Effects of hypericin-mediated photodynamic therapy on GM-CSF, MIF, VCAM-1 and ICAM-1 secretion in colorectal cancer cells in vitro

Anna Międzybrodzka 1, Aleksandra Kawczyk-Krupka 1, David Aebisher 2, Grzegorz Cieślar 1, Zenon Paweł Czuba 3

1 Department of Internal Medicine, Angiology and Physical Medicine, Center for Laser Diagnostics and Therapy, Faculty of Medical Sciences in Zabrze, Medical University of Silesia, Katowice, Poland
2 Department of Photomedicine and Physical chemistry, Faculty of Medicine, University of Rzeszow, Rzeszow, Poland
3 Department of Microbiology and Immunology, School of Medicine with the Division of Dentistry in Zabrze, Medical University of Silesia in Katowice, Zabrze, Poland

ABSTRACT

Introduction and aim. Photodynamic therapy with hypericin (HYP-PDT) is gaining importance as a potential treatment method for malignant neoplasms. The following study investigated whether HYP-PDT influences the secretion of certain factors of colon cancer cells in vitro.

Material and methods. Two colon cancer cell lines were used in this experiment: SW480 and SW620. The cells were properly prepared and then treated with photodynamic therapy with hypericin at concentrations of 0.25 µM and 0.5 µM and irradiation at the doses of 1 J/cm², 5 J/cm² and 10 J/cm². After using HYP-PDT, changes in the concentrations of four factors: GM-CSF, MIF, VCAM-1 and ICAM-1 were analyzed in the test tubes.

Results. In the case of SW480 cells: a notable decrease in GM-CSF, MIF, VCAM-1 and ICAM-1 secretion was noted after HYP-PDT. In the case of SW620 cells, after HYP-PDT: no effect on GM-CSF secretion was noted; it inhibited the secretion of VCAM-1 and MIF and increased the secretion of ICAM-1.

Conclusion. Photodynamic therapy with hypericin shows immunomodulatory potential in in vitro cell line experiments. This may indicate its possible ability to modify the course of malignant tumors and therefore requires further research.

Keywords. colorectal cancer, hypericin, photodynamic therapy

Introduction

Malignant tumors are the second leading cause of death, after cardiovascular diseases, for people worldwide. In 2020, nearly 2 million cases of colorectal cancer were diagnosed and 935,173 deaths from the disease were registered. Colon cancer ranks as the third most common malignancy in men after lung and prostate cancer and second after breast cancer among women. Thorough understanding of the biology and immunology of tumors and the mechanisms of cancer progression is crucial for the treatment process based on the selection of an appropriate therapy for a given patient, taking into account the total risk, type of molecular changes in tumour cells and effective biotransformation of the applied drug in a given patient. Knowing that the growth of cancerous tumors is controlled by a number of processes occur-
ring in the tumour niche as well as systemically, it is possible to conduct research using substances capable of influencing immunological processes. Tumour progression factors include many physiologically occurring proteins such as chemokines, cell adhesion factors, vascular growth factors and many other factors involved in promoting tumour growth, cell proliferation and angiogenesis. Furthermore, genetic mutations and disruption of many cell signalling pathways are crucial in tumour genesis and metastasis. These mechanisms lead, among others, to chronic inflammation, which within the tumour mass, together with local immune system activity, are elementary factors in tumour development and disease progression.1,2

Photodynamic therapy (PDT), which has already been proven effective in the treat-ment of skin, pancreatic and bladder cancer, as well as non-cancerous diseases such as autoimmune disorders (RA, SLE, psoriasis), acne vulgaris, macular degeneration, and infectious diseases, is one of the promising methods in cancer treatment.3-13 PDT is a relatively inexpensive and simple procedure, burdened with only few side effects and well tolerated by patients.14 It is based on an intracellular reaction occurring as a result of an interaction between three factors: a photosensitive substance, light and oxygen. As a result of absorption of light of a specific wavelength by a photosensitive sub-tance, its transition from the basic state to the excited triplet state takes place, which results in the transfer of a hydrogen atom or an electron from the photosensitizer to the substrate with formation of free radicals that react with oxygen. As a result, reactive oxygen species are formed (hydrogen peroxide, superoxide anion radicals, hydroxyl radicals) - type I reaction, or reaction of photosensitizer with triplet oxygen leading to singlet oxygen - type II reaction. PDT has a direct cytotoxic effect leading to cell necrosis or apoptosis, as well as stimulating the immune system and promoting inflammation.15-20 Effects on the secretory function of tumour cells, thus proliferation, angiogenesis and metastasis formation, is another interesting potential action of PDT.21-24 Among many photosen-sitizers used in PDT, hypericin (HYP), which is a natural plant pigment present in and extracted from certain plants of the genus Hypericum, most commonly St. John’s Wort, is of great interest. Due to its chemical structure, it is classified as an anthraquinone deriva-tive and belongs to naphthodianthrones. The chemical structure of hypericin is shown in Figure 1.

