The cells were directly transferred from BSA to the primary antibody (GSS and PGP1) of a certain concentration or ratio (PGP1 at the concentration of 10 µg/ml, and GSS in the ratio 1:100), and incubated for 1 h. After the incubation, the primary antibody was washed with PBS three times for 15 min. Under the same procedure, the cells were incubated with the secondary antibody. The secondary antibody was conjugated to the fluorescent dye Cy3 (in the dark, RT). The ratio in which the secondary antibody was added was 1:200, and in addition to the fluorescent dye it possessed, the dye was added to detect nuclei at a dilution of 1:1000 (DAPI). The secondary antibody was washed in the same manner, and then the coverslips were glued to the glass slides with polyvinyl alcohol medium, so that the cells were immersed in it. The preparations were dried and stored in the dark. The cells were visualized using an inverted fluorescent microscope (Nikon Ti-Eclipse) at 600x magnification, 24 h after treatment. Micrograph processing and quantification was performed by ImageJ software package (Wayne Rasband, ImageJ, http://rsb.info.nih.gov/ij/). Measuring cell fluorescence using ImageJ was described in detail

(https://theolb.readthedocs.io/en/latest/imaging/measuring-cell-fluorescence-using-imagej.html)

The results are presented as relative fluorescence per cell.

2.8. Relative gene expression analysis - qPCR

Isolation of total RNA from HCT-116, SW-480, and HT-29 control and cells treated with LAAO was performed by the published method (Chomczynski and Sacchi, 1987). RNA concentration in all samples was measured on a biophotometer. The samples were stored at -80 °C until analysis. The cells were seeded in a T25 cm^2 flask and treated with 5 ml of LAAO (2 and 5 µg/ml). Transcription of single-stranded RNA to complementary DNA was performed on Eppendorf Mastercycler PCR by commercial kits for Reverse Transcription (cDNA Reverse Transcription Kit, Applied Biosystems). Quantification of mRNA expression of target genes by qPCR (qPCR MasterMix, Applied Biosystems) on Quantitative Real-Time system (Applied Biosystems 7500/7500 Fast Real-Time PCR Software v2.0), was previously described in Nikodijević et al., 2019. The analysis was performed 24 h after treatment. Gene-specific primers are shown in Table S.1.

2.9. Statistical analysis

The data are expressed as mean ± standard error (SE). All analyses were examined in three individual experiments (in triplicate for each dose). Statistical significance was determined using the Student's t-test or the one-way ANOVA test for multiple comparisons. A p-value < 0.05 was considered significant.

3. Results

3.1. Cytotoxic effects of SV and LAAO

The effects of SV and LAAO on colon cancer (HCT-116, SW-480 and HT-29) and normal (HaCaT) cell viability were measured by MTT colorimetric assay. Both SV with all components

and LAAO induced a significant reduction of cell viability on all investigated cell lines (Fig. S.1). IC_{50} values were calculated from cell viability curves by using CalcuSyn program and expressed in μg/ml, 24 and 72 h after treatments (Table 1). The data are means ± SE of two independent experiments.

Table 1. SV and LAAO cytotoxic effect on HCT-116, SW-480, HT-29, and HaCaT cells expressed by IC_{50}.

	SV (μg/ml)		LAAO (μg/ml)	
Cell line	**24 h**	**72 h**	**24 h**	**72 h**
HCT-116	1.45±0.04	1.16±0.03	1.69±0.02	0.92±0.01
SW-480	1.19±0.01	2.16±0.03	1.84±0.06	1.51±0.01
HT-29	2.88±0.06	2.05±0.05	7.80±0.08	1.41±0.06
HaCaT	7.34±0.08	1.99±0.03	7.51±0.08	3.80±0.11

According to IC_{50} values, SV and LAAO induced pronounced time-dependent cytotoxicity, with the exception of SW-480 cells treated by SV. Normally, HaCaT cell line is less sensitive to treatments compared to cancer cells. The comparison of the sensitivity between different cancer cell lines from the colon evidently showed that the HT-29 cell line was the least sensitive 24 h after the applied treatment.

3.2. Influence of SV and LAAO on type of cell death

The type of induced cell death in the investigated treatments was determined by the cell staining with AO and EB, and observed on fluorescent microscope. Micrographs clearly showed morphological changes of the cells after the application of both treatments. Morphological changes on apoptotic cells were observed through different stages of cell condensation and marked as early (EA) and late (LA) stages of apoptosis. Necrotic cells (NC) were red-colored on micrographs (Fig. 1).

The venom from *Vipera ammodytes* induced a high percentage of apoptosis followed by necrosis especially in concentration of 2 μg/ml, compared to LAAO treatment. Necrosis was dominant type of cell death in treatment by 5 μg/ml of SV (Fig. 2, Table S.2). The results showed that LAAO induced apoptosis dominantly on all tested cell lines (early and late stages), while the percentage of necrosis was non-significant (Fig. 2, Table S.2).

3.3. Influence of LAAO on redox status

The concentration of superoxide anion radical in HCT-116 and SW-480 cells under the influence of LAAO significantly increased dose-dependently compared to controls (Fig. 3A). HT-29 cells were stable and maintained approximately the same level of $O_2^{\cdot -}$ in control and treated cells, with the exception of higher concentration (10 μg/ml), where the increase of tested parameter was evident (Fig. 3A).

The level of reduced glutathione GSH significantly increased under the dose-depended influence of LAAO on HCT-116 and SW-480 cell lines compared to controls. In HT-29 cells the smallest change in concentration of glutathione was observed, except in the highest concentration of 10 μg/ml, where the increase was significant (Fig. 3B).

The concentration of MDA significantly and dose-dependently increased in HCT-116 cells. In SW-480 and HT-29 cells the dose-dependent effect was not detected, and results showed a significant decrease of MDA concentration concerning control, except the lowest concentration in the HT-29 cell line, which was unchanged (Fig. 3C).

3.4. P-glycoprotein 1 and glutathione synthetase protein expression

Protein expression of PGP1 was determined by immunostaining and fluorescent microscope observation. The level of this protein decreased in all cells under the influence of LAAO, except

in HT-29 (in a concentration of 2 μg/ml), where it slightly increased compared to controls (Fig. 4 and 5).

The level of GSS protein expression increased in HCT-116 and HT-29 in relation to control. An increased level of GSS protein expression in the SW-480 cell line was nonsignificant.

3.5. Expression of biotransformation-related genes

The influence of LAAO on mRNA expression of genes involved in metabolic process and efflux of xenobiotics (*CYP1A1*, *CYP1B1*, *GSTP1*, *MRP1*, *MRP2*, and *PGP1*) in HCT-116, SW-480, and HT-29 cells was detected by the qPCR method.

