

Evaluation of apoptotic cell death mechanisms induced by hypericin-mediated photodynamic therapy in colon cancer cells

Aysun KILIÇ SÜLOĞLU¹, Elif KARACAOĞLU¹, Güldeniz SELMANOĞLU¹,

Hayriye AKEL², İbrahim Çağatay KARAASLAN^{2*}

¹Zoology Section, Department of Biology, Faculty of Science, Hacettepe University, Beytepe Campus, Ankara, Turkey

²Molecular Biology Section, Department of Biology, Faculty of Science, Hacettepe University, Beytepe Campus, Ankara, Turkey

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Abstract: Hypericin (HYP)-mediated photodynamic therapy (PDT) is a new alternative treatment strategy for colon cancer inducing various cell-death pathways. Apoptotic cell death is the desired cellular fate in cancer cells. Therefore, we investigated the apoptotic pathways by determining apoptosis-related proteins (survivin, caspase-3, caspase-9, Bcl-2, and Bax) at the mRNA level using real-time polymerase chain reaction (qPCR), and the percentage of survivin was determined by survivin ELISA in HT-29 and Caco-2 colon cancer cell lines. The downregulation of survivin was significant 16 h after PDT for both cells, while caspase-3 upregulation was apparent 24 h after PDT. Caspase-9 and caspase-3 upregulations were parallel to each other. There was no Bcl-2 expression in HT-29 cells, but we observed downregulation in Bcl-2 expression in Caco-2 cells after HYP photoactivation. The Bax expression downregulated significantly after 24 h incubation in HT-29 cells, while it was upregulated after 16 h incubation in Caco-2 cells. The present study provides evidence that HYP-induced alterations in apoptosis-related protein expression ended in different responses in HT-29 and Caco-2 colon cancer cells, and these may be used in the treatment of chemotherapy-resistant cancer types.

Key words: Hypericin, PDT, survivin, apoptosis, HT-29 cells, Caco-2 cells

1. Introduction

Colorectal cancer is a prominent death factor and the third most common cancer worldwide (Jemal et al., 2011). Early diagnosis and efficient treatment are significant for patient survival. Surgical resection is the main curative option for patients with metastatic type of colon cancer, but survival rates of patients with stage IV disease are fairly low after surgical resection (Fong et al., 1997; Gaston and Giacomantonio, 2014). Since treatment of severe cancer types with conventional methods is not possible, new strategies become important for diagnostic methods and patient survival (Fournier and Gordon, 2000). Photodynamic therapy (PDT) is one good candidate for new treatment strategies. PDT involves photosensitizer (PS), irradiation with a wavelength appropriate for PS, and molecular oxygen (Agostinis et al., 2011). Molecular oxygen is converted into singlet oxygen (1O_2) and other cytotoxic oxygen species, such as hydroxyl radical (OH^*) and superoxide anion radical (O_2^-). The type and dose of PS, the light source, and its intensity and cell type are important factors for determining the success of PDT. Following treatment with PDT, cell destiny leads to repair

of the damage, autophagy or apoptosis, and necrosis (Krammer and Verwanger, 2012).

Hypericin (HYP) is a natural PS extracted from *Hypericum perforatum*. Due to its low cytotoxicity and selective antitumor features, HYP has been used for the treatment of tumor tissues (Agostinis et al., 2002; Kiesslich et al., 2006; Barathan et al., 2013). It has been suggested that after treatment of HYP-mediated PDT, cells move towards cellular pathways according to the concentration of treated HYP. Low-dose concentrations of HYP induce growth stimulation via the p38 or JNK survival pathways. Unlike the effect of a low dose, a high dose of HYP leads to activation of stress-response pathways that trigger apoptosis or autophagic cell death. If the damage is too severe, the cell dies necrotically (Krammer and Verwanger, 2012).

In order to identify the correct HYP dose and irradiation time, appropriate marker genes should be monitored during the treatment process. Survivin is an anti-apoptotic protein that is a member of the inhibitor of apoptosis proteins (IAP) family (Deveraux and Reed, 1999). It is selectively expressed in tumor versus normal

* Correspondence: cagatayk@hacettepe.edu.tr

tissues (Jiang et al., 2001). This protein plays an important role in the regulation of cell division and suppression of apoptosis and is also involved in cellular processes such as cellular stress response, chemoresistance, and angiogenesis (Altieri, 2006). Survivin inhibits caspase proteins such as caspase-3, caspase-7, and caspase-9. In this way, apoptosis is prevented and tumor cell survival increases (Chu et al., 2010; Hernandez et al., 2011). Increased expression of the survivin protein is associated with tumor resistance to chemotherapeutic drug treatment, poor prognosis, and decreased patient survival (Ferrario et al., 2007). Therefore, survivin protein is a good candidate for monitoring tumor progression.