Hypericin is characterized by intensive light absorption at wavelengths above 550 nm, higher affinity for tumour tissues compared to normal tissue, insignificant toxicity in the dark and induces a strongly photocytotoxicity effect in tumour cells, with the strength of this cytotoxicity depending on the concentration of hypericin and the intensity of light and the presence of oxygen. To date, the mechanisms underlying the selective accumulation of hypericin in tumour tissue have not been fully understood, nor have all the mechanisms of its anticancer effects been clearly defined.25-26 The first reports on the photo-sensitizing properties of hypericin and the possibility of using it in photodynamic therapy and diagnostics date back to the 20th century.27-28 The first local application of hypericin as a photosensitizer in photodynamic therapy was described in 1996 in a patient with pleural mesothelioma.29 Promising effects in terms of inhibition of proliferation and destruction of tumour cells following hypericin-mediated photodynamic therapy have been observed among others in glioma, cutaneous melanoma, leukemias, breast, liver, and colorectal cancer.6,10-37

**Fig. 1. Hypericin – chemical structure**

**Aim**

Based on literature, author focused on determining the effects of HYP-PDT on colon cancer cells – showing the effectiveness of the therapy after using cytolethal doses and determination of the influence on selected molecular markers responsible for progression (growth, invasion and metastatic tumor) secreted by the cancer cells that have not been destroyed by HYP-PDT.

**Material and methods**

**SW480 and SW620 cell lines**

Two colorectal cancer cell lines SW480 and SW620, purchased from American Type Cell Culture (ATCC LGC Limited. Queens Road, Teddington, Middlesex, TW11 0LY, UK) in a frozen state, were used for the experiment. The SW480 cell line was derived from a primary adenocarcinoma in a 50-year-old Caucasian male. The cells were classified as type B in the Dukes classification, and show expression of carcinoen-associat-ed antigen (CEA), transforming factor TGF-P and the oncogenes myc, myb, ras, fos, sis, p53, abl, ros, Sr. The cell line SW620 was obtained from the same man, but the cells were collected one year later from a metastatic colorectal cancer lymph node. These cells are characterized by metastatic activity. They were classified as
Dukes' type C, and show expression of carcinogen-associated antigen (CEA) and the oncogenes: myc, myb, ras, fos, sis, p53, abl, ros, src.

Cell culture
The SW480 and SW620 cells were cultured in Leibovitz's L-15 medium supplemented with 10% inactivated fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin. Cultures were maintained in 25 and 75 cm² plastic bottles and incubated at 37°C with 100% humidity in a carbon dioxide-free atmosphere. The cells were grown in monolayer as adherent cells. The growth medium was changed 2-3 times per week. The prepared cells were resuspended in culture medium bringing the suspension to the desired density, calculated experimentally beforehand for both cell lines. The determined density was 5x10⁵/ml for SW480 cells and 2.5x10⁵/ml for SW620 cells. The cell suspension was then loaded into 96-well plates and incubated at 37°C under 100% humidity for 24 hours.

Incubation of cells with hypericin
After 24-hour incubation of the test cells, the growth medium was removed, the cells were washed with calcium and magnesium free PBS solution. They were then treated with hypericin (Hypericin Calbiochem) at 0.1 µM, 0.25 µM, 0.5 µM and 1 µM for 1 hour. After incubation with hypericin, the medium was removed, the cells were washed with calcium and magnesium free PBS twice, and then complete growth medium was added.

Exposure of cells to light
In the next stage of the experiment, cells were exposed to VIS visible light (400-750 nm) from an incoherent light source PDT TP-1 (Cosmedico Medizintechnik GmbH, Schwenningen, Germany) equipped with infrared filters and an orange filter, light emission in the 600-720 nm wavelength range was obtained. The additional filter was used to determine mitochondrial dehydrogenase activity. MTT was found to be membrane impermeable. These and other results suggest that MTT is taken up by cells through endocytosis and that lowered MTT formazan accumulates in the endosomal/lysosomal compartment and is then transported to the cell surface through exocytosis. Only living cells have the ability to reduce the yellow 3-[4,5- dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide to water-insoluble formazan. The reaction takes place in the cytoplasm with the participation of NADH/NAD⁺, NADPH/NADP⁺. A diagnostic kit from Sigma Chemical Co. (St. Louis, MO, USA) was used to determine mitochondrial dehydrogenase activity. After removal of the supernatant, culture medium with MTT at a concentration of 0.5 µM was added to the adhered cells. After removal of the medium, the resulting water-insoluble formazan was extracted with 100% DMSO. The microplates were shaken for 10 min and centrifuged. Then 150 µL of the supernatant with dissolved formazan was transferred to a 96-well polypropylene flat bottom plate. The absorbance of the extracted formazan was deter-mined at 550 nm vs. DMSO using an ELx 800 microplate reader (BioTek, Winooski, USA). From the results obtained, the percentage of living cells (viability) was calculated according to the formula:

\[ \text{Viability\%} = \frac{Ab \times 100\%}{Ak} \]

where:
\( Ab \) = absorbance of test sample; \( Ak \) = absorbance of control sample

Cellular uptake of hypericin
Cellular uptake of hypericin was assessed using an Olympus IX51 inverted microscope with a reflected fluorescence system (Olympus Corp) and a Color View III digital camera with Cell F imaging software (Soft Imaging System GmbH). 100 % DMSO was used in the stock solution of hypericin and hypericin is then dissolved in cell culture media at 0.1%. In the experiment, the intra-cellular fluorescence intensity of Hyp was deter-mined as a function of time with a flow cytometer (Becton Dickinson, LSR II) using the PerCP channel. A 488 nm excitation laser was used to stimulate Hyp fluorescence, and Hyp fluorescence emission was recorded at 600 nm.