The mRNA expression for metabolic enzymes (*CYP1A1*, *CYP1B1*, *GSTP1*) significantly increased in a dose-dependent manner under the influence of LAAO on treated HCT-116 cells compared to controls. In SW-480 cells, the level of mRNA for *CYP1B1* (in higher concentration) and *GSTP1* (in both applied concentrations) genes slightly increased, while the LAAO did not affect *CYP1A1* gene expression. In HT-29 cells, *CYP1A1* and *CYP1B1* significantly increased, while the *GSTP1* slightly increased compared to controls (Table 2).

Table 2. Expression of mRNA of genes involved in biotransformation process on HCT-116, SW-480, and HT-29 cells, under the influence of LAAO. The data are means ± SE of two independent experiments. *$p < 0.05$ compared to untreated cells. The result is presented as the fold change in mRNA expression in a target sample, relative to a control sample normalized to a reference gene. The relative gene expression was calculated according to the $2^{\wedge(-\Delta\Delta Ct)}$ method.

		LAAO (μg/ml)					
		CYP1A1	***CYP1B1***	***GSTP1***	***MRP1***	***MRP2***	***PGP1***
	Ctrl	**1**	**1**	**1**	**1**	**1**	**1**
HCT-116	2	7.52±0.04*	2.43±0.01*	1.34±0.01*	0.37±0.001*	1.55±0.01*	15.89±0.90*

	5	8.94±0.02*	4.59±0.001*	2.36±0.005*	1.05±0.001	1.91±0.01*	20.97±1.20*
SW-480	2	1.01±0.001	1.02±0.0001	1.22±0.001*	0.90±0.001*	1.20±0.01*	0.30±0.001*
	5	1.00±0.0001	1.71±0.001*	1.41±0.001*	1.06±0.001	1.42±0.01*	0.34±0.001*
HT-29	2	3.32±0.02*	3.81±0.0001*	1.08±0.001	1.74±0.001*	1.32±0.01*	0.71±0.001*
	5	2.35±0.01*	4.56±0.001*	1.02±0.001	1.56±0.001*	1.20±0.001*	0.65±0.001*

The efflux of metabolized treatment (substance) in the cells was examined by membrane transporters (MRP1, MRP2, PGP1). In HCT-116 and SW-480 cells, the expression of the *MRP1* gene decreased in smaller concentration of LAAO, while it remained almost unchanged in higher concentration. The expression of *MRP2* gene increased. In HT-29 cells, both tested genes increased (dose dependence was not observed) (Table 2). The expression of mRNA for *PGP1* gene significantly increased in HCT-116 cells. In SW-480 and HT-29, the decrease in *PGP1* expression was observed.

4. Discussion

Considering the fact that animal products and their active compounds conform some positive and useful effects in therapy against cancer (Nikodijević et al., 2019; Roy and Bharadvaya, 2020), the investigation in this field is necessary. Snake venoms have been less studied due to their destructive activity on normal cells, so in literature there are only a few data about anticancer properties of their components (Izidoro, 2014).

Our results point to significant cytotoxicity of SV and LAAO, due to inhibition of proliferation in all examined cell lines originating from colon cancer (HCT-116, SW-480, and HT-29), and lower sensitivity to normal HaCaT keratinocytes used as control for cytotoxicity.

The observed IC_{50} values and proapoptotic activity of LAAO on colon cancer cells indicate noticeable activity, compared to commercial cytostatic, 5-fluorouracil (IC_{50} values of 3.2, 10.07,

and 10.00 μM for HCT-116, SW and HT-29 respectively) after 72 h of treatment (Ikehata et al., 2014). Among different colon cancer cells, the HT-29 cell line is the least sensitive, while the HCT-116 was the most sensitive to LAAO treatment. Other authors reported similar sensitivity to the commercial cytostatic 5-fluorouracil, where HCT-116 cells were more sensitive compared to SW-480 and HT-29 (Ikehata et al., 2014). It has been reported that various mechanisms are included in different sensitivity of these cell lines, such as high expression of microRNA 21 in HT-29 (Deng et al., 2013) and different phenotype (Rodrigues et al., 1990). In most cancers, including colorectal, chromosomal instability (CIN) occurred, thus leading to an abnormal number of chromosomes and microsatellite instability – MIN (Rodrigues et al., 1990; Bhattacharyya et al., 1994). The SW-480 and HT-29 lines have the CIN phenotype (Loeb, 1991; Camps, 2006), mutation in p53 gene and preserved mismatch reparation system, while the HCT-116 has MIN phenotype, p53 wild-type, with damaged system for reparation (Rodrigues et al., 1990; Yao et al., 2005; Howells et al., 2007).

In addition to the cytotoxicity of SV and LAAO, the mechanism of their activity is very important for further investigation of potential targets and therapeutic application. Although the SV is a potent cytotoxic agent, our results observed by AO/EB microscope method, show that SV dominantly induced necrosis when applied in higher concentration. The occurrence of cancer cell necrosis in high percentages under the treatment by SV as a mixture of components is an unfavorable type of induced cell death and is not applicable as further therapy. Therefore, for further evaluation of antitumor potential, only the LAAO was considered, as a substance with dominant pro-apoptotic activity and insignificant necrotic activity.

The induction of apoptosis is commonly related to oxidative disbalance in cancer cells as a result of the application of cytotoxic substances (Milutinović et al., 2019). The concentrations of

monitored redox status parameters, $O_2^{\cdot -}$, and reduced glutathione increase and correlate with the observed cytotoxic and proapoptotic activity (Matés et al., 2008). In our paper, the changes in their concentrations under the influence of LAAO were least pronounced in the HT-29 line, and the most increased in the HCT-116. The increased $O_2^{\cdot -}$ mainly leads to induction of apoptosis through disturbance of mitochondria membrane integrity and activation of mitochondrial apoptotic pathway (Matés et al., 2008; Milutinović et al., 2019). The increased GSH, as part of the antioxidant defense system, indicates that the cells are protecting themselves from aggressive treatments that produce high levels of oxidative stress (Lushchak, 2012). This was confirmed by increased protein expression of GSS, the enzymes involved in glutathione *de novo* synthesis in the highest applied concentration of LAAO. Oxidative stress can lead to damages of cells and cellular components, such as lipids in cell membrane (Avery, 2011). The concentration of malondialdehyde (MDA), as an indicator of oxidative damage of membrane lipids, significantly dose-dependently increased in LAAO treated HCT-116 cells, compared to controls, which was not observed in SW-480 and HT-29 cells, thus suggesting no damage of the membrane. Rapid disintegration of the membrane due to oxidative stress leads to necrosis (Jaeschke and Ramachandran, 2018), which was not observed in the LAAO treatment.

