The Influence of Hypericin-Mediated Photodynamic Therapy on Interleukin-8 and -10 Secretion in Colon Cancer Cells

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Abstract

The aim of this study was to measure the secretion of interleukin (IL)-8 and -10 during an elicited immune response following sublethal doses of hypericin-mediated photodynamic therapy (HY-PDT) in experimental models of residual colon cancer cells in vitro. Investigations were performed on the cancer cell lines SW480 and SW620. Each cell line was exposed to 3 different concentrations of the photosensitizer HY and various doses of irradiation. The cell metabolic activity using an MTT assay was performed and then the measurement of IL-8 and IL-10 secretion was achieved using the Bio-Plex ProTMAssay. There was a statistically significant amplification of IL-8 secretion during HY-PDT in the SW620 cell line (at 1 J/cm^2: P = .01, 5 J/cm^2: P = .002, and 10 J/cm^2: P = .025) and a statistically significant decrease in IL-8 during HY-PDT in the SW480 cell line (at 1 J/cm^2: P = .05, 5 J/cm^2: P = .035, and 10 J/cm^2: P = .035). No statistically significant differences in IL-10 concentration were found following HY-PDT in the SW480 (at 1 J/cm^2: P > .4, 5 J/cm^2: P = .1, and 10 J/cm^2: P = .075) or in the SW620 cell line (at 1 J/cm^2: P > .4, 5 J/cm^2: P > .4, and 10 J/cm^2: P > .4). HY-PDT can both eliminate and control a primary tumor via cytotoxic effects, and at sublethal doses, it can affect IL release by colon cancer cells. In this experiment, this influence depended on the level of tumor cell metastatic activity.

Introduction

Photodynamic therapy (PDT) is a treatment option for cancers of the skin, head and neck, brain, lung, and bladder, as well as for premalignant and malignant lesions of the colorectum with high surgical risk.[1] Clinically approved photosensitizers (PS) for PDT are overwhelmingly porphyrins, chlorins, bacteriochlorins, and phthalocyanines that contain tetrapyrrole moieties, although other PS such as hypericin (a phenanthroperylene quinone) are also being investigated currently by us and others for clinical utility.[2] The cell-killing effect of PDT is predominantly the result of the generation of highly reactive and cytotoxic singlet oxygen ($^1O_2$) molecules from collision of endogenous triplet oxygen ($^3O_2$) with excited triplet state PS (a type II energy transfer process).[2] Electron transfer pathways (type I) can compete to some degree with type II processes leading to the production of other reactive oxygen species (ROS) such as hydroxyl radical

and superoxide, which are also cytotoxic.[2] The excited triplet state PS is generated by visible light irradiation of PS, ideally at longer visible wavelengths in the near-infrared optical

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window of tissue.[3] The generation of ROS from PS, oxygen and light, and subsequent cell death is collectively termed the photodynamic effect. An important distinction of the photodynamic effect in PDT is that it evokes both apoptotic and necrotic pathways in cells, both of which are implicated in eliciting an immune response.[4]

Hypericin (HY) is a natural product isolated from St. John's wort (*Hypericum*) that possesses photodynamic properties that were identified historically in the phenomenon of livestock hypericism.[5] HY has been explored for use as an antiviral agent, an antidepressant, and for fluorescence diagnosis, in addition to PDT.[6-9] Clinical use of HY as a PS for PDT was first reported in 1996 by Koren et al[10] for local treatment of malignant mesothelioma. Since then, PDT with HY has been reported to have potential for treatment of cutaneous T-cell lymphoma (*Mycosis fungoides*) and psoriasis,[11] bladder cancer,[12,13] nasopharyngeal tumors,[14,15] and ovarian cancer.[16] HY tends to accumulate in the endoplasmic reticulum and mitochondria, and following visible light irradiation, it induces immunogenic apoptosis by release of damage-associated molecular patterns (DAMPs), the most well-known being heat-shock protein HSP70.[2,17] There are recent data that indicate that HY-mediated PDT (HY-PDT) has antiangiogenic properties and could prevent tumor growth,[18] confirming earlier studies that suggest tumor vascular damage as a primary effect of HY-PDT.[19-21] The results of experiments performed on the different types of cancer cells suggest that the effect of cellular-targeted HY-PDT could be tumor specific. For instance, HY-PDT increases the level of vascular endothelial growth factor (VEGF) in bladder tumors and in nasopharyngeal carcinomas (NPCs) after HY-PDT,[22,23] although other researches provided evidence for reduced levels of VEGF in animals with NPCs.[24] There are also data that suggest that HY-PDT can reduce the rate of regrowth of colon cancer after treatment.[21]

