Potential of Melittin to induce apoptosis and overcome multidrug resistance in human colon cancer cell line

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Abstract

In this study, the anticancer effect of Melittin is shown through antiproliferative and proapoptotic effects on HT-29 cells, as well as ability to reverse multidrug resistance. Melittin directly affects the death receptor-dependent apoptotic pathway via increase of the Fas receptor protein expression, *Caspase 8* gene expression and activity of Caspase 8. Results of decreased Caspase 9 gene and protein expression, and multi-fold increased expression of *Bcl-2* gene suggest that mitochondria and inner apoptotic pathway are not involved in execution of Melittin induced apoptosis, as well as redox regulation of apoptosis based on decreased concentration of superoxide anion radicals and no affected glutathione level. Specially significance of this work are results on ability of Melittin to modulate the metabolizing and export system in cancer cells. Based on increased expression of all investigated genes related to biotransformation process, it can be assumed that *CYP1A1*, *CYP1B1*, *GSTP1* and *MRP2* are involved in metabolism of Melittin in HT-29 cells. P-glycoprotein is associated with the occurrence of resistance in anticancer therapy, so its reduced gene and protein expression by Melittin represents significant result in terms of possible therapeutic application and examination.

Keywords: Anticancer activity, Biotransformation, Caspase 8 and 9, Fas receptor

1. Introduction

The most common carcinoma in digestive tract is colorectal carcinoma (CRC), which takes the third place in frequency among human malignancies based on data from the World Health Organization (Sung *et al.* 2020).

Development of multi drug resistance (MDR) is one of the major causes of chemotherapy failures. The most investigated mechanisms of MDR are alternations of the genes and the proteins involved in the control of apoptosis, drug metabolism, such as the activation of enzymes from phase I (Cytochrome P450) and the glutathione detoxification system (phase II), as well as the efflux of drugs from the cells by transmembrane proteins (Guengerich 2007, Mansoori *et al.* 2017, Bukowski *et al.* 2020). ABC transporters (*ATP Binding Cassette*) are the most extensively studied efflux transporters associated with development of MDR (Szakács *et al.* 2006). The P-glycoprotein (PGP1, encoded by *MDR1* gene), MRP1 and 2 (*Multidrug Resistance-associated Protein 1 and 2*), and BCRP (*Breast Cancer Resistance Protein*) are most common members of the ABC family that efflux a large spectrum of xenobiotics (Dinic *et al.* 2015). Due to physiological role of colon epithelium, there are a high expression level of molecules involved in the metabolism of xenobiotics (Ranhotra *et al.* 2016). Among them, the MDR1 is the most expressed, followed by MRP1 and MRP2 (Berggren *et al.* 2007). Several attempts have been made to identify natural products with various chemical structures which can inhibit ABC transporters or supress the expression or activity of biotransformation enzymes associated with MDR (Wink *et al.* 2012).

Currently used therapy in the treatment of colorectal cancer involves the use of cytostatics with various side effects and nonselective activity. Thus, there is on-going need for discovery or synthesis of new, more adequate substances for improving current therapy approach (Piasek *et al.*

2009, Pagani 2010). Natural products, as well as those from animal sources, are widely available and cost-effective for production with numerous favourable pharmacological effects (Liu *et al.* 2014, Seca and Pinto 2018). Honey, propolis, royal jelly, bee pollen, bee bread, venom, and wax are the main European honeybee (*Apis mellifera*) products with potent health beneficial effects (Münstedt and Männle 2020, Nainu *et al.* 2021). The bee venom contains a large spectrum of constituents with confirmed anticancer potential (Son *et al.* 2007, Rady *et al.* 2017). Among them, Melittin is the most abundant component of honeybee venom and takes over 50% of total proteins (Raghuraman and Chattopadhyay 2007, Nikodijević *et al.* 2019) with significant pharmacological action (Gajski and Garaj-Vrhovac 2013, Rady *et al.* 2017). Understanding the specificity of molecular action of Melittin against cancer cells is key for developing and optimizing novel effective therapeutics from animal natural product. It is important to examine its potential for apoptosis induction, as the main process which regulates the occurrence and spread of cancer. Previously, the cytotoxic and proapoptotic activity of Melittin and melittin-conjugates have been reported on different types of cancer, such as glioma cells, hepatocellular carcinoma cells, leukemic cells, osteosarcoma, ovarian, prostate, renal and breast cancer cells (Li *et al.* 2006, Yang *et al.* 2007, Zhang *et al.* 2007, Moon *et al.* 2008, Park *et al.* 2011, Jo *et al.* 2012, Gajski and Garaj-Vrhovac 2013, Rady *et al.* 2017, Yavari *et al.* 2020).

Although Melittin showed pharmacological action in various cancer cell lines, there is still a lack of evidence for its anticancer and proapoptotic activity in different CRC cell lines. It is only reported that bee venom shows cytotoxic effects in HCT-116 and SW480 CRC cells (Zheng *et al.* 2015, Nikodijević *et al.* 2019), while Melittin inhibited the growth of CT26 CRC cells via inhibition of protein translation and synthesis (Soliman *et al.* 2019). Melittin induced significant

cell death in HCT-116 cells (Bei *et al.* 2015) and pronounce cytotoxicity and apoptosis in HCT-116 and SW480 cells (Nikodijević *et al.* 2019).

In accordance with the literature data mentioned above, the aim of this study was to investigate the proapoptotic activity of Melittin in the less sensitive, HT-29 CRC cells. Additionally, the potential of Melittin to modulate cancer cell resistance stay poorly understood. Potential of Melittin to overcome MDR was confirmed in HCT-116 and SW480 cells (Nikodijević *et al.* 2019), but in the other CRC cells it's still unknown. Also, according to our knowledge there are insufficient data about Melittin metabolism and biotransformation in the CRC cells, which deserves detailed investigation.