The levels of colorectal cancer progression factors
The levels of four different cancer progression factors: granulocyte-macrophage colony stimulating factor (GM-
Effects of hypericin-mediated photodynamic therapy on GM-CSF, MIF, VCAM-1 and ICAM-1 secretion in colorectal cancer cells in vitro

GM-CSF, macrophage migration inhibitory factor (MIF), vascular cell adhesion molecule (VCAM-1) and intercellular adhesion molecule (ICAM-1) were analyzed in the cell culture filtrates. The levels of the tested parameters were determined using the Bio-Plex Pro Assay kit based on xMAP suspension technology (Bio-Rad Laboratories Inc, USA). Assays were performed for control samples without hypericin and light exposure, with 0.25 µM and 0.5 µM hypericin without light exposure, without hypericin and irradiated with light at 1 J/cm², 5 J/cm² and 10 J/cm², and test samples for 0.25 µM and 0.5 µM hypericin and light exposure at 1 J/cm², 5 J/cm² and 10 J/cm². Measurements were performed according to the Bio-Plex kit manufacturer’s instructions, starting 24 hours after illumination of the test samples. First, cell culture supernatants were incubated for one hour with reagents containing magnetic beads conjugated with antibodies corresponding to the progression factors studied. After the incubation and washing period, biotinylated detection antibodies were added and samples were further incubated for 30 minutes. Magnetic beads were then washed and streptavidin-phycoerythrin (PE) solution was added to each well for 10 minutes. Then, after washing with buffer to remove un-bound streptavidin-PE, the beads were re-suspended in buffer. Beads bound to each cytokine were analyzed in a Bio-plex Array Reader (Bio-Plex 200 System). Fluorescence intensity and cytokine concentrations were assessed using BioPlex Manager software. Standard curves for each cytokine were calculated using a reference cytokine sample provided with the kit. For each type of test sample, tests were performed in triplicate.

Statistical methods
Measurement results were presented as mean and standard deviation (SD). Statistical significance was determined using Student’s t-test (Microsoft Office, Warsaw, Poland). The obtained results were presented in a descriptive form and as tables and graphs. The significance level was set at < 0.05. Graphs contain a description of the coordinate axes and the study groups, a description of the data, numerical values and a graphical representation of standard deviations and statistical significance for the groups compared. The tables show mean values, standard deviation and level of statistical significance measured by Student’s t-test.

Results
The presence of photosensitizer in the cells
The presence of hypericin in the cells was assessed using an inverted fluorescence microscope. Figures 2 and 3 show photographs taken for both cell lines, showing the fluorescence of the analyzed cells and thus the intracellular accumulation of hypericin. For proper imaging of the cells, a fluorescein isothiocyanate (FITC) filter was used at 200x magnification. Additionally, the extent of cellular hypericin uptake was assessed using a flow cytometer (Becton Dickinson, LSR II) in the PerCP channel by measuring the fluorescence intensity induced by cellular exposure to radiation from a 488 nm laser.

Figure 4 shows graphs representing the escalation of fluorescence of hypericin-treated cells and the correlation between fluorescence intensity and the time and photosensitizer concentration.

![Fluorescence microscope image of SW480 cell line. Red fluorescence indicates the presence of hypericin](image1)

![Fluorescence microscope image of SW620 cell line. Red fluorescence indicates the presence of hypericin](image2)

![Diagrams presenting the dependence of the intensity of fluorescence of the tested cells on the time of their incubation with hypericin and on the concentration of photosensitizer: a) for SW480 cells; b) for SW620 cells](image3)
Viability of the cells – MTT assay for measuring cell metabolic activity
SW480 cell line
Cell viability in the SW480 cell line after light exposure at 1 J/cm², 5 J/cm² and 10 J/cm² at different doses of hypericin showed no significant differences compared to the control. No significant cytotoxic effect was observed. The results are shown in Figure 5.

SW620
Cell viability of the SW620 cell line after light exposure at doses of 1 J/cm², 5 J/cm² and 10 J/cm² and application of hypericin remained within 100% in most samples. A light dose of 1 J/cm² and a hypericin concentration of 1 µM led to a reduction in cell viability by 81.88% (p<0.01). A 10 J/cm² dose with 1 µM hypericin resulted in a decrease in viability to 55.57% (p<0.01). In view of the significant cytotoxic effect of hypericin at 1 µM, this concentration was not used to assess the secretion of the progression factors tested, as living cells are required for this analysis. The results are shown in Figure 6.