One of the main problems in anticancer therapy lies in the development of cancer cell resistance to drugs (Housman et al., 2014). The metabolism of drugs in cancer cells has an important role in their future activity, whereby more or less toxic compounds may be formed or ejected from the cell (Gao et al., 2013). Our study presents an expression profile for membrane transporter genes, potentially responsible for the development of resistance to the LAAO, as a potent cytotoxin in three different colon cancer cell lines. It has previously been reported that PGP1 is the highest expressed in colon cancers, followed by MRP1 and MRP2 (Berggren et al., 2007). Our results

indicate differences in membrane transporter gene expression between different cell lines originated from the colon. The *PGP1* mRNA expression increased in HCT-116 cells, while in SW-480 and HT-29 it decreased on both gene and protein levels. The increase of *PGP1* on gene-level indicates that HCT-116 may develop resistance to the investigated treatment in the future, or after a long time of exposure to the treatment, because the level of PGP1 protein expression reduces after 24 h. This explains several previously published results where HCT-116 cells recover after prolonged exposure to different treatments (Ćurčić et al., 2012; Alimpić et al., 2015). On the other hand, decreased PGP1 gene and protein expression in SW-480 and HT-29 cells is a precious outcome in terms of developing LAAO as an inhibitor of membrane transporter and overcoming of resistance. Many natural substances mainly phenols from plants possess this ability to reduce the expression or activity of efflux pumps, which can be used to improve the efficacy of the anticancer treatment (Sjostedt et al., 2017; Milutinović et al., 2019). Other investigated transporters were unequally expressed in different colon cell lines treated by LAAO. Besides P-gp, *MRP2* mRNA expression also increased in HCT-116 cells as well as in SW-480, while *MRP1* decreased or unchanged in SW-480. Both, *MRP1* and *2* increased in HT-29, suggesting that these transporters may have a role in the ejection of LAAO from HT-29 cells and be responsible for the lowest sensitivity to the treatment.

For their activity, the mentioned transporters mainly required previously metabolized drugs by CYP and other enzymes, conjugated to glutathione (as part of the glutathione detoxification system) by *GSTP1* (Bredel, 2001). According to the results and increased mRNA expression for genes involved in the biotransformation process and drug metabolism, it is evident that LAAO is a substrate for these enzymes. The most pronounced expression of the *CYP* genes (*CYP1A1* and *CYP1B1*) in HCT-116 cells indicates the need for an intensive metabolism of LAAO. However,

the results of observed cytotoxicity in this cell line may in that case suggest formation of more active metabolite or production of ROS (Reactive oxygen species) as a mediator of apoptosis and cytotoxicity.

This study provides significant results about insufficiently investigated L-amino acid oxidases from snake venom, as potent cytotoxic and proapoptotic agents for colon cancer cells. Additionally, our results highlight the main mechanism of their activity and achieved anticancer properties, and induction of apoptosis mediated by changes in redox status. A novel result is that the LAAO changes mRNA expression of genes associated with biotransformation processes and the development of drug resistance. The result indicates different expression profiles in different colon cancer cells and potential prediction for long-term treatment, with minimum possibility for occurrence of drug resistance.

Acknowledgments

This work was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia (Agreement no. 451-03-9/2021-14/200122; 451-03-68/2020-14/200378 and 451-03-9/2021-14/ 200007).

Declaration of interest statement

There is no conflict of interests.

References

Alimpić, A., Pljevljakušić, D., Savikin, K., Knezević, A., Ćurčić, M., Veličković, D., Stević, T., Petrović, G., Matevski, V., Vukojević, J., Marković, S., Marin, P., Duletić-Laušević, S., 2015. Composition and biological effects of *Salvia ringens* (Lamiaceae) essential oil and extracts. Industrial Crops and Products. 76, 702–709. https://doi.org/10.1016/j.indcrop.2015.07.053.

Auclair, C., Voisin, E., 1985. Nitroblue tetrazolium reduction. In: Greenwald RA. Handbook of methods for oxygen radical research. 1st ed. Boka Raton: CRC Press, 123.

Avery, S.V., 2011. Molecular targets of oxidative stress. Journal of Biochemistry. 434 (2), 201–210. https://doi.org/10.1042/BJ20101695.

Baker, M.A., Cerniglia, G.J., Zaman, A., 1990. Microtiter plate assay for the measurement of glutathione and glutathione disulfide in large numbers of biological samples. Analytical Biochemistry. 190 (2), 360–365. https://doi.org/10.1016/0003-2697(90)90208-q.

Baskić, D., Popović, S., Ristić, P., Arsenijević, N.N., 2006. Analysis of cyclohexamide-induced apoptosis in human leukocytes: Fluorescence microscopy using annexin V/propidium iodide versus acridin orange/ethidium bromide. Cell Biology International. 30 (11), 924–932. https://doi.org/10.1016/j.cellbi.2006.06.016.

Berggren, S., Gall, C., Wollnitz, N., Ekelund, M., Karlbom, U., Hoogstraate, J., Schrenk, D., Lennerna, H., 2007. Gene and protein expression of P-glycoprotein, MRP1, MRP2, and CYP3A4 in the small and large human intestine. Molecular Pharmaceutics. 4 (2), 252–257. https://doi.org/10.1021/mp0600687.

Bhattacharyya, N.P., Skandalis, A., Ganesh, A., Groden, J., Meuth, M., 1994. Mutator phenotypes in human colorectal carcinoma cell lines. Proceedings of the National Academy of Sciences of the United States of America. 91 (14), 6319–6323. https://doi.org/10.1073/pnas.91.14.6319.

Bredel, M., 2001. Anticancer drug resistance in primary human brain tumors. Brain Research Reviews. 35 (2), 161–204. https://doi.org/10.1016/S0165-0173(01)00045-5.

423 Buege, J.A., Aust, S.D., 1987. Microsomal lipid peroxidation. Methods in Enzymology. 52, 302–
424 310. https://doi.org/10.1016/s0076-6879(78)52032-6.

425 Camps, J., Armengol, G., del Rey, J., Lozano, J.J., Vauhkonen, H., Prat, E., Egozcue, J., Sumoy,
426 L., Knuutila, S., Miro, R., 2006. Genome-wide differences between microsatellite stable and
427 unstable colorectal tumors. Carcinogenesis. 27 (3), 419–428.
428 https://doi.org/10.1093/carcin/bgi244.