2. Material and Methods

2.1. Chemicals

Dulbecco's Modified Eagle Medium (DMEM) were obtained from Capricorn, Germany, USA. Ethidium bromide (EB), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and NBT (Nitro Blue Tetrazolium) were obtained from SERVA, Germany. Acridine Orange (AO) was obtained from Acros Organics, New Jersey, USA. Primary antibody GSS, was obtained from Sigma Aldrich. The High-Capacity cDNK Reverse Transcription Kit and secondary antibody conjugated with Cy3, Diamidino-2-phenylindole (DAPI), and primary antibody PGP1 were from Thermo Fisher Scientific, US. Primers were synthesized by Eurofins Genomics, Ebersberg. Sensi FASTTM SYBR Lo-ROX Kit was purchased from Bioline, London, UK. Caspase 8 colorimetric assay kits, Fas and Caspase 9 antibody were obtained from R&D Systems, USA, Canada. All other solvents and chemicals were of analytical grade.

2.2. Storage and dissolution of Melittin

Melittin (dry powder) was used from Sigma Aldrich (CAS No. 20449-79-0) and stored at -20 °C. Initially, Melittin was dissolved in few drops of distilled water before use, while the concentration necessary to perform the experiments were made in DMEM.

2.3. Culturing the cell line

The human colorectal adenocarcinoma, HT-29 cell line, and fetal lung fibroblasts MRC-5 (American Type Culture Collection, USA) were cultivated in DMEM according to the protocols in Milutinović et al. (2020). After reaching confluency of 70-80%, cells were used for experiments.

2.4. MTT cell viability assay

The cytotoxic activity of Melittin on HT-29 and MRC-5 cells was determined by colorimetric MTT cell viability method. Healthy MRC-5 cells were used as a control for cytotoxicity. The procedures of cell seeding, treating, and results processing were previously described in detail in Nikodijević *et al.* (2021). Cells were treated with Melittin in concentration range of 0.1 to 50 μg/ml. The assay was performed 24 and 72 h after treatment. CalcuSyn program was used to determine the IC_{50} values form dose curves.

2.5. Determination of cell death type

For determination of cell death type, the HT-29 cells were cultured and stained with Acridine orange/ethidium bromide (AO/EB) dye according to protocol in Nikodijević *et al.* (2021). The HT-29 cells were treated by Melittin (1 and 5 μg/ml), while in the control cells only the DMEM was replaced. After treatment, the cells were observed and captured immediately after staining on inverted fluorescent microscope (Nikon Ti-Eclipse), 400x magnification. A percentage of viable cells, the cells in different states of apoptosis (early and late), and necrotic cells, were calculated according to the protocol described previously in Nikodijević *et al.* (2021).

2.6. Immunocytochemistry

Level of protein expression (Fas receptor, Caspase 9, PGP1 and glutathione synthetase (GSS) was performed by immunofluorescence (Higuchi *et al.* 2001). HT-29 cells were cultured and prepared for microscopy according to the procedure described in Nikodijević *et al* (2021). The cells were treated with Melittin (1 and 5 µg/ml), while in the control cells were added DMEM only. The level of cellular fluorescence (observed on microscope Nikon Ti-Eclipse), in control and treated cells was quantified on fluorescence micrography using ImageJ software (Wayne Rasband, ImageJ, http://rsb.info.nih.gov/ij/). The results are presented as relative fluorescence per cell.

2.7. Caspase 8 activity assays

The enzyme activity of Caspase 8 was measured using Caspase 8 colorimetric assay kit. HT-29 cells were seeded in 6-well plates (10^6 cells/well) and treated by Melittin dissolved in 2 ml of DMEM (1 and 5 µg/ml), while in the control cells were added DMEM only. Experimental procedure was performed according to the manufacturer protocol, described in Milutinović *et al.* (2015). The level of enzymatic activity is directly proportional to the colour intensity, i.e., the obtained absorbances.

2.8. Quantitative mRNA analysis by qPCR method

Isolation of total RNA from HT-29 control and cells treated with Melittin (1 and 5 µg/ml) was performed by isolation protocol based on the reaction of phenol/chloroform (Tan and Yap 2009). RNA concentration in all samples was measured on Eppendorf biophotometer. Reverse transcription and quantification of mRNA expression for human *Bax*, *Bcl-2*, *Caspase 8*, *Caspase 9*, *CYP1A1*, *CYP1B1*, *GSTP1*, *MRP1*, *MRP2* and *MDR1* genes was performed using a commercial kit (details in *Chemicals*). *β-actin* was employed as a housekeeping-reference gene, and results were obtained according to the formula described in Nikodijević *et al.* (2021). Gene-specific primer sequences are presented in Table S.1.

2.9. Concentrations of redox status parameters

2.9.1. Superoxide anion radical level

The level of superoxide anion radical ($O_2^{\cdot -}$) was measured by NBT assay (Auclair and Voisin 1985). The cells were seeded in a 96-well plate (10^4 cells/well). After 24 h of incubation, the cells were treated by Melittin (1 and 5 µg/ml), while the untreated cells served as a control. The experimental procedure was previously described in detail in Nikodijević *et al.* (2021). The results were firstly expressed in nmol/ml and then calculated according to the number of viable cells (data obtained by MTT) in each applied concentration.

2.9.2. Gluthatione level

The concentration of glutathione (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 by Melittin (1 and 5 µg/ml) after 24 h. The experimental procedure was previously described in detail in Nikodijević *et al.* (2021). 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).

2.9.3. Malondialdehyde concentration

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 by Melittin (1 and 5 µg/ml) after 24 h. The experimental procedure was previously described in detail in Nikodijević *et al.* (2021). 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.

2.10. Statistical analysis

The data are expressed as mean ± standard error (SE). The Student's t-test and one-way ANOVA (SPSS for Windows, ver. 17, 2008, Chicago, IL) were used to determine a statistical significance (p value < 0.05 was considered significant).

3. Results

3.1. Cytotoxic effects of Melittin

The cytotoxic effect of Melittin was shown through significantly reduced HT-29 cell viability in relation to non-treated cells (Figure S.1). IC_{50} values, calculated from cell viability curves, indicate strong cytotoxicity of the investigated treatment, 11.38 μg/ml for 24 h and 13.22 μg/ml for 72 h after treatment. In the highest applied concentration melittin induce cytotoxicity also on normal MRC-5 cells but compared to carcinoma cells showed higher IC_{50} values (28.96 μg/ml for 24 h, and 23.30 μg/ml for 72 h) and some selectivity (Figure S.1).