The levels of the analyzed progression factors
In this study, the cytokines GM-SCF, MIF, VCAM-1 and ICAM-1 were selected for analysis due to their relatively high concentrations in cancer cell supernatants and their potential role in the process of tumor development. Bio-Plex Pro Assay kit was used to determine the concentration of individual cytokines released from the examined cancer cell lines treated with photodynamic therapy with hypericin. The results of the experiment were presented using descriptive statistics, tables and graphs. In this study it has been observed that light alone and hypericin alone also can affect cytokine release by cells. Statistically significantly lower concentration of cytokines were noted after light doses of 5 and 10 J/cm². A significant effect of hypericin alone was observed in SW480 cells. At the hypericin concentration of 0.25 µM lower concentrations of ICAM-1 were noted, while at the concentration of 0.5 µM lower concentrations of GM-CSF and ICAM-1 were noted. Moreover, the two cell lines were compared by taking control samples (without hypericin and without irradiation) to determine whether cells from primary colorectal cancer (SW480) and metastatic cells (SW620) secrete cytokines to a similar extent (Tab. 1–4).

Granulocyte-macrophage colony-stimulating factor (GM-CSF)
GM-CSF concentration was determined using photodynamic therapy in light doses of 1, 5 and 10 J/cm² and hypericin in concentrations of 0.25 and 0.5 µM. The use of Hyp-PDT did not affect the concentration of GM-CSF secreted by SW620 cells. PDT caused a statistically significant decrease in GM-CSF secretion in SW480 cells compared with control (p<.05). The results of the tested samples are shown in Figure 7 for the SW480 cell line and in Figure 8 for the SW620 cell line. Moreover, the study of control samples showed that SW480 cell line secrete GM-CSF in higher concentrations (p<0.02) than SW620 cell line. GM-CSF results are given in pg/ml. The results are shown in Table I.
Effects of hypericin-mediated photodynamic therapy on GM-CSF, MIF, VCAM-1 and ICAM-1 secretion in colorectal cancer cells in vitro

Fig. 8. Diagram presenting the concentration of GM-CSF in the tested supernatants from SW620 cancer cells under certain conditions: without Hyp and light (control), after treatment with Hyp PDT at various concentrations and after irradiation at various doses

Table 1. Descriptive statistics showing the difference in the level of GM-CSF secretion between the SW480 and SW620 cell lines in the control samples

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Num. of trials</th>
<th>Average concentration (pg/ml)</th>
<th>SD</th>
<th>Compared cell lines</th>
<th>The Mann-Whitney U test</th>
</tr>
</thead>
<tbody>
<tr>
<td>SW480</td>
<td>5</td>
<td>11.37</td>
<td>2.12</td>
<td>SW480 and SW620</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>SW620</td>
<td>5</td>
<td>2.13</td>
<td>0.57</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Macrophage migration inhibitory factor (MIF)

MIF concentration was determined using photodynamic therapy in light doses of 1, 5 and 10 J/cm² and hypericin in concentrations of 0.25 and 0.5 μM. After Hyp-PDT treatment, lower MIF levels were found in SW480 cell line (p<0.05) compared to controls. In SW620 cell line, MIF secretion decreased after Hyp-PDT at hypericin concentrations of 0.25 μM and 0.5 μM with light doses of 5 and 10 J/cm² (p<0.05). MIF concentration is given in pg/ml. The results are shown in Figure 9 for SW480 cell line and Figure 10 for SW620 cell line. Moreover, the study of control samples (without light and hypericin) showed that there is no statistically significant difference in MIF secretion between SW480 and GM-CSF cell lines (p>0.05). The results are shown in Table 2.

Table 2. Descriptive statistics showing the difference in the level of MIF secretion between the SW480 and SW620 cell lines in the control samples

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Num. of trials</th>
<th>Average concentration (pg/ml)</th>
<th>SD</th>
<th>Compared cell lines</th>
<th>The Mann-Whitney U test</th>
</tr>
</thead>
<tbody>
<tr>
<td>SW480</td>
<td>5</td>
<td>190.71</td>
<td>45.76</td>
<td>SW480 and SW620</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>SW620</td>
<td>5</td>
<td>148.88</td>
<td>5.29</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Vascular cell adhesion molecule-1 (VCAM-1)

VCAM-1 concentration was determined using photodynamic therapy in light doses of 1, 5 and 10 J/cm² and hypericin in concentrations of 0.25 and 0.5 μM. PDT caused a statistically significant decrease in VCAM-1 secretion in SW480 cells at all light doses and hypericin concentrations (p<0.05). For SW620 cells, there were significantly lower VCAM-1 levels in the samples treated with photodynamic therapy with 0.25 and 0.5 concentration of hypericin and light doses of 5 and 10 J/cm² compared to controls. VCAM-1 concentration is given in pg/ml. The results are shown in Figure 11 for SW480 cell line and Figure 12 for SW620 cell line. Moreover, the study of control samples (without light and hypericin) showed that SW480 cell line secrete higher concentrations of VCAM-1 (p<0.02) than SW620 cell line. The results are shown in Table 3.

Table 3. Descriptive statistics showing the difference in the level of VCAM-1 secretion between the SW480 and SW620 cell lines in the control samples

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Num. of trials</th>
<th>Average concentration (pg/ml)</th>
<th>SD</th>
<th>Compared cell lines</th>
<th>The Mann-Whitney U test</th>
</tr>
</thead>
</table>

Intercellular adhesion molecule-1 (ICAM-1)

ICAM-1 concentration was determined using photodynamic therapy in light doses of 1, 5 and 10 J/cm² and
hypericin in concentrations of 0.25 and 0.5 µM. The use of PDT resulted in a statistically significant decrease in ICAM-1 secretion in SW480 cell line at 10 J/cm² light dose and 0.25 and 0.5 µM hypericin concentrations (p<0.05). A significant increase in ICAM-1 secretion in SW620 cells (p<0.05) was noted after light of 5 J/cm² alone and with Hyp at a concentration of 0.25 µM. ICAM-1 concentration is given in pg/ml. The results are shown in Figure 13 for SW480 cell line and Figure 14 for SW620 cell line. Moreover, the study of control samples (without light and hypericin) showed that SW480 cell line secrete higher concentrations of ICAM-1 (p<0.02) than SW620 cell line. The results are shown in Table 4.