429 Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium
430 thiocyanate-phenol-chloroform extraction. Analitical Biochemistry. 162 (1), 156–159.
431 https://doi.org/10.1016/0003-2697(87)90021-2.

432 Ćurčić, M., Stanković, M., Radojević, I., Stefanović, O., Čomić, Lj., Topuzović, M., Đačić, D.,
433 Marković, S., 2012. Biological effects, total phenolic content and flavonoid concentrations of
434 fragrant yellow onion (*Allium flavum* L.). Medicinal Chemistry. 8 (1), 46–51.
435 https://doi.org/10.2174/157340612799278441.

436 Deng, J., Lei, W., Fu, J.C., Zhang, L., Li, J.H., Xiong, J.P., 2014. Targeting miR-21 enhances the
437 sensitivity of human colon cancer HT-29 cells to chemoradiotherapy *in vitro*. Biochemical and
438 Biophysical Research Communications. 443 (3), 789–795.
439 http://dx.doi.org/10.1016/j.bbrc.2013.11.064.

440 Du, X.Y., Clemetson, K.J., 2002. Snake venom l-amino acid oxidases. Toxicon. 40 (6), 659–665.
441 https://doi.org/10.1016/S0041-0101(02)00102-2.

442 Gao, F., Zhang, J.M., Wang, Z.G., Peng, W., Hu, H.L., Fu, C.M., 2013. Biotransformation, a
443 promising technology for anti-cancer drug development. Asian Pacific Journal of Cancer
444 Prevention. 14 (10), 5599–5608. http://dx.doi.org/10.7314/APJCP.2013.14.10.5599.

445 Georgieva, D., Arni, R.K., Betzel, C., 2008. Proteome analysis of snake venom toxins:
446 pharmacological insights. Expert Rev. Proteomics. 5 (6), 787–797.
447 https://doi.org/10.1586/14789450.5.6.787.

448 Guengerich, F.P., 2008. Cytochrome P450 and chemical toxicology. Chemical Toxicology
449 Research. 21 (1), 70–83. https://doi.org/10.1021/tx700079z.

450 Haber, M., Smith, J., Bordow, S.B., Flemming, C., Cohn, S.L., London, W.B., Marshall, G.M.,
451 Norris, M.D., 2006. Association of high-level *MRP1* expression with poor clinical outcome in a
452 large prospective study of primary neuroblastoma. Journal of Clinical Oncology. 24 (10), 1546–
453 1553. https://doi.org/10.1200/JCO.2005.01.6196.

454 Hall, M.D., Okabe, M., Shen, D.W., Liang X.J., Gottesman, M.M., 2008. The role of cellular
455 accumulation in determining sensitivity to platinum-based chemotherapy. Annual Review of
456 Pharmacology and Toxicology. 48 (2008), 495–535.
457 https://doi.org/10.1146/annurev.pharmtox.48.080907.180426.

458 Higuchi, H., Bronk, S.F., Takikawa, Y., Werneburg, N., Takimoto, R., El-Deiry, W., Gores, G.J.,
459 2001. The bile acid glycochenodeoxycholate induces TRAIL-Receptor 2/DR5 expression and
460 apoptosis. Journal of Biological Chemistry. 276 (42), 38610–38618.
461 https://doi.org/10.1074/jbc.M105300200.

462 Hoffmann, U., Kroemer, H.K., 2004. The ABC Transporters MDR1 and MRP2: Multiple
463 functions in disposition of xenobiotics and drug resistance. Drug Metabolism Reviews. 36 (3-4),
464 669–701. https://doi.org/10.1081/DMR-200033473.

Housman, G., Byler, S., Heerboth, S., Lapinska, K., Longacre, M., Snyder, N., Sarkar, S., 2014. Drug resistance in cancer: An overview. Cancers. 6 (3), 1769–1792. https://doi.org/10.3390/cancers6031769.

Howells, L.M., Mitra, A., Manson, M.M, 2007. Comparison of oxaliplatin- and curcumin-mediated antiproliferative effects in colorectal cell lines. International Journal of Cancer. 121, 175–183. https://doi.org/10.1002/ijc.22645.

Ikehata, M., Ogawa, M., Yamada, Y., Tanaka, S., Ueda, K., Iwakawa, S., 2014. Different effects of epigenetic modifiers on the cytotoxicity induced by 5-fluorouracil, irinotecan or oxaliplatin in colon cancer cells. Biological & Pharmaceutical Bulletin. 37 (1), 67–73. https://doi.org/10.1248/bpb.b13-00574.

Izidoro, L.F.M., Sobrinho, J.C., Mendes, M.M., Costa, T.R., Grabner, A.N., Rodrigues,V.M., da Silva, S.L., Zanchi, F.B., Zuliani, J.P., Fernandes, C.F.C., Calderon, L.A., Stábeli, R.G., Soares, A.M., 2014. Snake venom L-amino acid oxidases: Trends in pharmacology and biochemistry. BioMed Research International. 2014:196754. http://dx.doi.org/10.1155/2014/196754.

Jaeschke, H., Ramachandran, A., 2018. Oxidant stress and lipid peroxidation in acetaminophen hepatotoxicity. Reactive Oxygen Species. (Apex). 5 (15), 145–158. http://dx.doi.org/10.20455/ros.2018.835.

Kerb, R., Hoffmeyer, S., Brinkmann, U., 2001. ABC drug transporters: hereditary polymorphisms and pharmacological impact in MDR1, MRP1 and MRP2. Pharmacogenomics. 2 (1), 51–64. https://doi.org/10.1517/14622416.2.1.51.

485 Komarova, N.L., Wodarz, D., 2005. Drug resistance in cancer: Principles of emergence and
486 prevention. Proceedings of the National Academy of Sciences. 102 (27), 9714–9719.
487 https://doi.org/10.1073/pnas.0501870102.

488 Latinović, Z., Leonardi, A., Šribar, J., Sajevic, T., Žužek, M.C., Frangež, R., Halassy, B.,
489 Trampuš-Bakija, A., Pungerčar, J., Križaj, I., 2016. Venomics of *Vipera berus berus* to explain
490 differences in pathology elicited by *Vipera ammodytes ammodytes* envenomation: Therapeutic
491 implications. Journal of Proteomics. 146 (2016), 34–47.
492 http://dx.doi.org/10.1016/j.jprot.2016.06.020.