Photodynamic therapy also stimulates the presentation of tumor antigens and stimulates the release of proinflammatory and immunosuppressive cytokines.[25] Tumor-associated antigens affect tumor cells by stimulating the release of factors associated with angiogenesis and metastasis including the release of interleukin (IL)-8 and IL-10.[25] Therefore, our specific aim was to validate the occurrence of IL release in colon cancer cell models, to ascertain cytokine-dependent colon cancer cells progression.

Interleukin 8 is a mix of ser-IL-8 and ala-IL-8 that is produced, particularly under hypoxic conditions, by endothelial cells, monocytes, keratinocytes, and fibroblasts.[26] IL-8 is a chemotactic cytokine that is highly specific for neutrophils that are activated in inflamed tissue. IL-8 also stimulates angiogenesis and has been implicated as a factor in colon tumor metastasis. It has been demonstrated that the production of IL-8 by colon cells is associated with metastasis of colon tumor cells, stimulating angiogenesis and the growth of colon cancer.[27,28] IL-10 is an anti-inflammatory cytokine that

inhibits the immune response by inhibiting cytokine release by macrophages, lymphocytes, and monocytes, and by suppressing antigen presentation by reducing monocytic major histocompatibility complex (MHC) class II expression.[29] Production of IL-10 occurs in T-cells primarily Th2 and Treg, macrophages, dendritic cells, and B lymphocytes. As with IL-8, IL-10 is a factor in the progression of colorectal cancer, and elevated levels of IL-10 have been implicated in an increase in morbidity.[30,31] Our objective in this work was to evaluate the production of IL-8 and IL-10 in response to in vitro HY-PDT in human colon adenocarcinoma cell lines SW480 and SW620 that differ in their degree of malignancy. In this study, HY-PDT was performed with a particular emphasis on cytotoxicity and the effect of sublethal doses of HY-PDT on secretion of IL-8 and IL-10, which have been implicated as factors responsible for the process of progression and metastasis of colorectal cancer.

Materials and Methods

Cell Lines

Two human colon cancer cell lines—SW480 and SW620—which differ in their degree of malignancy (SW480 originated from adenocarcinoma of the colon and SW620 originated from lymph node metatasis[32] and considered to have greater malignancy) were used for the experiments. The cell lines were purchased from ATCC (American Type Cell Culture-ATCC LGC Limited) and kept frozen prior to use. These 2 cell lines have been used previously in oncological experiments to assess mechanisms of action of chemotherapeutics and anticancer agents.[33] SW480 (ATCC, Cat. No. CCL-228) cells from a 50-year-old Caucasian male with primary colorectal adenocarcinoma expresses carcinoembryonic antigen (CEA), TGF-β transformant, and oncogenes: myc, myb, ras, fos, sis, p53, abl, ros, and Sr. The SW480 line is characterized by local malignancy, and according to Dukes qualifications, the line cells were qualified as type B. SW620 (ATCC, Cat. No. CCL-227) cells from a 51-year-old Caucasian male derived from metastatic cells in the colon cancer lymph node and expresses the CEA of oncogenes: myc, myb, ras, fos, sis, p53, abl, ros, and src. The SW620 line is characterized by high metastatic activity, and according to Dukes qualifications, the line cells were qualified as type C.

Methodology

Preparation of Cell Cultures. The cells were obtained frozen in liquid nitrogen in cryotube vessels in a freezing medium. For culturing cells of lines SW480 and SW620, a culture medium was used with the following composition: Leibovitz's L-15 with the addition of 10% inactivated fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin. The culture was grown in 25 cm^2 and 75 cm^2 plastic bottles

(Nunc A/S) in a culture medium, and then incubated at 37 °C under 100% humidity without carbon dioxide. The cells adhered in the form of a monolayer, and exchange of growth medium was performed 2 to 3 times per week. Cells adhering to the bottom of the culture flasks were detached from the medium with a solution of 0.25% trypsin solution and 0.53 mM ethylenediaminetetraacetic acid (EDTA) followed by incubation for 5 to 10 minutes at 37 °C. After the trypsinization process, fresh growth medium was added to the cell suspension and centrifuged for 5 to 7 minutes (125 × g).