Caspase-3 is an executioner caspase that catalyzes the cleavage of many cellular proteins, and this cleavage is followed by apoptosis-related cellular alterations. Caspase-3 is a fundamental protein frequently used to investigate apoptotic pathways (Devarajan et al., 2002). During apoptosis, cytochrome-c (cyt-c) releases from mitochondria into the cytosol and induces the caspase activation pathway starting with procaspase-9 activation. This leads to activation of procaspase-3 and cell death (Hostanska et al., 2003). It is commonly accepted that Bcl-2 and Bcl-XL, which are members of the BCL-2 family, inhibit the release of cyt-c, while proapoptotic members such as Bid, Bax, and Bad facilitate the release of cyt-c. Therefore, decreasing the expression of Bcl-2 is a way of increasing the activity and sensitivity of PDT in particular cell lines (Fadeel et al., 1999).

In this study, we aimed to evaluate the apoptotic cell death mechanisms in HT-29 and Caco-2 cells after HYP-mediated PDT. For this purpose, survivin, caspase-3, caspase-9, Bcl-2, and Bax gene expressions and the levels of survivin in cell lysates were measured.

2. Materials and methods

2.1. Cell culture conditions and PDT procedure

HT-29 (passage numbers 7–10) and Caco-2 (passage numbers 13–20) cells were obtained from the Foot and Mouth Disease Institute (HÜKÜK), Ankara, Turkey. The cells were maintained in Dulbecco's Modified Eagle medium (DMEM) (HyClone Laboratories, Inc., Logan, UT, USA) supplemented with 10% fetal bovine serum (PAA Laboratories, Linz, Austria) at 37 °C and 5% CO₂ in a humidified incubator. The cell lines were routinely tested for mycoplasma and other contamination throughout the experiment and only used if they were contamination-free. HYP HPLC grade (AppliChem, Germany) was prepared as a stock solution in dimethyl sulfoxide (DMSO) (final concentration, <0.1%). HYP concentrations were chosen according to results of a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity test (Kılıç

Süloğlu et al., 2015). The cells were incubated with 0.04, 0.08, and 0.15 µM HYP concentrations in dark conditions for 24 h. Prior to irradiation, the medium containing HYP was replaced with fresh HYP-free DMEM. A dark control group at 0.15 µM HYP incubation without irradiation was also included. The irradiation device consisted of twelve L18W/830 fluorescent tubes (Osram, Berlin, Germany) with maximum emission in the range 530–620 nm and included 8 fans to prevent overheating. The light dose was measured with TES 1335 luxmeter (Rotronic, Taipei, Taiwan). HYP was activated by light at a total dose of 4 J/cm². The groups are control group (no HYP treatment but photoactivated), DMSO group (DMSO treatment only and photoactivated), and HYP treatment groups (0.04, 0.08, and 0.15 µM HYP treatment and photoactivated). As the control group and DMSO group had no differences in any measured parameters, DMSO group data were not shown. Analysis was performed 16 h and 24 h after irradiation, and the experiments were performed in triplicate.

2.2. Total protein assay

HT-29 and Caco-2 cells were seeded at a density of 75 × 10⁴ viable cells per 75 cm² culture flask for control and HYP treatment groups. After HYP activation, cells were washed with ice-cold phosphate-buffered saline (PBS), scraped off, and solubilized in ice-cold RIPA buffer containing 25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS (Pierce Biotechnology 89901, Rockford, IL, USA) with protease inhibitor cocktail (Roche 11873580001, Mannheim, Germany). Cell lysates were centrifuged at 13,000 rpm for 30 min, and supernatant was separated and stored at –80 °C. Protein concentrations of cell lysates were measured at 562 nm with a microplate spectrophotometer (µQuant, Bio-Tek Instruments, Inc., Winooski, VT, USA) and bicinchoninic acid protein analysis kit (Pierce Biotechnology 23227, Rockford, IL, USA).