493 Leslie, E.M., Deeley, R.G., Cole, S.P., 2005. Multidrug resistance proteins: role of P-
494 glycoprotein, MRP1, MRP2, and BCRP (ABCG2) in tissue defense. Toxicology and applied
495 pharmacology. 204 (3), 216–237. https://doi.org/10.1016/j.taap.2004.10.012.

496 Loeb, L.A., 1991. Mutator phenotype may be required for multistage carcinogenesis. Cancer
497 Research. 51 (12), 3075–3079.

498 Lushchak, V.I., 2012. Glutathione homeostasis and functions: Potential targets for medical
499 interventions. Journal of Amino Acids. 2012, 736837. https://doi.org/10.1155/2012/736837.

500 Matés, J.M., Segura, J.A., Alonso, F.J., Márquez, J., 2008. Intracellular redox status and
501 oxidative stress: implications for cell proliferation, apoptosis, and carcinogenesis. Archives of
502 Toxicology. 82 (5), 273–299. https://doi.org/10.1007/s00204-008-0304-z.

503 Milutinović, M.G., Maksimović, V.M., Cvetković, D.M., Nikodijević, D.D., Stanković, M.S.,
504 Pešić, M., Marković, S.D., 2019. Potential of *Teucrium chamaedrys* L. to modulate apoptosis
505 and biotransformation in colorectal carcinoma cells. Journal of Ethnopharmacology. 240,
506 111951. https://doi.org/10.1016/j.jep.2019.111951.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Immunological Methods. 65 (1-2), 55–63. https://doi.org/10.1016/0022-1759(83)90303-4.

Nikodijević, D.D., Milutinović, M.G., Cvetković, D.M., Ćupurdija, M.Đ., Jovanović, M.M., Mrkić, I.V., Jankulović-Gavrović, M.Đ., Marković, S.D., 2019. Impact of bee venom and melittin on apoptosis and biotransformation in colorectal carcinoma cell lines. Toxin Reviews. https://doi.org/10.1080/15569543.2019.1680564.

Podolski-Renić, A., Jadranin, M., Stanković, T., Banković, J., Stojković,S., Chiourea, M., Aljančić, I., Vajs, V., Tešević, V., Ruždijić, S., Gagos, S., Tanić, N., Pešić, M., 2013. Molecular and cytogenetic changes in multi-drug resistant cancer cells and their influence on new compounds testing. Cancer Chemotherapy and Pharmacology. 72 (3), 683–697. https://doi.org/10.1007/s00280-013-2247-1.

Rodrigues, N.R., Rowan, A., Smith, M.E., Kerr, I.B., Bodmer, W.F., Gannon, J.V., Lane, D.P., 1990. p53 mutations in colorectal cancer. National Academy of Sciences of the United States of America. 87 (19), 7555–7559. https://doi.org/10.1073/pnas.87.19.7555.

Roy, A., Bharadvaja, N., 2021. Venom-derived bioactive compounds as potential anticancer agents: A review. International Journal of Peptide Research and Therapeutics. 27, 129–147. https://doi.org/10.1007/s10989-020-10073-z.

Sandusky, G.E., Mintze, K.S., Pratt, S.E., Dantzig, A.H., 2002. Expression of multidrug resistance-associated protein 2 (MRP2) in normal human tissues and carcinomas using tissue microarrays. Histopathology. 41 (1), 65–74. https://doi.org/10.1046/j.1365-2559.2002.01403.x.

Sjostedt, N., Holvikari, K., Tammela, P., Kidron, H., 2017. Inhibition of breast cancer resistance protein and multidrug resistance associated protein 2 by natural compounds and their derivatives. Molecular Pharmacology. 14 (1), 135–146. https://doi.org/10.1021/acs.molpharmaceut.6b00754.

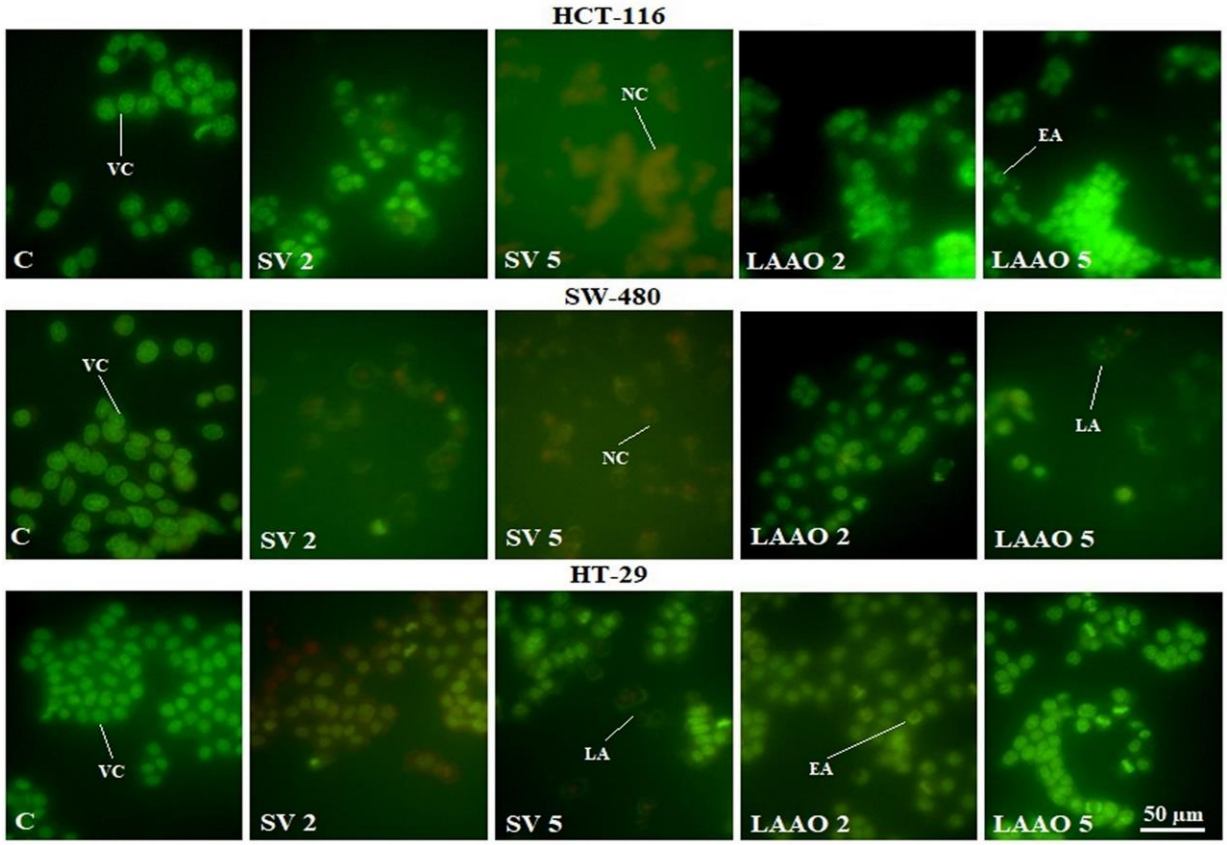
Sung, H., Ferlay, J., Siegel, R.L., Laversanne, M., Soerjomataram, I., Jemal, A., Bray, F., 2020. Global cancer statistics 2020: Globocane estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA: A Cancer Journal for Clinicians. 71 (3), 209–249. https://doi.org/10.3322/caac.21660.