Incubation of Cells With the Photosensitizer Hypericin. The cells were suspended in the culture medium, bringing the suspension to a suitable density determined experimentally for each of the lines: SW480 (5 × 10^5/mL) and SW620 (2.5 × 10^5/mL). The cells were then adhered on 96-well plates, incubated at 37 °C under 100% humidity for 24 hours. The culture medium was removed, and the monolayer of cells was rinsed and treated with selected concentrations of HY (from Hypericin Calbiochem) in concentrations ranging from 0.1 μM to 1.0 μM. After 1 hour of incubation, the monolayer was aspirated with limited exposure to light. The cells were washed 2 times with phosphate-buffered saline. An incubation time of 1 hour was established by fluorescence intensity to avoid cytotoxicity providing a sublethal dose.[34,35]

Cell Exposure to Light. After adding the culture medium, exposure to light with energy doses of 0 J/cm^2, 5 J/cm^2, and 10 J/cm^2 was initiated. A PDT TP1 photodynamic therapy lamp (Cosmedico Medizintechnik GmbH) was used for irradiation, which, with the orange filter installed, emits radiation in the range of 600 to 720 nm with surface power density 1.5 W/cm^2 measured below a 1.5-cm-thick water filter at the surface of the monolayer. After irradiation, the culture was kept at 37 °C, 100% humidity without CO_2 for 24 hours.

Metabolic Cell Activity (Cytotoxicity Test) Using the MTT Method

The metabolic activity status was assessed by a colorimetric assay for assessing cell metabolic activity (MTT assay). This method is based on the ability of viable cells to reduce the yellow 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazole bromide (Sigma-Aldrich) to water-insoluble violet formazan. The reaction takes place in the cytoplasm via NADH/NAD$^+$ and NADPH/NADP$^+$. Prior to the MTT assay, the supernatant was gently removed from the pre-adhered cells in the microplate wells. The wells were topped with growth medium supplemented with MTT at a concentration of 0.5 mg/mL (1.2 mM). Subsequently, they were incubated for 4 hours at 37 °C. Again, the cell medium was removed and the water-insoluble blue formazan produced was extracted with 100% dimethyl sulfoxide (DMSO). The microplates were shaken for 10 minutes on a shaker and centrifuged. A volume of 150 μL supernatant from each well with dissolved formazan was transferred to a 96-well flat-bottom polypropylene plate from Corning Inc (Costar; Corning). The absorbance of the extracted formazan was determined at a wavelength of 550 nm with regard to DMSO using a BioTek EON microplate reader from BioTek Instruments, Inc. Based on the results, cytotoxicity (CT) was calculated as the percentage of cells killed according to the formula:

$$CT\% = 100 - (A_b \times 100\ \%) / A_k$$

where A_b denotes absorbance of the test sample and A_k denotes absorbance of control samples.

Evaluation of Hypericin Absorption Measured by Flow Cytometry. Hypericin cell penetration was detected with an inverted research microscope Olympus IX51 with reflected fluorescence system (Olympus Corp) and Color View III digital camera with imaging software Cell F (Soft Imaging System GmbH). The solvent used for the 0.1% HY stock solution was DMSO. The fluorescence intensity of HY in cells as a function of time was determined using a flow cytometer (Becton Dickinson, LSR II) using the PerCP channel. In order to excite the fluorescence of HY, an excitation laser at 488 nm was used and HY fluorescence emission was recorded at 651 nm.