3.2. Apoptosis induced by Melittin

Proapototic activity of Melittin was determined by the AO/EB microscopic method. Micrographs (Figure 1), showed morphological changes (chromathin condensation and fragmentation, decrease in cell volume and nucleus size, membrane blebbing) of the HT-29 cells after the treatment by Melittin (1 and 5 μg/ml). The cells morphology and colour changes on micrographs indicate the presence of apoptosis in different stages, labeled as early (EA) and late (LA) stage. The percentage of cells in apoptosis increased in a dose-dependent manner, compared to control (Table 1). Necrotic cells were not detected in applied concentrations (Figure 1).

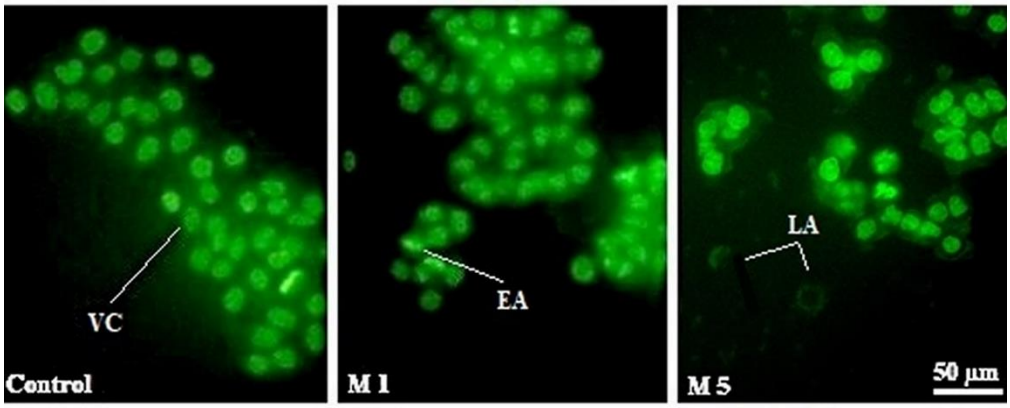

Figure 1. Morphological changes on HT-29 control, and cells treated by Melittin (M1 - 1 µg/ml and M5 – 5 µg/ml), after 24 h. VC – viable cells, EA – Early Apoptosis, LA – Late Apoptosis. Green fluorescence is product by AO dye.

Table 1. Percentage of viable (VC), apoptotic (EA - Early Apoptosis; LA -Late Apoptosis) and necrotic (N) HT-29 cells, 24 h after treatment by Melittin (1 and 5 µg/ml).

Melittin µg/ml	VC	EA	LA	NC
0	96.57±1.33	3.43±0.32	/	/
1	93.21±0.37*	6.79±0.36*	/	/
5	83.41±0.94*	14.75±1.39*	1.84±0.46*	/

The data means ± SE of three independent experiments. *$p < 0.05$ compared to untreated cells

3.3. Molecular mechanisms of Melittin-induced apoptosis

3.3.1. Protein expression of proapoptotic molecules

Protein expression of Fas receptor, as a first activated molecule in the external, death receptor-mediated apoptotic pathway, and Caspase 9, which mediates in inner apoptotic pathway, in HT-29 control, and cells treated by Melittin was determined by imunocytochemistry Results showed the significant increase of Fas receptor protein expression in treated HT-29 cells (1 and 5 µg/ml of Melittin) compared to control, non-treated cells (Figure 2).

Protein expression of Caspase 9 is significantly decreased in HT-29 cells treated by Melittin in concentration of 1 µg/ml and unchanged in concentration of 5 µg/ml, compared to control (Figure 2).

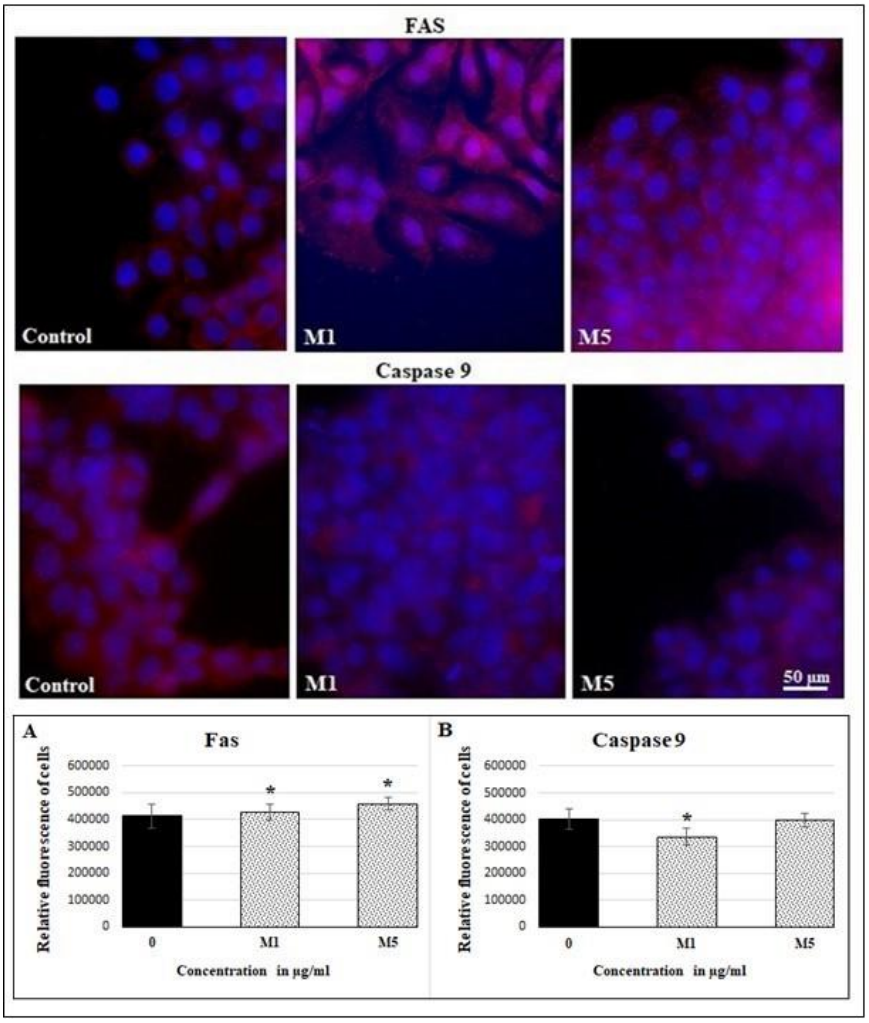

Figure 2. Protein expression of FAS and Caspase 9 in HT-29 cells. Quantified relative fluorescence from micrographs by the ImageJ program (level of protein expression) for Fas receptors (A) and Caspase 9 (B) in HT-29 control, and cells treated by Melittin (M1 - 1 µg/ml and M5 – 5 µg/ml), after 24 h. Proteins are colored by Cy3 (red), nucleus are colored by DAPI (blue). The data means ± SE of three independent experiments. *$p < 0.05$ compared to untreated cells