Torigoe, T., Izumi, H., Ishiguchi, H., Yoshida, Y., Tanabe, M., Yoshida, T., Igarashi, T., Niina, I., Wakasugi, T., Imaizumi, T., Momii, Y., Kuwano, M., Kohno, K., 2005. Cisplatin resistance and transcription factors. Current Medicinal Chemistry. Anti-cancer Agents. 5 (1), 15–27. https://doi.org/10.2174/1568011053352587.

Wang, Y., Zhong, J., Bai, J., Tong, R., An, F., Jiao, P., He, L., Zeng, D., Long, E., Yan, J., Yu, J., Cai, L., 2018. The application of natural products in cancer therapy by targeting apoptosis pathways. Current Drug Metabolism. 19 (9), 739–749. https://doi.org/10.2174/1389200219666180511154722.

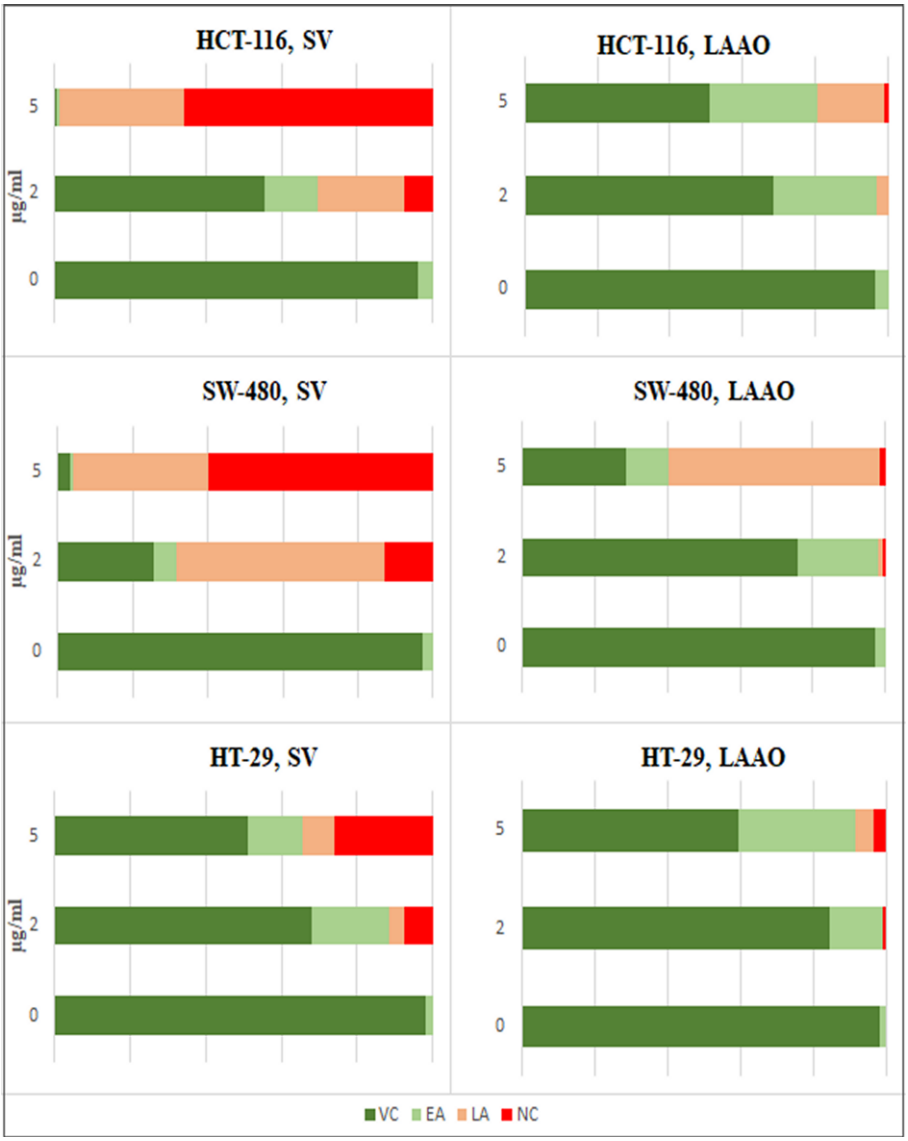
Yao, K., Gietema, J.A., Shida, S., Selvakumaran, M., Fonrose, X., Haas, H.B., Testa, J., O'Dwyer, P.J., 2005. In vitro hypoxia-conditioned colon cancer cell lines derived from HCT116 and HT29 exhibit altered apoptosis susceptibility and a more angiogenic profile in vivo. British Journal of Cancer. 93 (12), 1356–1363. https://doi.org/10.1038/sj.bjc.6602864.

Figure captions



549

550 **Fig. 1.** Morphology of HCT-116, SW-480 and HT-29 control (C) and cells treated by LAAO and
551 SV in a concentration of 2 and 5 µg/ml, detected by AO/EB double staining, 24 h after
552 treatments (VC – viable cells, EA – Early Apoptosis; LA -Late Apoptosis; NC – Necrosis).



553

554 **Fig. 2.** Percentage of viable (VC), apoptotic (EA - Early Apoptosis; LA -Late Apoptosis) and
555 necrotic (NC) HCT-116, SW-480 and HT-29 cells after 24h of treatment with SV (2 and 5
556 ug/ml) or LAAO (2 and 5 µg/ml).