2.3. Survivin protein measurement

Survivin levels in cell lysates were quantified using a sandwich enzyme-linked immunosorbent assay kit (ELISA) according to the manufacturer's guidelines (Cell Signaling Technology, Danvers, MA, USA). Equal amounts of protein samples were loaded into survivin-antibody-coated wells, and absorbances were read at 450 nm with a microplate spectrophotometer (µQuant, Bio-Tek Instruments, Inc., Winooski, VT, USA). Data was expressed as percentage of survivin.

2.4. RNA isolation and cDNA synthesis

Total RNA was isolated from HT-29 and Caco-2 cells using an RNeasy Mini kit (Qiagen, Valencia, CA, USA). First-strand cDNA was synthesized from 0.5 µg of RNA with the Superscript III First-Strand Synthesis system (Invitrogen, Carlsbad, CA, USA) using 0.5 µg of oligo-dT. Real time-

PCR experiments were performed on the ABI 7500 Fast Real Time-PCR system and with a Quantifast SYBR Green PCR kit. PCR conditions were as follows: PCR initial heat activation at 95 °C for 5 min followed by 40 cycles of denaturation at 95 °C for 10 s and combined annealing–extension at 60 °C for 30 s. All reactions were performed in triplicate and repeated at least 2 times. Cyclophilin A gene (PPIA) was used as an internal reference to normalize the target transcripts using the $2^{-\Delta\Delta CT}$ method. Real-time PCR primers designed for PPIA, survivin, Bax, and caspase-3 are listed in the Table. The other primers for caspase-9 (Hs CAS9 1 SG QuantiTect Primer assay) and Bcl-2 (Hs BCL2 1 SG QuantiTect Primer assay) were obtained from Qiagen (Valencia, CA, USA). Data were given as the relative expression ratio (gene expression/reference gene expression).

2.5. Statistical analysis

Statistical analysis was performed using SPSS 22 for Windows. Data were expressed as mean \pm standard error (SE), and statistical significance was assigned at the $P \leq 0.05$ level. The homogeneity of variance and normal distribution between groups were evaluated by general linear model procedure and Kolmogorov–Smirnov nonparametric test. Parameters were analyzed by one-way ANOVA. To identify the sources of significant main effect, post hoc comparisons (Tukey) were used (Sokal et al., 1995).

3. Results and discussion

There have been limited reports to provide a relationship between gene expressions of apoptosis-associated proteins such as survivin, Bax, Bcl-2, caspase-3, and caspase-9 after response to HYP-mediated PDT in Caco-2 cells. The aim of this study was to evaluate these proteins in order to predict HYP resistance of colon cancer cells and to determine modifications in their mRNA expressions. There were several reports about HYP photoactivation and results in HT-29 cells, but in the current study we investigated this in Caco-2 cells for the first time.

Based on the MTT cytotoxicity assay, we determined the HYP concentrations of 0.04 μ M, 0.08 μ M, and 0.15 μ M. These concentrations caused 20%–42% inhibition of metabolic activity and induced a significant increase in apoptotic index in HT-29 and Caco-2 cells. The percentages of inhibition were 82.54 ± 1.57 (0.04 μ M HYP), 68.85 ± 4.88 (0.08 μ M HYP), and 57.09 ± 5.63 (0.15 μ M HYP) after 16 h incubation and 81.98 ± 2.35 (0.04 μ M HYP), 66.64 ± 3.96 (0.08 μ M HYP), and 55.51 ± 1.67 (0.15 μ M HYP) after 24 h incubation in HT-29 cells. For Caco-2 cells the percentages of inhibition were 84.57 ± 4.02 (0.04 μ M HYP), 71.61 ± 5.30 (0.08 μ M HYP), and 61.50 ± 3.43 (0.15 μ M HYP) after 16 h incubation and 87.66 ± 3.95 (0.04 μ M HYP), 74.83 ± 1.45 (0.08 μ M HYP), and 65.01 ± 1.27 (0.15 μ M HYP) after 24 h incubation (Kılıç Süloğlu et al., 2015). In this study, further analysis was performed in order to explain apoptotic mechanisms and related protein alterations due to HYP photoactivation. We have studied the effect of HYP-mediated PDT on survivin, caspase-3, caspase-9, Bcl-2, and Bax gene expressions and cellular levels of survivin and total protein content.