Determination of IL-8 and IL-10 Concentration in Supernatants From SW480 and SW620 Cell Cultures. To measure concentrations of IL-8 and IL-10 released from cancer cells after HY treatment and/or irradiation, the Bio-Plex Pro Assay kit based on xMAP suspension array technology (Bio-Rad Laboratories Inc) was used. Measurements were taken 24 hours after irradiation according to the manufacturer's procedure. The cell culture supernatants were incubated with antibody-conjugated magnetic beads for 60 minutes. Following the incubational period and washing, biotinylated detection antibodies were added and incubated for 30 minutes. Next, the beads were washed and streptavidin-phycoerythrin (PE) was added to each well for 10 minutes. Then, after washing with buffer to remove the unbound streptavidin-PE, the beads were suspended in buffer. The beads bound to each cytokine were analyzed in the Bio-plex Array Reader (Bio-Plex 200 System). The fluorescence intensity was evaluated using Bio-Plex Manager software, and cytokine concentrations were automatically calculated with this software. Standard curves for each cytokine were generated using kit-supplied reference cytokine sample. For each type of test sample, the IL-8 and IL-10 assays were performed in triplicate.

Statistical Method for the Evaluation of Results. Microsoft Excel spreadsheet and Student's *t* test were used for calculations. Mean values and standard deviations were calculated.

Interleukin concentrations were characterized by descriptive statistics such as cardinality (N), arithmetic
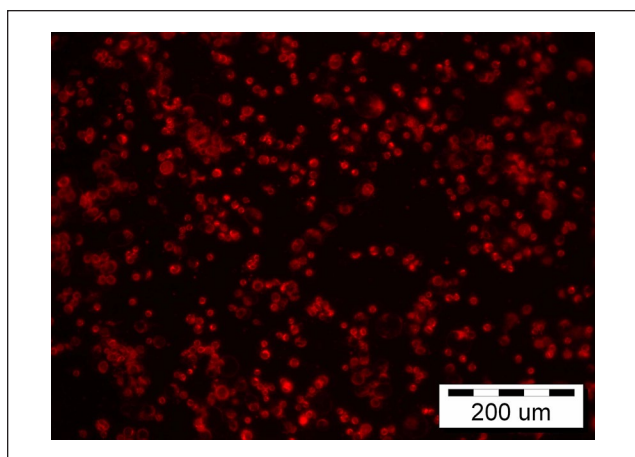
Figure 1. Photograph from an inverted fluorescence microscope after the absorption of hypericin (0.5 μM) by SW480 line cells. A fluorescein isothiocyanate (FITC) filter was used at 200× magnification.
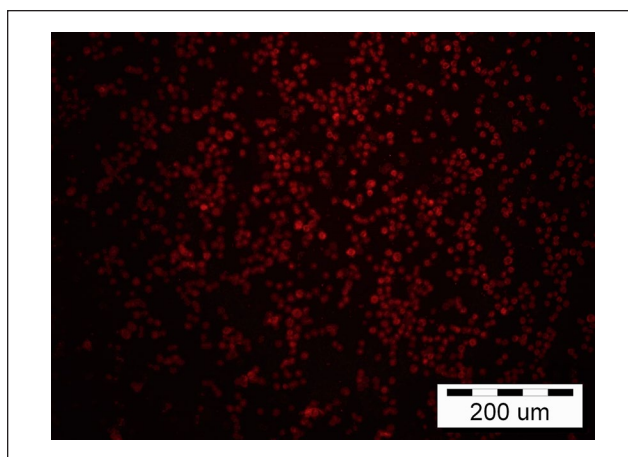
Figure 2. Photograph from an inverted fluorescence microscope after hypericin (0.5 μM) absorption by the SW620 cell line. A fluorescein isothiocyanate (FITC) filter was used at 200× magnification.

mean (mean), standard deviation (SD), minimum, lower quartile (Q1), median, upper quartile (Q3), and maximum. The effects of PDT and HY on IL concentrations in individual cell lines were analyzed by means of linear regression, including light intensity and the dose of HY (as numeric variables) as well as their interaction (labeled ":" between variable names), and then reducing model to optimal using the stepwise reverse method. The value of .05 was assumed as the level of significance. All calculations were made in the R statistical package (v 3.4.3).

Results

Fluorescence and Fluorescence Intensity of Hypericin Absorbed by SW480 and SW620 Cultured Cells

The conducted experiment showed that HY is absorbed by cells without affecting cell viability (Figures 1 and 2).