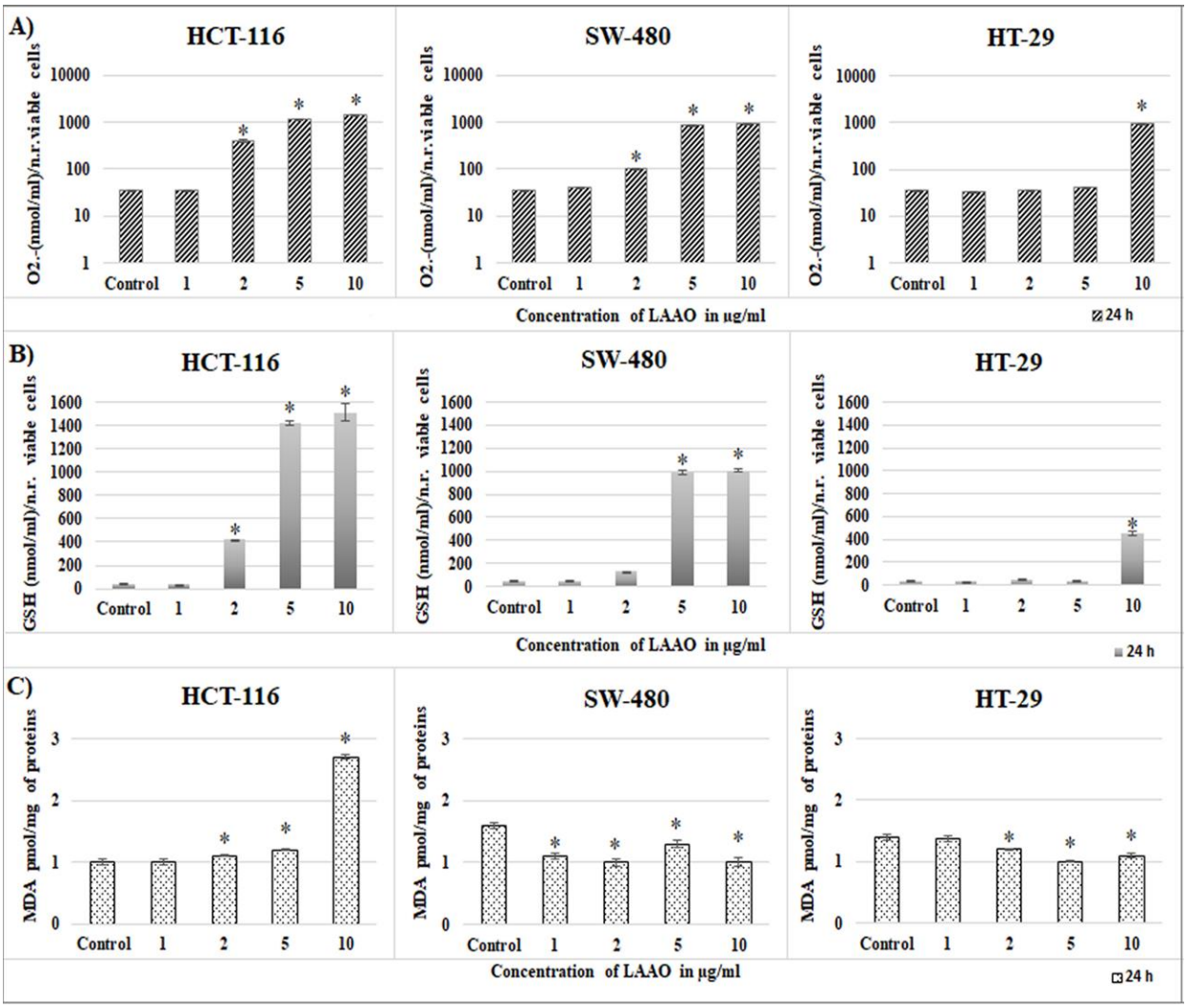
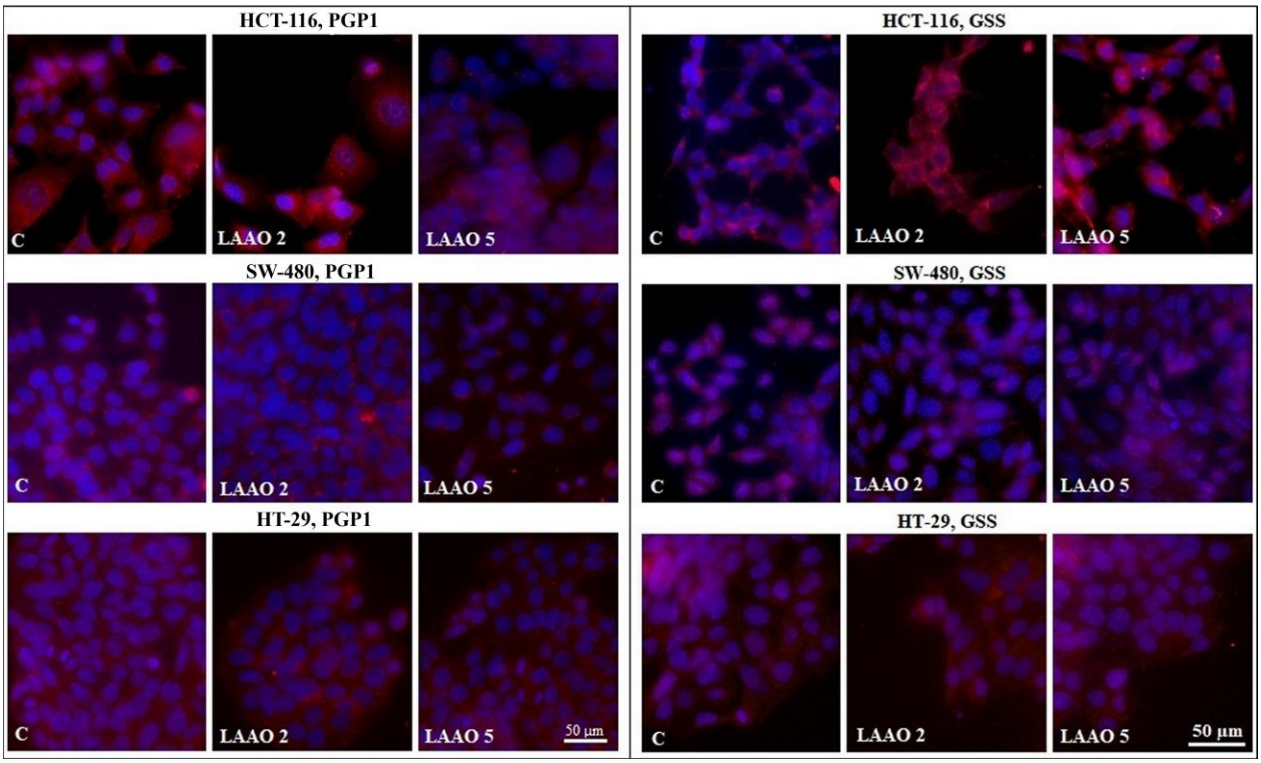


Fig. 3. Influence of LAAO on redox status parameters on HCT-116, SW-480, and HT-29 cells, after 24 h; **A)** The concentration of superoxide anion radical, presented in nmol/ml and calculated per number of viable cells; **B)** The concentration of GSH, presented in nmol/ml and calculated per number of viable cells; **C)** The concentration of MDA, presented in pmol/mg of protein. The data are means ± SE of two independent experiments. *$p < 0.05$ compared to untreated cells.