The protein assay measures the protein content of viable cells, which is an indirect measurement of cell viability, and evaluates cell toxicity with supporting other cytotoxicity assays (Fotakis and Timbrell, 2006). According to Kılıç Süloğlu et al. (2015), 0.04, 0.08, and 0.15 μ M HYP induced cytotoxicity in HT-29 and Caco-2 cells. In this study, the decrease in protein content depending on HYP concentration was parallel to increased cytotoxicity. The protein content decreased substantially in 0.04, 0.08, and 0.15 μ M HYP groups of HT-29 cells 24 h after PDT (Figure 1A). In Caco-2 cells, the protein content also decreased in HYP groups compared to the control group. In contrast to HT-29 cells, the protein content increased after 24 h incubation in Caco-2 cells except in the 0.15 μ M HYP group, which indicated higher cell number and lower cytotoxicity in these groups (Figure 1B).

Survivin (BIRC5) is a member of the IAPs family that is expressed in many different cancer cell types (Coumar

Table. Real-time primer sequences.

Gene	Real-time primer sequences
PPIA	Forward: 5'- GGTCCTCAAAGACAGCAGAAA -3' Reverse: 5'- GTCACCACTCTGACACATAAA -3'
Survivin	Forward: 5'- CCACCGCATCTCTACATTCA -3' Reverse: 5'- CCAAGTCTGGCTCGTTCTC -3'
Bax	Forward: 5'- TTCTGACGGCAACTTCAACT -3' Reverse: 5'- CAGCCCATGATGGTTCTGAT -3'
Caspase-3	Forward: 5'- CTCTGGAATATCCCTGGACAAC -3' Reverse: 5'- ACATCTGTACCAGACCGAGA -3'

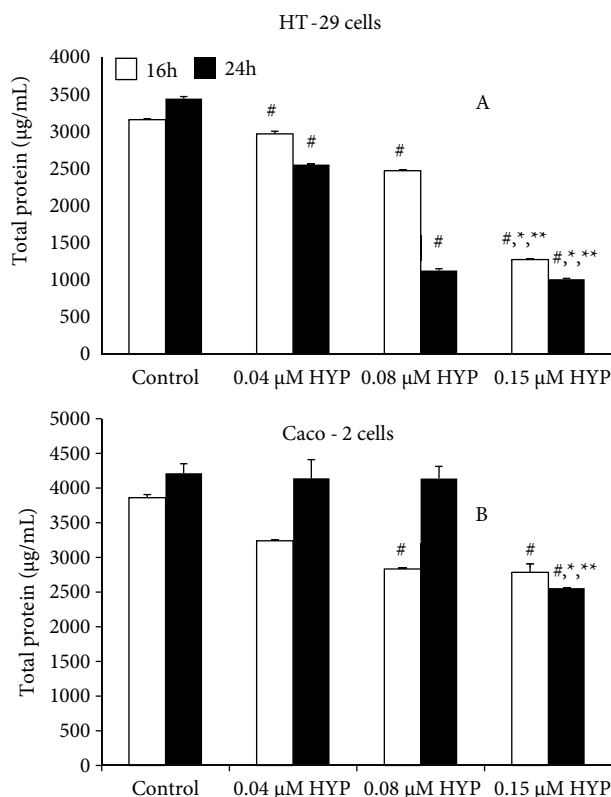


Figure 1. The amount of total protein (µg/mL) after photoactivation of HYP for 16 h and 24 h in A) HT-29 and B) Caco-2. Data is expressed as mean ± SE from three independent experiments performed in triplicate. Significantly different from #: control group, *: 0.04 µM HYP group, **: 0.08 µM HYP group.

et al., 2013). Survivin contributes several processes related to the regulation of mitosis, apoptosis, and cellular stress response, but it cannot inhibit caspases directly (Mikešová et al., 2013). Survivin prevents apoptosis by contributing to the inhibition of caspase-3 and caspase-7. Survivin is also expressed in transformed cell lines in a cell-cycle-dependent manner in order to regulate the cell cycle in G2 and M phases (Suzuki et al., 2000). Previous studies by Kılıç (2012) revealed that HYP-mediated PDT induced G2/M arrest in the cell cycle of HT-29 and Caco-2 cells, which might be related to fluctuation in survivin expression. However, they did not investigate the alterations in mRNA and protein levels of survivin expression after HYP photoactivation.