Table 3. Descriptive statistics showing the difference in the level of VCAM-1 secretion between the SW480 and SW620 cell lines in the control samples

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Number of trials</th>
<th>Average concentration (pg/ml)</th>
<th>SD</th>
<th>Compared cell lines</th>
<th>The Mann-Whitney U test</th>
</tr>
</thead>
<tbody>
<tr>
<td>SW480</td>
<td>5</td>
<td>20.72</td>
<td>2.78</td>
<td>SW480 and SW620</td>
<td>&lt; 0.02</td>
</tr>
<tr>
<td>SW620</td>
<td>5</td>
<td>8.24</td>
<td>0.80</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Discussion

The use of HYP-PDT in the treatment of colorectal cancer has attracted the interest of researchers for decades. Of particular interest is the immunological effect induced as a consequence of hypericin in photodynamic therapy. In the case of cancer therapy, the ability to influence cellular and immunological processes taking place in the tumour niche is extremely important. In this paper we publish for the first time the results...
of a study on the effect of hypericin used in photodynamic therapy on in vitro GM-CSF, MIF, VCAM-1 and ICAM-1 secretion in colorectal cancer cells. GM-CSF is a haemopoietic cytokine whose primary function is to stimulate the growth of granulocytes and macrophages and to influence lymphocytes. In addition, GM-CSF promotes the differentiation of haemopoietic stem cells into dendritic cells and stimulates the growth and proliferation of many other cell types, including eosinophils, monocytes, neutrophils and keratinocytes. GM-CSF is produced and secreted by various groups of cells, including activated B cells, T cells, mast cells, endothelial cells, macrophages and fibroblasts, most commonly in response to inflammatory stimuli. In addition to its function as a haemopoietic growth factor, GM-CSF also has various functions in mature haemopoietic cells, including enhancing phagocytosis and production of pro-inflammatory cytokines, promoting leukocyte adhesion and chemotaxis, and promoting antigen presentation. Antigen-presenting cells play a key role in triggering the anti-tumour immune response. Professional primary antigen presenting cells are dendritic cells. GM-CSF stimulates the growth and activity of dendritic cells by effectively enhancing anti-tumour immunity. In addition, GM-CSF modulates collagen metabolism of the extracellular matrix, can promote migration and proliferation of endothelial cells, thus participating in the process of angiogenesis. GM-CSF has also been shown to act as a tumour-derived factor that promotes cancer cell proliferation and migration in various solid tumors and cancer cell lines, thus promoting tumour growth and progression. Constitutive expression and secretion of GM-CSF protein have been observed in some cancer cell research models, and elevated serum GM-CSF levels are considered a potential diagnostic and prognostic marker correlating with poor prognosis in colorectal cancer patients. Tumour cells producing GM-CSF promote specific anti-tumour immunity by teaching CD4+ and CD8+ T lymphocytes to recognize circulating tumour-specific antigens, thereby inducing a systemic immune response. In addition to its immune system-stimulating functions, GM-CSF also has direct effects on tumour progression and invasion. In the pathomechanism of breast cancer, it has been shown that GM-CSF participates in the formation of tumour associated macrophages, thus participating in the positive coupling of tumour growth and metastasis formation, while reduced GM-CSF expression inhibits the metastatic process. In our study, we recorded an evident reduction in GM-CSF secretion by colorectal cancer cells of the SW480 cell line, escalating with increasing HYP concentration and light intensity. Furthermore, we showed that SW480 cells secrete more GM-CSF than SW620 cells. A similar result regarding the inhibition of GM-CSF secretion was observed by Du et al., who studied the effect of HYP-PDT on nasopharyngeal carcinoma (NPC). The aim of their study was to analyze the upregulation of matrix metalloproteinase-9 (MMP-9) expression in well-differentiated cells of NPC-derived HK1 cell lines under the influence of HYP-PDT and, among other things, they found that MMP-9 expression was reduced after HYP-PDT by inhibiting GM-CSF production. Colorectal cancer cells can independently synthesize MMP-9, which is involved in the processes of progression and metastasis by, among other things, degrading the extracellular matrix and affecting the formation of organelles associated with cell movement. Moreover, elevated expression of the MMP-9 enzyme in colorectal cancer cells is associated with increased tumour aggressiveness and invasive potential. Thus, HYP-PDT-mediated inhibition of GM-CSF secretion may, by reducing MMP-9 expression, significantly reduce tumour growth processes. Various mechanisms for the effect of GM-CSF on tumour progression have been postulated. Chen et al. identified the function of GM-CSF as a factor that promotes colon cancer progression by inducing epithelial to mesenchymal transition (EMT), which involves the transformation of cells through the loss of cell-to-cell adhesion capacity and through the breakdown of tight and gap junctions, allowing tumour cell expansion. MIF is a pro-inflammatory cytokine with multidirectional effects. Its main functions are stimulation of interleukin-6, interleukin-1β and TNF-α secretion and stimulation of MHC type II molecules expression on macrophages. Furthermore, MIF promotes cell proliferation, inhibits apoptosis and regulates immune cell processes. Many solid tumors overexpress MIF, which may be involved in regulating the production and expression of various factors, e.g. by increasing VEGF secretion to promote tumorigenesis. The serum level of MIF in colorectal cancer patients and colorectal cancer cells is significantly higher than in healthy individuals and correlates with the presence and extent of metastasis and with an increased risk of metastases, so it may have some potential as a marker for the clinical diagnosis of liver metastases in colorectal cancer. Li et al. demonstrated that the proinflammatory cytokine MIF secreted by infiltrating lymphocytes may contribute to the local progression and promotion of metastasis of nasopharyngeal carcinoma through the induction of MMP-9 in the indirect pathway. Furthermore, it may stimulate invasion of nasopharyngeal carcinoma cell lines in vitro, and infiltrating lymphocytes in NPC may be responsible for cancer cell invasion and metastasis. The inhibition of the activity of the p53 protein, which acts as a transcription product of the TP53 anti-oncogene is involved in many cellular processes aimed at repairing damaged DNA or inducing cell apoptosis in response to DNA damage, is another tumour-promoting mechanism de-
scribed by MIF. The loss of p53 protein function is among the most common turning points in tumorigenesis. Locally high MIF levels cause activation of T cells and enhance the activity of macrophages, which release oxygen radicals and nitric oxide in response to a stimulus. MIF is able to block the p53 response and inhibit nitric oxide-induced macrophage apoptosis.