3.3.2. Caspase 8 activity

Enzime activity of initiator caspase in external apoptotic pathway, Caspase 8, in HT-29 cells, under the influence of Melittin was monitored by the colorimetric method. The results showed the significant increase of Caspase 8 activity in HT-29 cells, dose-dependly in relation to control (Figure 3).

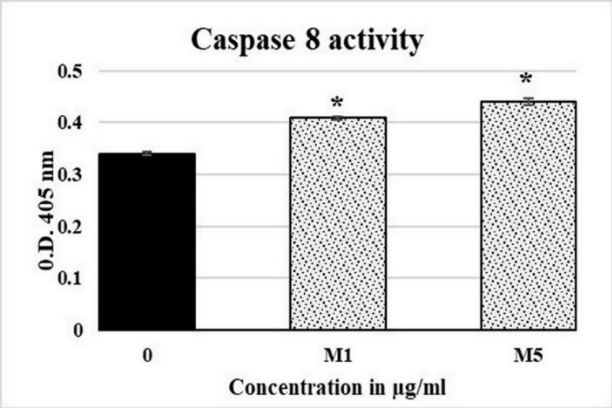

Figure 3. Activity of Caspase 8 in HT-29 control, and cells treated by Melittin (M1 - 1 µg/ml and M5 – 5 µg/ml), after 24 h. The data means ± SE of three independent experiments. *$p < 0.05$ compared to untreated cells

3.3.3. Expression of mRNA for apoptosis-related genes

Expression of mRNA for apoptosis-related genes (proapoptotic *Bax*, antiapoptotic *Bcl-2*, *Caspase 8* and *Caspase 9*), were determined by qPCR method. Results showed significant increase of mRNA expression for all monitored genes compared to control, except the Caspase 9 that was significantly decreased (Table 2).

Table 2. Expression of mRNA for apoptosis-related genes in HT-29 cells treated by Melittin (M1 - 1 µg/ml and M5 – 5 µg/ml), after 24 h.

	Bax	***Bcl-2***	***Caspase 8***	***Caspase 9***
Control	**1**	**1**	**1**	**1**
M1	2.17±0.01*	4.99±0.02*	2.41±0.01*	0.002±0.0001*
M5	2.79±0.01*	23.75±0.01*	2.55±0.02*	0.43±0.001*

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 (data ± SE of three independent experiments, calculated according to the $2^{\wedge(-\Delta\Delta Ct)}$ method; *p < 0.05 compared to untreated cells).

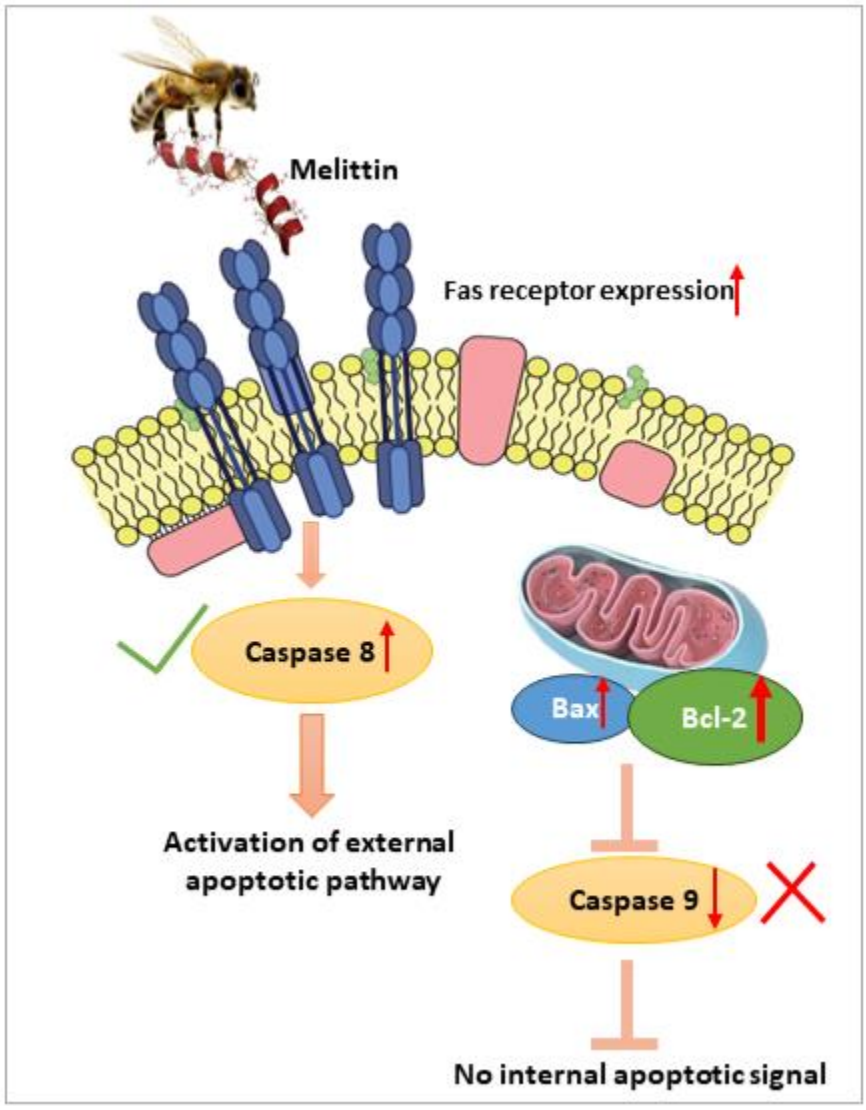

Figure 4. Potential mechanism of the proapoptotic action of Melittin in HT-29 cells – schematic view.