In this study, alterations in percentage of survivin in HT-29 and Caco-2 cell lysates after PDT with HYP at 16 h and 24 h incubation are shown in Figure 2. No significant differences were observed among the control, DMSO, and dark control groups. According to the results, percentage of survivin decreased in a dose- and incubation-time-dependent manner in both HT-29 and Caco-2 cells. In

HT-29 cells, percentage of survivin in the 0.04, 0.08, and 0.15 µM HYP groups decreased significantly compared to the control group for 16 h and 24 h incubation. In HT-29 cells, percentage of survivin in the 0.08 µM HYP group diminished significantly compared to the control and 0.04 µM HYP group 16 h after PDT (Figure 2A). Similarly, for 24 h incubation, percentage of survivin in all dose groups decreased compared to the control group. In Caco-2 cells, percentage of survivin in the 0.08 and 0.15 µM HYP groups decreased significantly compared to the control group at both 16 h and 24 h incubation (Figure 2B). Additionally, the 0.15 µM HYP group was significantly different from the 0.04 µM HYP group 24 h after PDT.

In order to evaluate cell-death pathways, we performed qPCR to investigate gene expressions of survivin, caspase-3, caspase-9, Bcl-2, and Bax. According to the results, HYP photoactivation induced a decrease in survivin expression in HT-29 cells. The decrease was statistically significant in 0.08 and 0.15 µM HYP groups compared to the control after 16 h and 24 h with the exception of upregulation in 0.08 µM HYP after 24 h (Figure 3A). The decline in

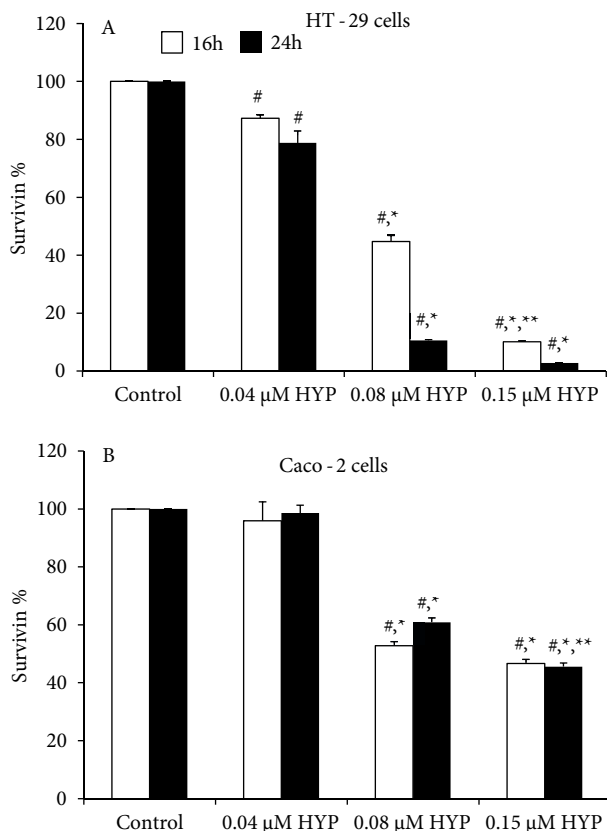


Figure 2. The percentage of survivin after photoactivation of HYP for 16 h and 24 h in A) HT-29 and B) Caco-2 cells. Data is expressed as mean ± SE from three independent experiments performed in triplicate. Significantly different from #: control group, *: 0.04 µM HYP group, **: 0.08 µM HYP group.

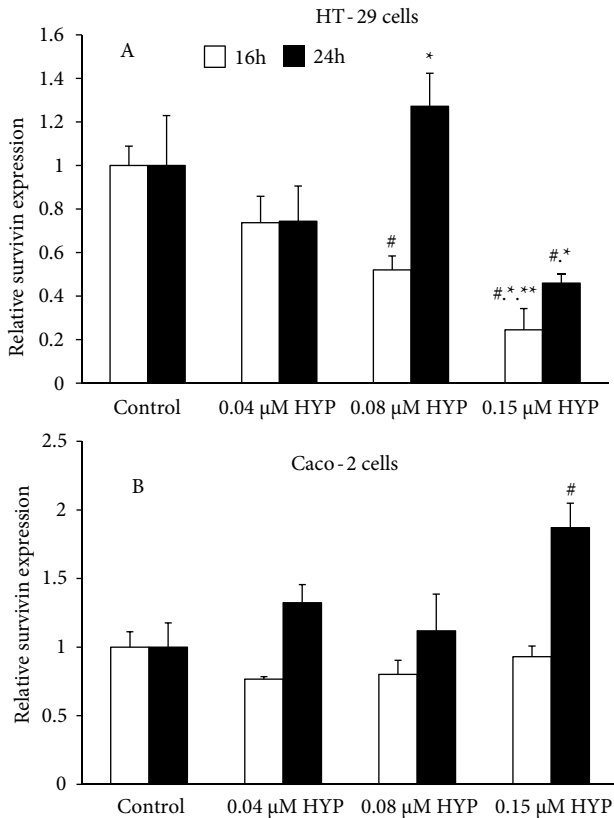


Figure 3. Relative expression ratio of survivin in A) HT-29 and B) Caco-2 cells determined by real-time reverse transcription PCR. Data is expressed as mean \pm SE from three independent experiments performed in triplicate. Significantly different from #: control group, *: 0.04 μ M HYP group, **: 0.08 μ M HYP group.