In another study, Wilson et al. argued that MIF promotes intestinal cancer formation, through angiogenesis, and genetic deletion of MIF results in reduced tumour microvessel density. MIF has been associated with numerous cancers, including kidney, prostate, colorectal and glioma, and many reports have documented its key role in activating tumour immuno-suppression. In our study, we recorded a significant reduction in MIF secretion by the colorectal cancer cells tested in both SW480 and SW620 cell lines after hypericin-mediated photodynamic therapy. Thus, it can be assumed that therapies inhibiting the MIF function could contribute to an improved efficacy of anticancer treatment.

Adhesion molecules, glycoproteins belonging to the immunoglobulin superfamily, have also been analyzed as factors of progression. Vascular cell adhesion molecule-1 (VCAM-1) is constitutively expressed on tissue macrophages, stromal cells and epithelial cells and on the surface of stimulated endothelial cells. VCAM-1 mediates the adhesion of lymphocytes, monocytes, eosinophils and basophils to vascular endothelium and mediates leukocyte and endothelial cell signal transduction. There is evidence that VCAM-1 is involved in cancer progression and metastasis, by mediating the adhesion of tumour cells to vascular endothelial cells and promoting metastatic processes, including angiogenesis. Moreover, it has been identified as one of the most important factors able to promote and sustain the development of tumour vasculature. Generation of new blood vessels plays a key role in tumour progression and is characterized by invasion, migration and proliferation of endothelial cells. The above processes depend strictly on the interplay between cells and components of the extracellular matrix. Several experiments have documented significantly higher levels of VCAM-1 in the serum of cancer patients or in cancer cells in vitro compared with controls. Overexpression of VCAM-1 stimulates tumour neovascularization, resulting in tumour growth and metastasis formation. The severity of VCAM-1 expression correlates with disease stage and the presence of metastases and is highly pronounced in endothelial cells, especially in capillaries. Maurer et al. demonstrated that colorectal cancer cells overexpress VCAM-1 and ICAM-1. Therefore, it seems logical to undertake studies on ways to inhibit VCAM-1 expression to see if blocking this factor would affect the course of the cancer. In our study, we observed an inhibitory effect of HYP-PDT on VCAM-1 secretion by the colorectal cancer cells in both assessed cell lines.

ICAM-1 is located superficially in the membrane of immune, endothelial and epithelial cells. Physiologically, its expression remains low and is strongly induced by various pro-inflammatory cytokines. ICAM-1 plays an important role in many physiological processes, including immune cell effector functions, T-cell activation, leukocyte movement and elimination of pathogens and dead cells. In addition, ICAM-1 regulates leukocyte movement and adhesion interactions with the vessel wall and directs leukocyte passage through the endothelial layer and mediates intra- and extracellular signals. ICAM-1 may similarly act in transformed epithelial and tumour cells, is involved in the activation of pro-inflammatory cascades and mediates multiple signaling pathways such as adhesion, angiogenesis and tumour cell transmigration and immune escape. ICAM-1 expression has been found in the vast majority of cells in the tumour microenvironment and has been correlated with aggressive and invasive tumour phenotypes. High expression of ICAM-1 has also been documented in metastases. Publications on the effect of photodynamic therapy on the expression and secretion of adhesion molecules VCAM-1 and ICAM-1 are scarce. In an experiment with colon cancer cell lines SW480 and SW620 analyzing the effect of photodynamic therapy with aminolevulinic acid under normoxia and CoCl2-induced hypoxia on the in vitro secretion of adhesion molecules VCAM-1 and ICAM-1 by colon cancer cells, no significant effect of this therapy on these progression factors was observed. On the other hand, a group of Chinese researchers presented a study in which photodynamic therapy increased VCAM-1 expression in glioma cells with a concomitant increase in tumour growth, while PDT combined with a monoclonal antibody directed against VCAM-1 significantly inhibited tumour growth and prolonged survival and significantly reduced VCAM-1 expression. Another report documented a reduction in the number of metastases and a significant decrease in adhesion molecules, including ICAM-1, among colorectal cancer cells tested after PDT with Photofrin and benzoporphyrin derivative mono-acid ring A. In our study, we recorded an inhibitory effect of HYP-PDT on ICAM-1 secretion by SW480 cells, and in the case of SW620 cells, we observed an inducing effect of HYP-PDT on ICAM-1 secretion at a low dose of hypericin and upon irradiation at 5 J/cm2. Furthermore, we demonstrated that SW480 cells produced ICAM-1 at higher concentrations than SW620 cells.