Cellular Uptake of Hypericin

The uptake of HY was monitored by flow cytometry under conditions that did not alter cell growth or appearance and did not affect cell viability. At various times of incubation with 1 μM, 0.5 μM, and 0.25 μM HY, the cells were analyzed for their red fluorescence. Under these conditions, which are not toxic to the cells, there was a significant increase in HY uptake by the cells as a function of concentration and time. Flow cytometry is a widely accepted method in the field of research regarding cellular uptake of HY. Using this method, we can not only assess the grade of cellular uptake of HY but also observe morphological differences and characterize photodynamic action. For

instance, this method was used to analyze the cellular uptake of HY in human kidney 293 cells with overexpression of glutathione S-transferase P1-1 to modulate their sensitivity to PDT.[36] Immunofluorescence labeling and flow cytometry were also performed on RINm5F insulinoma cells following HY loading and photoactivation.[37] Flow cytometry, together with the microscopy, can be very useful in suggesting morphological differences and in the observation of cells survival. The cellular pharmacokinetic profile of HY is a particularly important application with potential implications for PDT.

For both cell lines, it was observed that fluorescence intensity increased with time and the HY concentration varied linearly with fluorescence intensity (Figure 3a and b).

Metabolic Cell Activity (HY-PDT Cytotoxicity)