3.4. Impact of Melittin on biotransformation and MDR-related genes and proteins

3.4.1. Expression of mRNA for biotransformation and MDR-related genes

Expression of mRNA for genes involved in metabolism of xenobiotics, as well as genes related to MDR (*CYP1A1*, *CYP1B1*, *GSTP1, MRP1, MRP2* and *MDR1*), were determined by qPCR method. Results showe the significant increase of mRNA expression for all investigated genes involved in phase I (*CYP1A1* and *CYP1B1)* and phase II (*GSTP1*) of biotransformation (Table 3). Gene expression for membrane transporter MRP2 is also increased, while other two investigated transporters (*MRP1* and *MDR1*) were supressed (Table 3).

Table 3. Expression of mRNA for biotransformation and MDR-related genes in HT-29 cells treated by Melittin (M1 - 1 μg/ml and M5 – 5 μg/ml), 24 h after treatments.

	CYP1A1	***CYP1B1***	***GSTP1***	***MRP1***	***MRP2***	***MDR1***
Control	**1**	**1**	**1**	**1**	**1**	**1**
M1	8.00±0.02	1.97±0.01	3.94±0.05	0.89±0.001	4.20±0.01	0.02±0.001
M5	14.03±0.05	1.85±0.001	5.58±0.01	1.07±0.02	14.83±0.05	0.01±0.001

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 (data ± SE of three independent experiments, calculated according to the $2^{\wedge(-\Delta\Delta Ct)}$ method; *$p < 0.05$ compared to untreated cells).*3.4.2. Protein expression of PGP1 and GSS*

Protein expression of PGP1 and Glutathione synthetase (GSS), the second enzyme in the GSH biosynthesis pathway, in HT-29 control, and cells treated by Melittin was determined by imunocytochemistry Results showed the significant decrease of both investigated protein expression in treated HT-29 cells (1 and 5 μg/ml of Melittin) in relation to control (Figure 5).

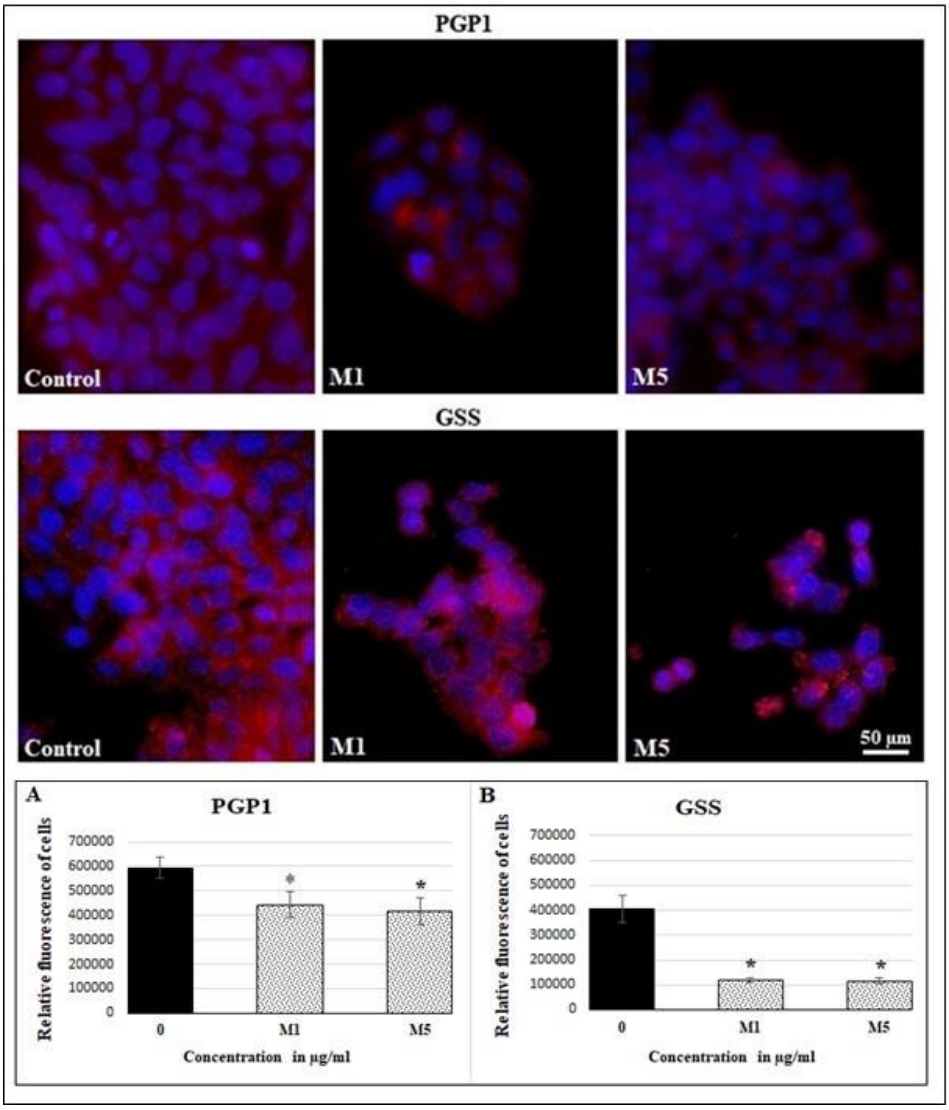

Figure 5. Protein expression of PGP1 and GSS in HT-29 cells. Quantified relative fluorescence from micrographs by the ImageJ program (level of protein expression) for PGP1 (A) and GSS (B) in HT-29 control, and cells treated by Melittin (M1 - 1 µg/ml and M5 – 5 µg/ml), after 24 h. Proteins are colored by Cy3 (red), nucleus are colored by DAPI (blue). The data are means ± SE of three independent experiments. *$p < 0.05$ compared to untreated cells.