survivin expression was apparent in HT-29 cells for both 16 h and 24 h incubation, while in Caco-2 cells survivin expression started to increase after 24 h incubation. However, only the increase in the 0.15 μ M HYP group was statistically significant compared to the control after 24 h incubation (Figure 3B). The decrease in survivin expression was apparent in HT-29 cells for both 16 h and 24 h incubation, while in Caco-2 cells survivin expression started to increase after 24 h incubation. This elevation in Caco-2 cells was in concordance with previous results in which we found that the apoptotic index decreased after HYP photoactivation in Caco-2 cells after 24 h incubation. Interestingly, the apoptotic index decreased suddenly in the 0.08 μ M HYP group in HT-29 cells, which may be due to the fact that the cells started to resist apoptosis and tried to survive.

Caspases have been found as inactive proenzymes in the cell. Whenever the cell receives an apoptosis signal, procaspases become activated. The caspase family has 14 members in mammals, including caspases -2, -8, -9, and -10 as initiators and caspases -3, -6, and -7 as executioners

(Hostanska et al., 2003). Poly (ADP-ribose) polymerase (PARP) fragmentation, which is an indication of activation of the effector caspase-3, was the most important step during HYP-mediated cell death in cervical HeLa cells (Assefa et al., 1999). PARP cleavage was also observed in our study, in both HT-29 and Caco-2 cells, after HYP photoactivation (data not shown). It was reported that HYP caused increased caspase-9 and caspase-3 activity in leukemia U937 cells (Hostanska et al., 2003). Similarly, our observations suggest that caspase-9 activates caspase-3 and initiates apoptotic cell death in HT-29 and Caco-2 cells.

According to these results, relative gene expression of caspase-3 was downregulated after 16 h incubation and upregulated after 24 h incubation, especially in the 0.04 and 0.08 μ M HYP groups in HT-29 cells. They were statistically significant, and the results were compatible with each other (Figure 4A). The apparent increase in caspase-3 and caspase-9 gene expressions in 0.08 μ M HYP group indicated induced apoptotic cell death, while the decrease in expression of these genes likely pointed to necrotic cell death due to increased HYP concentration. In Caco-2 cells, upregulation of the caspase-9 gene relative

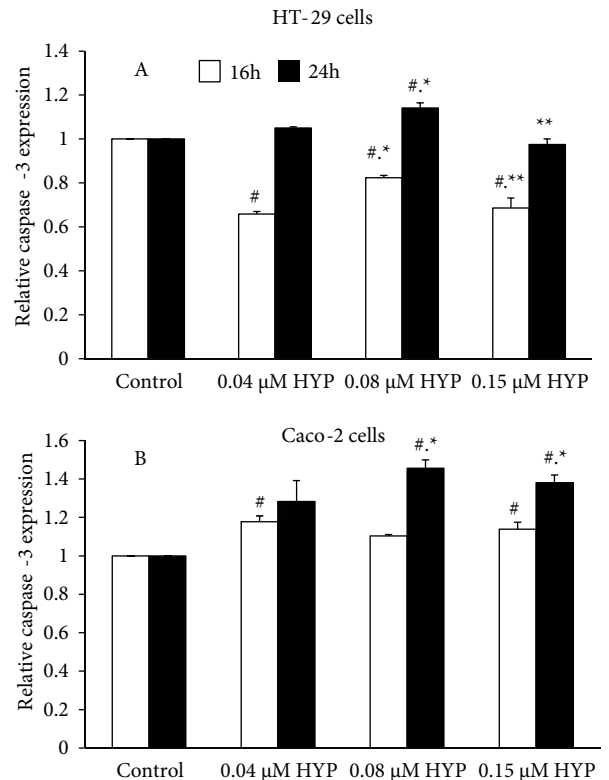


Figure 4. Relative expression ratio of caspase-3 in A) HT-29 and B) Caco-2 cells determined by q-PCR. Data is expressed as mean \pm SE from three independent experiments performed in triplicate. Significantly different from #: control group, *: 0.04 μ M HYP group, **: 0.08 μ M HYP group.