There are few papers in the literature analyzing the effect of HYP-PDT on the secretion of progression factors. In a study by Majernic et al., the effect of HYP-PDT on the expression of proangiogenic factors was analyzed using different research models. They compared cell culture in a 2D model, 3D model and in experimentally
generated micro-tumors. The cytotoxic effect of HYP-PDT HP was confirmed and the effect was shown to be highly dependent on the research model used. Significant differences in gene expression of the analyzed proangiogenic factors were observed depending on the experimental model and it was found that the level of gene expression did not correlate with the level of protein expression, especially in the 2D model, which was considered to be an effect of damage to organelles responsible for proteosynthesis. It was shown that cells cultured in 2D cell models were significantly more sensitive to treatment than cells in the 3D model. It was concluded that for this type of analysis, the selection of an appropriate experimental model is crucial. Our experiment was performed in a classical 2D culture. There is evidence for limitations of this research model, such as insufficient signaling between cells and between the cell and the extracellular matrix. Important signaling pathways may be disrupted or bypassed under such experimental conditions. The hypoxic state, which is one of the hallmarks of cancerous tumours, can fundamentally affect the effect of a given therapy. Cell culture conditions, depending on in vitro and in vivo experimental models, can significantly affect growth factor gene expression.22

Analyzing the available literature on the use of hypericin in photodynamic therapy for colorectal cancer in vitro and in vivo animal studies, publications proving the effectiveness of this method and showing its multidirectional effects can be found. Blank et al. documented in their in vivo and in vitro experiment on C26 colon cancer cells that photodynamic therapy with hypericin reduced cell viability in a dose-dependent and light-dependent manner and observed extensive vascular damage and tumour necrosis as a result of HYP-PDT.72 After PDT combining the effects of hypericin with hyperforin on HT-29 colon cancer cell model, increased production of reactive oxygen species (ROS) enhancing anti-tumour activity, induction of apoptosis, inhibition of cell cycle and blockade of expression of matrix metalloproteinases 2 and 9 were reported.36 Under the influence of photodynamic therapy with hypericin, decreased gene expression of dysadherin, an anti-adhesion molecule known for its pro-cancerogenic and metastasis-promoting function, and changes in the organization of F-actin belonging to cytoskeleton proteins were observed.35 Austrian researchers performed an in vivo experiment on mice that were injected intravenously with low-dose hypericin and irradiated with red light. After 60 days, the effects of HYP-PDT were evaluated and it was noted that PDT at a standard dose and with a 4-hour interval between drug administration and irradiation led to complete tumor eradication in the entire study group.74

Due to the high potential of hypericin and its complex mechanisms of action in photodynamic therapy at the molecular level, attempts have been made to combine it with other drugs and various inhibitors to maximize its effects. A group of Slovak re-searchers presented the results of a study on HT-29 colon cancer cells proving that combination treatment including HYP-PDT and MK-886, an inhibitor of lipoxygenase, an enzyme involved in multiple immune mechanisms, can improve the therapeutic efficacy of PDT; moreover, it induces changes in the cell cycle and significantly enhances apoptosis under the influence of hypericin photodynamic therapy.75 Other researchers from the same Slovak Centre showed that pretreatment with proadifen (a non-selective cytochrome P450 enzyme inhibitor with proven apoptotic effects in HT-29 colon adenocarcinoma) affected the function of ABC membrane transporters MRPs (multidrug resistance protein) and BCRP (breast cancer resistance protein) leading to increased accumulation of hypericin in HT-29 colon adenocarcinoma cells and enhanced HYP-PDT effect.76