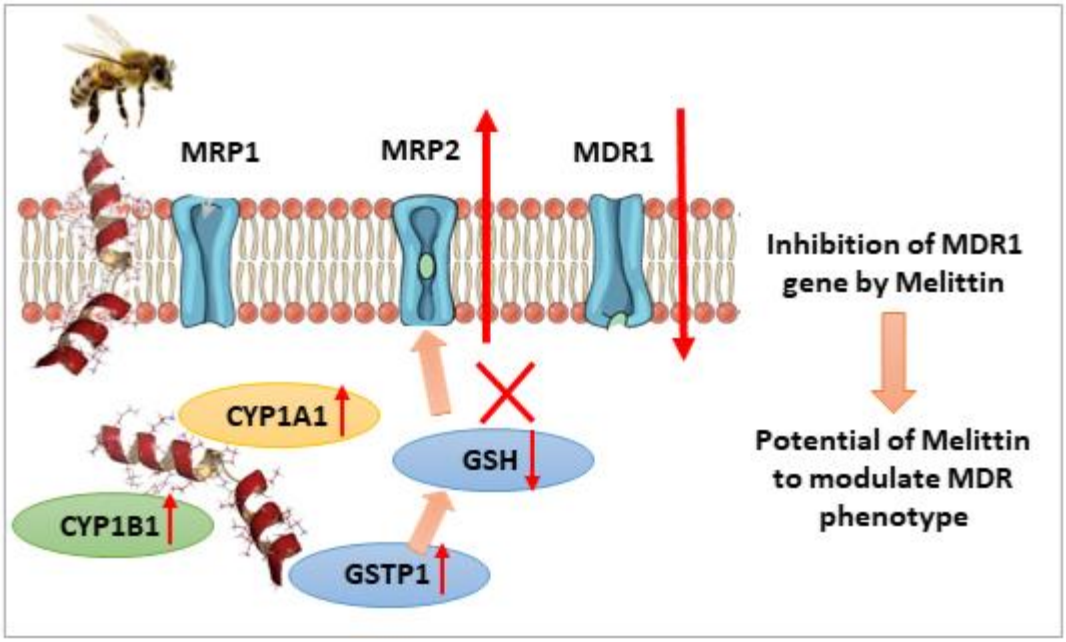

Figure 6. Biotransformation of Melittin and potential to modulate MDR in HT-29 cells - schematic view.

3.5. Impact of Melittin on redox status parameters

The concentrations of superoxide anion radical ($O_2^{\cdot -}$), glutathione (GSH), and malondialdehyde (MDA) in control HT-29 cells and cells treated by Melittin, were determined by the colorimetric methods. The results showed that the concentration of $O_2^{\cdot -}$ is significantly decresed under the influence of Melittin on HT-29 cells, while GSH level is nonsignificantly changed compared to control. Melittin significantly increased level of MDA in concentrations of 1, 2 and 5 μg/ml (Figure 7).

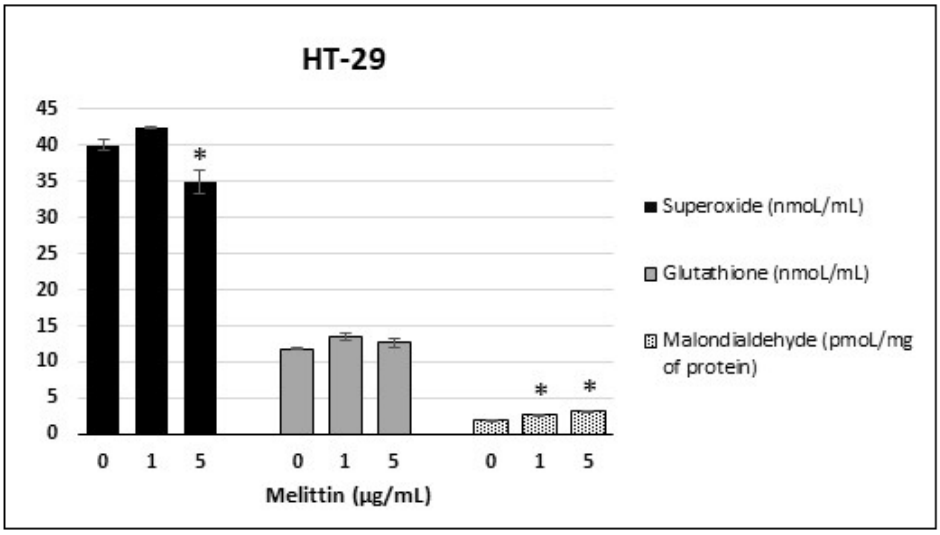

Figure 7. Impact of Melittin on concentration of $O_2^{\cdot -}$, GSH and MDA in HT-29 cells, 24 h after treatment. The data means ± SE of three independent experiments. *$p < 0.05$ compared to untreated cells.

4. Discussion

Malignant diseases are one of the leading health problems and among the most common causes of mortality in the human population regardless of gender (Sung *et al.* 2020). The increased frequency of malignancy requires evaluation the new and modify existing cytostatics, considering side effects and increasingly frequent occurrence of the cancer cell resistance to therapy (Piasek *et al.* 2009, Pagani 2010, Mills *et al.* 2018). Natural products deserve the most attention in this field of research, the most common plants and plant components, which show a high antitumor potential and at the same time a beneficial effect on healthy cells (Milutinović *et al.* 2019; Huang *et al.* 2023; Gligor *et al.* 2023). Except plants, different animal products and their constituents were examined in this field, like silk of Indian meal moth and spider silk, which show high potential as biomaterials useful in antitumor therapy (Milutinović *et al.* 2020, Kozlowska *et al.* 2017). Venoms, especially those produced in animals, are also an important source of antitumor substances (Roy and Bharadvaya 2020). Bee venom, as one of the most frequently investigated venoms for tumor

therapy, shows anticancer properties (Gajski and Garaj-Vrhovac 2013), including proapoptotic effects in human colon cancer cells (Zheng *et al.* 2015, Nikodijević *et al.* 2019). Some of our previous results about composition of bee venom showed that the most abundant component of bee venom is the lytic peptide - Melittin (Nikodijević *et al.* 2019). Literature data also indicate that Melittin, as a major bioactive component, has the greatest contribution to the bee venom antitumor effects (Gajski and Garaj-Vrhovac 2013, Ceremuga *et al.* 2020).