expression was parallel to the increase in caspase-3 expression, depending on the HYP concentration after 16 h and 24 h incubation. In the 0.15 μM HYP group the increase was statistically significant compared to the control group after 16 h and 24 h incubation, and the 0.08 μM HYP group was different from the control and 0.04 μM HYP groups after 24 h incubation (Figure 4B). Caspase-9 expression was downregulated after 16 h incubation and upregulated after 24 h incubation in HT-29 cells (Figure 5A). Additionally, a statistically significant increase in caspase-9 expression was observed in the 0.15 μM HYP group compared to the control and 0.04 μM HYP groups after 16 h in Caco-2 cells (Figure 5B).

Induced cell-death type can be influenced by the genetic basis of a tumor cell. For example, due to the overexpression of anti-apoptotic Bcl-2 protein, which is expressed in more than half of all cancer types, resistance may have occurred after HYP-mediated PDT treatment, and balance may tend toward necrotic cell death (Reed, 1998). Therefore, decreasing the expression of Bcl-2 is a way to increase the activity and sensitivity of PDT in particular cell lines (Fadeel et al., 1999). In a previous study,

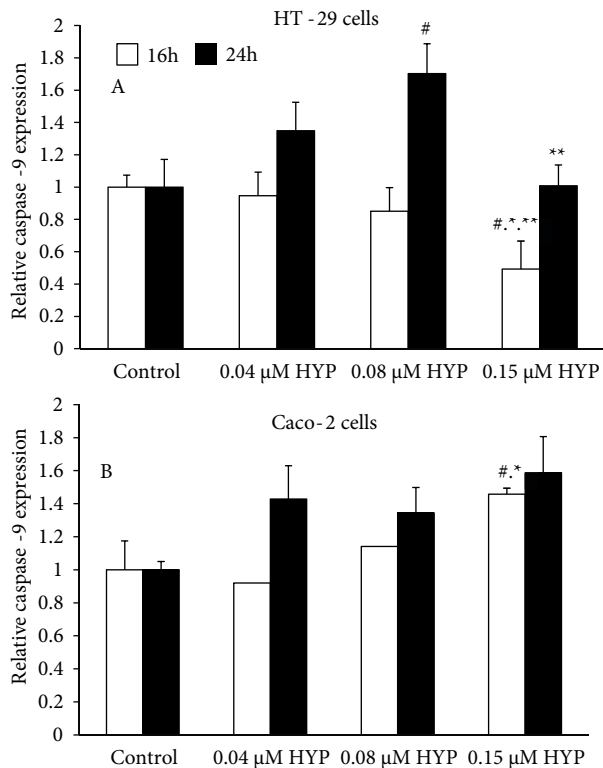


Figure 5. Relative expression ratio of caspase-9 in A) HT-29 and B) Caco-2 cells determined by q-PCR. Data is expressed as mean \pm SE from three independent experiments performed in triplicate. Significantly different from #: control group, *: 0.04 μM HYP group, **: 0.08 μM HYP group.

it was revealed that HT-29 cells with no Bcl-2 expression increased anti-apoptotic protein Bcl-XL expression after HYP-mediated PDT (Mikes et al., 2007). Our findings also support the results showing HT-29 cells do not express Bcl-2 gene. On the other hand, Bcl-2 expression increased in Caco-2 cells when they were confluent under in vitro conditions (Gauthier et al., 2001). Similarly, we observed Bcl-2 expression in Caco-2 cells. To our knowledge, this is the first study showing the HYP-mediated PDT affected the Bcl-2 expression in Caco-2 cells. The downregulation of Bcl-2 expression was statistically significant in the 0.04 and 0.08 μM HYP groups, compared to the control, for 24 h incubation after HYP phototactivation. In addition, the decrease was important in the 0.15 μM HYP group, compared to all other groups, for 16 h incubation time (Figure 6).

In colon cancer cells, the ratios of anti-apoptotic protein Bcl-2 and proapoptotic proteins (Bak and Bax) dominate the apoptotic potential of a cell. Bcl-2 upregulation is related to the worst prognosis in colorectal tumorigenesis, while Bax expression tumors have a better prognosis (Cerdea et al., 2006). Suppressed Bcl-2 and induced Bax expression are associated with altered apoptotic profiles in Caco-2 cells (Cerdea et al., 2006). Therefore, decreasing Bcl-2 and increasing Bax, could be a significant target in PDT in order to increase survival rates for colon cancer.