Although photodynamic therapy is considered an effective method for treating cancer, cancer cells may use certain resistance mechanisms, including up-regulation of apoptosis inhibitors, to overcome the cytotoxic effects of PDT. One such inhibitor of apoptosis is survivin, whose expression is blocked by the small-molecule inhibitor YM155. Application of HYP-PDT together with YM155 sensitizes HT-29 colon cancer cells to HYP-PDT and induces apoptosis.77 In another report, after application of HYP-PDT with yeast-derived manumycin A, increased sensitivity of HT-29 cells to oxaliplatin and decreased HT-29 cell viability, suppression of cell proliferation, significantly lower levels of apoptosis inhibitors (cIAP1, cIAP2, XIAP, survivin) were observed. This combination resulted in induction of apoptosis and enhanced phagocytosis of cancer cells.78 These results were confirmed by Chinese researchers, according to whom autophagy and reactive oxygen species are mainly responsible for the increased chemosensitivity to oxaliplatin of cancer cells during HYP-PDT. HYP-PDT at high doses induced autophagic cell death and induced high concentrations of reactive oxygen species and increased cytoplasmic reticulum stress.79,80 The ABCG2 protein, which belongs to the ABC membrane transporters, is another important factor responsible for multidrug resistance in tumors and lower PDT efficacy. The use of a compound called Ko143, which is an ABCG2 inhibitor, increases the efficacy of HYP-PDT as observed in in vitro studies using 2D and 3D models of HT29 and HT116 colon cancer cells.81 In another study, HYP-PDT induction of MRP-1 protein expression in HT-29 colon cancer cells was observed, suggesting that hypericin may
The use of hypericin in photodynamic therapy is limited by its hydrophobic properties. In order to improve water solubility and enhance the photodynamic effect, hypericin can be bound to a carrier that will facilitate transport into and within the cell and prevent the formation of aggregates inside the cell. A symmetric tri-block copolymer containing poly(ethylene oxide) and poly(propylene oxide), Pluronic P123 (P123), proved to be an interesting nanocarrier for hypericin. The effect of Hyp/P123 was analyzed for colorectal cancer cells Caco-2 and HT-29. A beneficial effect and good efficacy of HYP-PDT was observed after the application of the nanocarrier, which played a significant role in the penetration of Hyp across the cell membrane.84 In another case, to pre-vent the accumulation of hypericin in a non-target site and to reduce its side effects, Hyp was bound to superparamagnetic iron oxide nanoparticles (SPION) and guided to the target by an external magnetic field. It was evaluated that this strategy could improve the efficacy and specificity of the treatment and could reduce toxic side effects.85 It has been demonstrated in animal studies that the use of magnetic carriers accumulates the drug more effectively in tumour cells than traditional routes of administration.86 In another in vitro study, Nano formulation of transferrin with hypericin (HTfNP) was successfully used, preventing premature release of hypericin and improving its availability by better targeting to the tumour site.87 Similarly, significantly improved performance of HYP-PDT used in a study with HT-29 colon adenocarcinoma cells was achieved by using non-polymeric nanogels formed by a low molecular weight gelator as a hypericin nanocarrier. The nanogels provided free, efficient intracellular transport of hydrophobic hypericin.88 Chinese researchers have developed a biodegradable nanoparticle, Hyp-NP, to deliver hypericin to the tumour site. It was documented that Hyp-NP showed efficient cellular accumulation with localization in the cytoplasm and retained affinity for fluorescence, it may prove useful to enhance the efficacy of Hyp-PDT.

**Conclusion**

On the basis of the experiments author proved that determination of the growth, invasion and metastasis of the colon cancer cells factors might be useful in the disease progress prognosis, choosing optimal treatment and verifying its effectiveness, as well as in monitoring the patients after the therapy. At the same time, the results confirming the destructive effect of HYP-PDT on the colon cancer cells, show a possibility of extending the indication for photodynamic therapy using HYP, qualification of precancerous changes, including adenoma and early stages of the colon cancer. HYP-PDT influences the secretion of growth, migration, angiogenesis and metastasis factors by the colon cancer cells, mainly by their suppression. In our study, we recorded an evident reduction in GM-CSF secretion by colorectal cancer cells of the SW480 cell line, escalating with increasing HYP concentration and light intensity. Furthermore, we showed that SW480 cells secrete more GM-CSF than SW620 cells. In our study, we recorded a significant reduction in MIF secretion by the colorectal cancer cells tested in both SW480 and SW620 cell lines after hypericin-mediated photodynamic therapy. Thus, it can be assumed that therapies inhibiting the MIF function could contribute to an improved efficacy of anticancer treatment. We noticed an inhibitory effect of HYP-PDT on VCAM-1 secretion by the colorectal cancer cells in both assessed cell lines and an inhibitory effect of HYP-PDT on ICAM-1 secretion by SW480 cells, and in the case of SW620 cells, we observed an inducing effect of HYP-PDT on ICAM-1 secretion at a low dose of hypericin and upon irradiation at 5 J/cm2. Furthermore, we demonstrated that SW480 cells produced ICAM-1 at higher concentrations than SW620 cells. These effects of photodynamic therapy using HYP determines the possibility of application of adjuvant therapy, including cell based immunotherapy with respect to the factors responsible for the invasiveness of the colon cancer.

**Data availability**

Data available on request from the authors.

**Ethics approval**

Not applicable.

**References**


Effects of hypericin-mediated photodynamic therapy on GM-CSF, MIF, VCAM-1 and ICAM-1 secretion in colorectal cancer cells in vitro


27. French CS, Smith JH, Virgin HI, Airth RL. Fluorescence-Spectrum Curves of Chlorophylls, Phaeophytins,


Effects of hypericin-mediated photodynamic therapy on GM-CSF, MIF, VCAM-1 and ICAM-1 secretion in colorectal cancer cells in vitro