Our study deal data about antitumor properties of Melittin on HT-29 colon cancer cells – cytotoxicity and induced changes in activated apoptosis- and biotransformation-related pathways under the influence of Melittin compared to untreated cells. Summary of results we tried to show schematically (Figures 4 and 6). Considering the literature reports about the less sensitivity of HT-29 cells compared to other CRC cells (Ikehata *et al.* 2014), the investigation of the mechanisms of action was of great importance. Our results showed that Melittin inhibited HT-29 cell growth and induced significant cytotoxicity based on IC_{50} values for 24 and 72 h. By comparing the observed IC_{50} values of Melittin in HCT-116 and SW-480 cell lines (in range 8.89-10.84 in two investigated periods, 24 and 72 h) (Nikodijević *et al.* 2019), it is evident that the HT-29 cell line is the least sensitive to Melittin. The lower sensitivity of the HT-29 cells to cytostatic in some literature data is associated with a high expression of microRNA 21 (Deng *et al.* 2013). Although Melittin showed higher IC_{50} values in HT-29 cells compared to other CRC lines, it exhibited better cytotoxicity in comparison with commercial cytostatic 5-fluorouracil in the same cell line (Ikehata *et al.* 2014). Previously, it has been reported that Melittin inhibited growth of cells originated from different organs, like U937 cell line from human leukaemia cancer (Moon *et al.* 2008), human prostate cancer cell lines LNCaP, DU145, and PC-3 (Park *et al.* 2011), human ovarian cancer cell lines SKOV3 and PA-1 (Jo *et al.* 2012), MCF-7 and MDA-MB-231 cells from human breast cancer

(Yavari *et al.* 2020), etc. Several mechanisms of action of Melittin in different types of cancer cells were investigated, including inhibition of cancer cell proliferation, induction of apoptosis lysis of cell membrane, etc (Gajski and Garaj-Vrhovac 2013, Zarrinnahad *et al.* 2018). However, the mechanism of action of Melittin remains unexplored in HT-29 cells. In addition to cytotoxicity of Melittin on HT-29 cells, in the first step of investigation we have examined potential selectivity of Melittin related to healthy cells, fetal lung fibroblasts MRC-5. These cells were used as a control only for cytotoxicity, where the results showed the presence of selectivity and higher IC_{50} values compared to that observed in HT-29 cells. Our previously published data also reported selectivity and weaker effect of Melittin on healthy HaCaT cells compared to cancer cell lines (Nikodijević et al., 2019), which we partially confirmed by results on healthy MRC-5 lung fibroblasts, especially in the targeted concentrations that we examined (1 and 5 μg/ml).

Understanding the mechanism of proapoptotic action of Melittin may be of crucial importance for antitumor therapy. The morphological changes that occurred in HT-29 cells under the influence of Melittin mainly correspond to the stages of early apoptosis, rather than late apoptosis and necrosis. An induction of apoptosis, as the most favourable mechanism of action in the cancer treatment, with the complete absence of necrosis, is considered a favourable effect. Moreover, apoptosis was further confirmed by morphological and biochemical hallmarks on gene and protein level. These biomarkers clearly showed that Melittin induced apoptosis via external, death receptor dependent pathway in HT-29 cells (Figure 4). Melittin also induced apoptosis through activation of death receptor on cell membrane and inhibition of JAK/STAT3 pathway in ovarian cancer cells SKOV3 and PA-1 (Jo *et al.* 2012), which corelate to our detected mechanism of proapoptotic activity of Melittin. Acording to our results, the main mechanism of apoptosis induction was due increased the Fas receptor protein expression, *Caspase 8* gene expression and

activity of Caspase 8 in HT-29 cells. On the other hand, Melittin decreased caspase 9 gene and protein expression, which suggested that mitochondria and inner apoptotic pathway are not involved in execution of apoptosis, regardless of changes in expression of Bcl-2 member of protein family in HT-29 cell line. Also, anti-apoptotic gene expression of *Bcl-2* was multi-fold increased in the Melittin treated cells. It prevents activity of pro-apoptotic Bax to form pores on mitochondrial membrane and release pro-apoptotic factors, like cytochrome c (Luo *et al.* 2020). Although, gene expression of *Bax* was increased, the balance between these two parameters (Bax *vs* Bcl-2) is important, where the balance is disturbed in favour of the anti-apoptotic protein. Summary of results indicate to potential mechanism was presented on scheme (Figure 4).

Since Melittin is a substance from venom we further examined whether the induced apoptosis is redox dependent. Many literatures data show prooxidative activity of Melittin, such as induction of increased reactive oxygen species formation and oxidative stress in leukocytes (Stuhlmeier 2007), human hepatoma HepG2 cells (Wang *et al.* 2021), gastric cancer SGC-7901 cells (Kong *et al.* 2016), as well as increased hydroxyl radicals and hydrogen peroxides production in human melanoma cells (Tu *et al.* 2008). Our results showed that Melittin did not significantly change the level of $O_2^{\cdot -}$ and GSH in HT-29 cells, indicating that the Melittin induced redox independent cell death in HT-29 cells. The decreased GSS protein expression indicated that there was not *de novo* synthesis of GSH, assuming that *de novo* synthesis is supressed in HT-29 cell line. Recent findings also reported that Melittin did not affect redox status (catalase and free radicals' content) in Melittin injected mice (El Bakary *et al.* 2020).

Although decreased or unchanged $O_2^{\cdot -}$ and GSH levels suggested absence of oxidative stress, the level of MDA, as sensitive indicator of oxidative stress, was increased in treated HT-29 cells. Gajski *et al.* (2016) suggested that Melittin increased formation of reactive oxygen species,

reduced GSH level, increased lipid peroxidation and phospholipase C activity, showing induction of oxidative stress in human peripheral blood lymphocytes. Also, Han *et al.* (2002) stated that bee venom and Melittin increased lipid peroxides formation in rabbit renal proximal tubule cells. Our data confirmed that Melittin significantly increased the level of MDA, which is well-established marker of oxidative damage of membrane lipids.

The cytotoxic action of Melittin may relate to its lytic property, affinity for cell membrane lysis. As an amphipathic helical peptide, Melittin can incorporate into the phospholipid bilayer, leading to cell membrane disruption and intense cytotoxicity (Ceremuga *et al.* 2020). The affinity of Melittin to disrupt cell membranes is reported on gastric (AGS) and colon cancer cells (COLO205, HCT-15), where it leads to swelling and formation of granulations (Soliman *et al.* 2019). According to the literature, lipid peroxides can be formed under the influence of Melittin as a consequence of its entry in cells, because the integrity of the membrane is disrupted. Namely, the entry of Melittin through the cell membrane may leads to increased production of MDA in cancer cells (A375 and MCF7 cells), compared to untreated cells (Shaw *et al.* 2019).