According to our results, in HT-29 cells downregulation of Bax expression was statistically significant in the 0.04, 0.08, and 0.15 μM HYP groups, compared to the control group, after 24 h incubation. In addition, the upregulation of Bax expression in the 0.04 μM HYP group after 16 h incubation was not statistically significant (Figure 7A). Caco-2 cells responded differently to PDT; Bcl-2 expression decreased while Bax expression increased, especially in the 0.15 μM HYP group for 16 h and 24 h incubation time (Figure 7B).

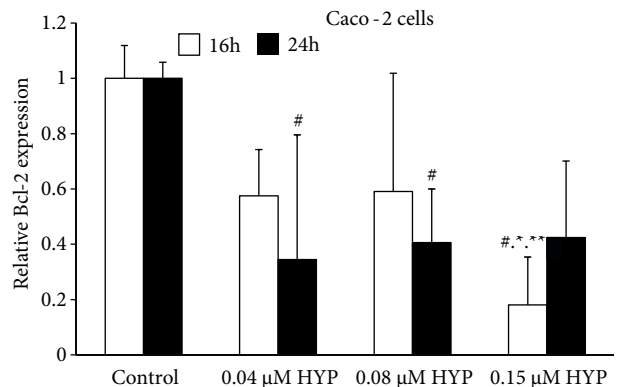


Figure 6. Relative expression ratio of bcl-2 in Caco-2 cells determined by q-PCR. Data is expressed as mean \pm SE from three independent experiments performed in triplicate. Significantly different from #: control group, *: 0.04 μM HYP group, **: 0.08 μM HYP group.

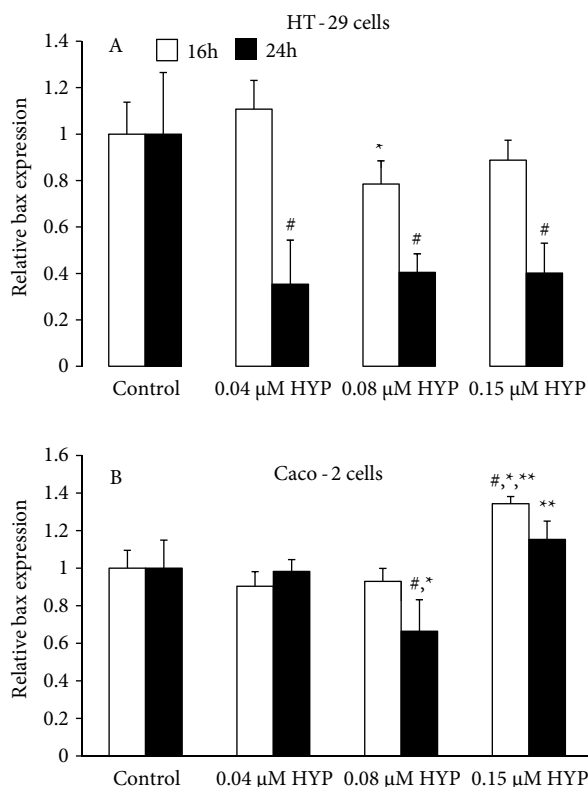


Figure 7. Relative expression ratio of Bax in A) HT-29 and B) Caco-2 cells determined by q-PCR. Data is expressed as mean \pm SE from three independent experiments performed in triplicate. Significantly different from #: control group, *: 0.04 μ M HYP group, **: 0.08 μ M HYP group.

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In conclusion, observed gene expression alterations related to apoptosis in HT-29 and Caco-2 cells may result in different responses to HYP-mediated PDT, development of resistance, or activation of repair mechanisms. Additionally, these changes may help to determine the type and time of cell death. We observed for the first time the different actions of Caco-2 cells faced with HYP-mediated PDT in apoptotic pathways. Up- and down-regulations of anti-apoptotic survivin gene expressions are comparable with each other after HYP photoactivation, as expected. As a limitation of this study, it is difficult to say that survivin and other apoptosis-associated gene expressions are directly related to each other after HYP photoactivation. The alterations in response to HYP activation in two colon cancer cell lines may be due to cells that have developed resistance to apoptosis and their tendency towards necrotic cell-death or the activation of caspase-independent mechanisms. Further investigations are essential to shed light on the details of cell-death mechanisms induced by HYP photoactivation.

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