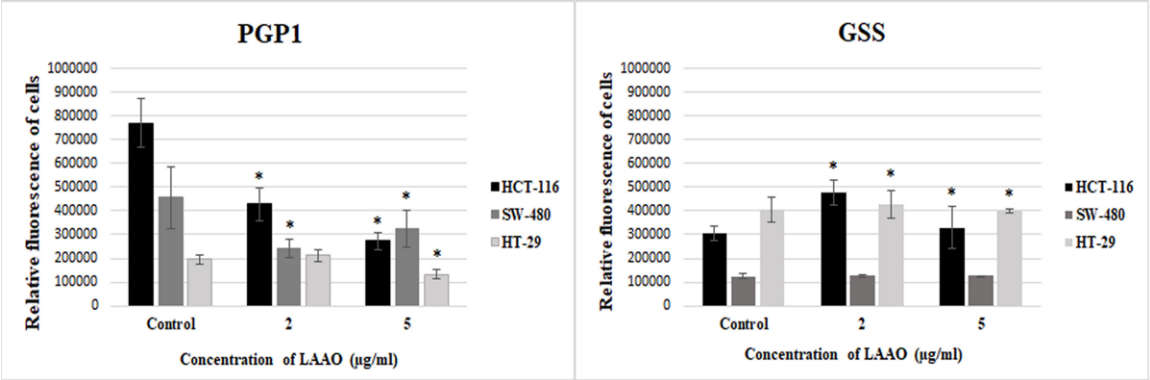


564

565 **Fig. 4.** Protein expression of P-gp on HCT-116, SW-480, and HT-29 control (C) and cells treated
566 with LAAO (2 and 5 µg/ml), 24 h after treatments. The cells were visualized using an inverted
567 fluorescent microscope at 600x magnification. The nuclei are colored blue (DAPI color), P-gp is
568 red (secondary antibody conjugated to Cy3). The relative intensity of fluorescence in control and
569 treated cells was measured by the ImageJ program.

570

571



572

573 **Fig. 5.** The level of P-gp and GSS on HCT-116, SW-480, and HT-29 control and cells treated
574 with LAAO (2 and 5 µg/ml), 24 h after treatments. The data are means ± SE of two independent
575 experiments. *$p < 0.05$ compared to untreated cells.

576

577 Supplementary material

578 **Table 1.** Specific human gene primers.

Genes	Forward sequence	Reverse sequence
β-actin	5′-AAGCAGGAGTATGACGAGTCCG-3′	5′-GCCTTCATACATCTCAAGTTGG-3′
CYP1A1	5′-TAGACACTGATCTGGCTGCAG-3′	5′-GGGAAGGCTCCATCAGCATC-3′
CYP1B1	5′-TGATGGACGCCTTTATCCTCTC-3′	5′-CATAAAGGAAGGCCAGGACATA-3′
GSTP1	5′-TCAAAGCCTCCTGCCTATAC-3′	5′-AGGTGACGCAGGATGGTATT-3′
MRP1	5′-ACCCTAATCCCTGCCCAGAG-3′	5′-CGCATTCCTTCTTCCAGTTC-3′
MRP2	5′-ATACCAATCCAAGCCTCTAC-3′	5′-GAATTGTCACCCTGTAAGAG-3′
PGP1	5′-GCCTGGCAGCTGGAAGACAAATACACAAAATT-3′	5′-CAGACAGCAGCTGACAGTCCAAGAACAGGACT-3′

579

580

581

582

583

584

Table 2. Effect of SV and LAAO on HCT-116, SW-480, and HT-29 cells. Results are presented as percentage of viable (VC), apoptotic (early-EA, late-LA) and necrotic (N) cells, 24 h after treatments by SV and LAAO (2 and 5 µg/ml). The results are presented as the mean of three independent experiments ± standard error. Statistically significant difference ($p < 0.05$): * in relation to control values; # HCT-116 in relation to SW-480 cells; & HCT-116 in relation to HT-29 cells; $ HT-29 in relation to SW-480 cells.

SV					LAAO				
µg/ml	**VC**	**EA**	**LA**	**NC**	**µg/ml**	**VC**	**EA**	**LA**	**NC**
HCT-116									
0	96.23±0.05	3.77±0.01	/	/	**0**	96.23±0.05	3.77±0.01	/	/
2	55.34±0.02*#	14.33±0.01*#	22.75±0.03*#	7.58±0.01*#	**2**	68.63±0.05*#	28.10±0.02*#	3.27±0.01*#	/
5	0.35±0.01*#	1.06±0.001*	32.86±0.05*#	65.73±0.05*#	**5**	51.03±0.03*#	29.33±0.02*#	18.77±0.02*#	0.87±0.001*#
SW-480									
0	97.23±0.01	2.77±0.01	/	/	**0**	97.23±0.01	2.77±0.01	/	/
2	25.58±0.3*$	6.20±0.5*$	55.43±0.5*$	12.79±0.01*$	**2**	75.89±0.05*$	22.19±0.1*$	1.28±0.01*$	0.64±0.001*$
5	3.09±0.01*$	1.03±0.001	36.09±0.1*$	59.79±0.5*$	**5**	28.57±0.02*$	11.64±0.1*$	58.20±0.5*$	1.59±0.001*$
HT-29									
0	98.15±0.01	1.85±0.05	/	/	**0**	98.15±0.01	1.85±0.05	/	/
2	68.11±0.23*&	20.27±0.15*&	3.98±0.05*&	7.64±0.50*&	**2**	84.51±0.05*&	14.55±0.01*&	/	0.94±0.001*&
5	50.99±0.25*&	14.74±0.05*&	8.38±0.01*&	25.89±0.30*&	**5**	59.58±0.15*&	31.84±0.22*&	5.31±0.05*&	3.27±0.001*&

Figure 1. The effects of LAAO (A) and SV (B) on HCT-116, SW-480, HT-29 colorectal carcinoma cell viabilities, and normal HaCaT cells (C). The results are presented as the mean of three independent experiments ± standard error. * Statistically significant difference ($p < 0.05$) in relation to control values.