The presence of upregulated biotransformation process, high activity of biotransformation enzymes, upregulated expression and activity of membrane transporters which eject treatment from the cells may contribute to less sensitivity of HT-29 cells to many treatments. This data supports the fact that HT-29 cells, like many other cell lines originating from colon tissue, may have innate resistance (Ranhotra *et al.* 2016). So, it was of great importance to examine, for the first time, the influence of Melittin on the parameters of biotransformation and drug-efflux in HT-29 cells. Moreover, there is a particular need for inhibition of these parameters, expression and/or their activity, with the aim to reduce the risk of resistance development. The metabolism of drugs in tumour cells determines their future, whether a more or less toxic compound will be formed, as

well as whether it will be retained or ejected from the cell (Gao *et al.* 2013, Housman *et al.* 2014). An overexpression of biotransformation enzymes from phase I (CYP1A1 and CYP1B1), contribute to the hydrolysis, reduction and oxidation to create more soluble forms of xenobiotics (Guengerich 2007), while enzyme in phase II (GSTP1 – glutathione S-transferase pi 1), involved in reactions of glucuronidation, sulfonation, acetylation, methylation, and conjugation with glutathione, can contribute to MDR development (Szakács *et al.* 2006). According to increased expression of all investigated genes related to biotransformation process, it can be assumed that those biotransformation enzymes (*CYP1A1*, *CYP1B1*, and *GSTP1*) are necessary for Melittin metabolism in HT-29 cells. Additionally, *MRP2* was significantly increased, but it is not clear whether Melittin is conjugated with GSH as a substrate for MRP2 transporter and exported (Figure 6). This transporter was increased in Melittin treated HCT-116 cells, too (Nikodijević *et al.* 2019), while it was decreased in SW480 cells.

However, the reduced *MDR1* gene and PGP1 protein expression represents a significant result in terms of possible therapeutic application suggesting potential opportunity for co-treatments with cytostatic that are PGP1 substrates. Inhibition of efflux pumps, i.e. membrane transporters, especially PGP1, is one of the most important strategies for overcoming resistance to cytostatics (Robey *et al.* 2019). Many natural substances, mainly phenols from plants, possess ability to reduce the expression or activity of efflux pumps, which can be used to improve the effectiveness of cytostatic through reducing its elimination and increasing cytostatic retention in the cell (Sjostedt *et al.* 2017, Milutinović *et al.* 2019). Currently, there is a few data on this topic for animal venoms and their components (Nikodijević *et al.* 2019, Nikodijević *et al.* 2021). In study by Nikodijević *et al.* (2019), the authors showed that bee venom and Melittin modulates metabolizing enzymes and export pumps in colon cancer cell lines HCT-116 and SW-480

(Nikodijević *et al.* 2019). The potential of Melittin to enhance efficiency of chemotherapeutics and overcome MDR in colon cancer cells is favourable results and deserve future detailed examination. Future studies should focus on the method of synergistic action of Melittin with other anti-cancer drugs. The results obtained in our study will attract the attention of researchers due to promising therapeutic effects of Melittin.

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Declaration of interest statement

There is no conflict of interests.

Data availability statement

Data will be available on demand.

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Supplementary material

Table S.1 Gene specific primers

	Forward primer	Reverse primer
β-actin	5′-AAGCAGGAGTATGACGAGTCCG-3′	5′-GCCTTCATACATCTCAAGTTGG-3′
Bax	5′-GGACGAACTGGACAGTAACATGG-3′	5′-GCAAAGTAGAAAAGGGCGACAAC-3′
Bcl-2	5′-CTACGAGTGGGATGCGGGAGATG-3′	5′-GGTTCAGGTACTCAGTCATCCACAG-3′
Cas8	5′-AGAGTCTGTGCCCAAATCAAC-3′	5′-GCTGCTTCTCTCTTTGCTGAA-3′
Cas9	5′-GAGTCAGGCTCTTCCTTTG-3′	5′-CCTCAAACTCTCAAGAGCAC-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′
MDR1	5′-GCCTGGCAGCTGGAAGACAAATACACAAAATT-3′	5′-CAGACAGCAGCTGACAGTCCAAGAACAGGACT-3′

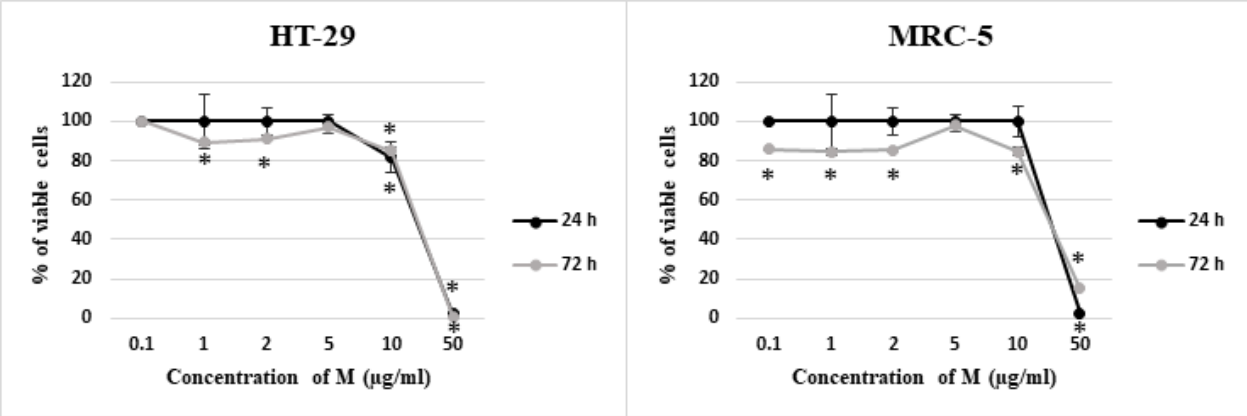

Figure S.1. Impact of Melittin (M) on HT-29 and MRC-5 cell viability, 24 and 72 h after treatment.