Hypericin in the dark possessed no or minimal toxicity. The viability of the SW480 and SW620 cell lines after using HY in concentrations from 0.1μM to 1μM did not change in the absence of irradiation. For cells in the absence of irradiation (0 J/cm^2), the average % cytotoxicity for SW480 cells was 1.8% ± 1.1% and average % cytotoxicity for SW620 cells was 1.9% ± 1.2% ($P > .4$; Figure 4). Furthermore, when using light at a dose of 5 J/cm^2 (SW480 2.4% ± 1.1% cytotoxicity, SW620 4.0% ± 1.7% cytotoxicity; Figure 5) and at 10 J/cm^2 and 0.1 μM to 0.25 μM concentrations of HY, no cytotoxic effect was observed with the exception of 1.0 μM and 10 J/cm^2 where the cytotoxicity of SW480 was 12.3% ± 2% and 31% ± 3% for SW620 (Figure 6). Cytotoxic effects of hypericin on SW480 and SW620 cell lines using light at an energy dose of and 20 J/cm2 and 40 J/cm2 in different doses of HY are presented in Figures 7 and 8.
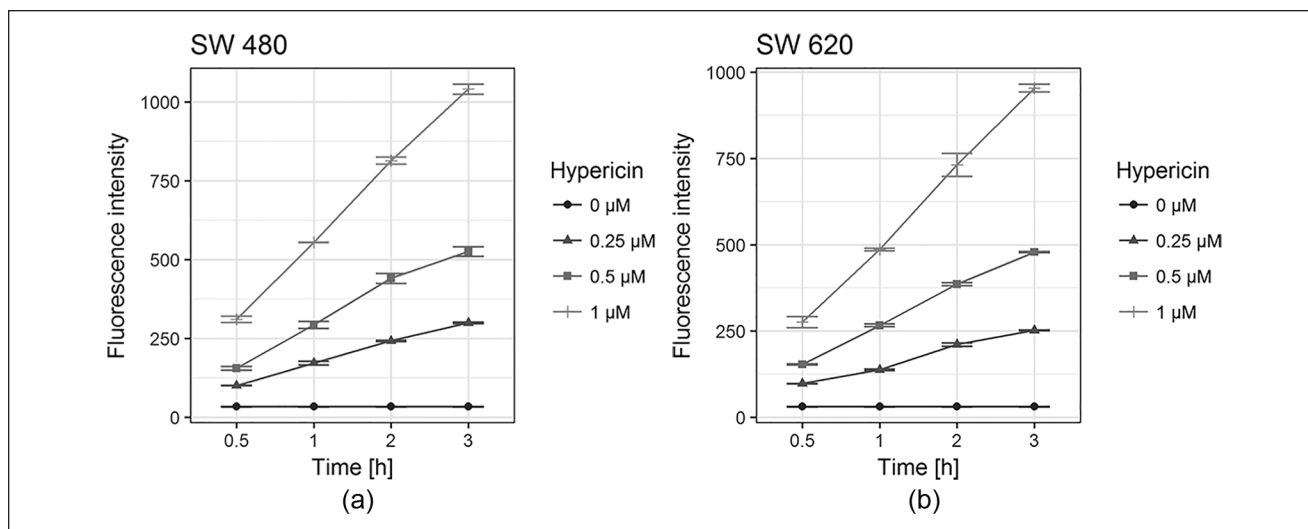
Figure 3. (a) Fluorescence intensity (PerCP channel) of SW480 line cells as a function of time. (b) Fluorescence intensity (PerCP channel) of SW620 line cells as a function of time.
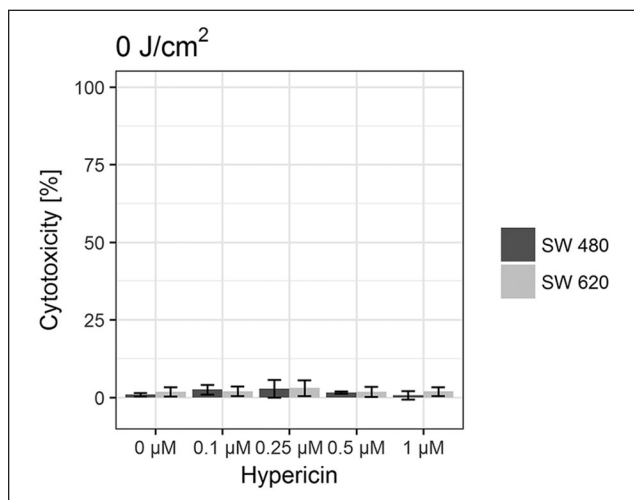
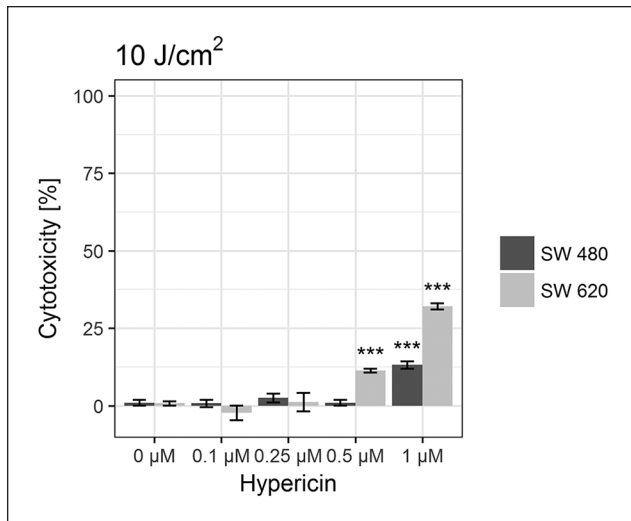**Figure 4.** Cytotoxic effects of hypericin on SW480 and SW620 cell lines using light at a dose of 0 J/cm^2.
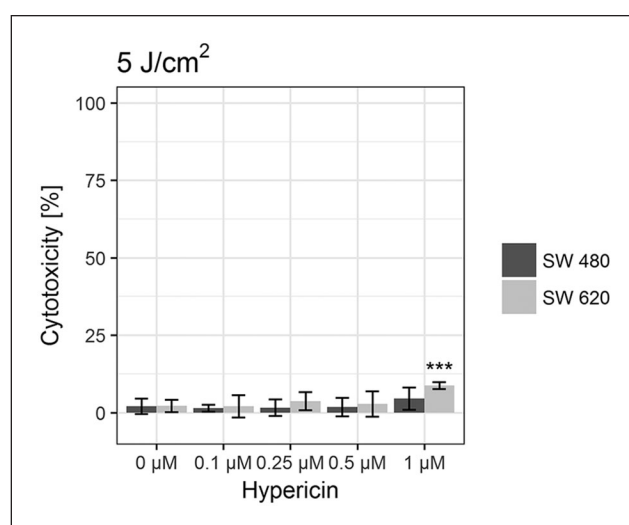
Figure 5. Cytotoxic effects of hypericin on SW480 and SW620 cell lines using light at a dose of 5 J/cm^2.

Measurement of IL-8 and IL-10 Concentration in Treated Cells

Concentrations of IL-8 and IL-10 released from SW480 and SW680 cells after HY and HY-PDT treatment (0, 0.25, and 0.5 μM) with and without irradiation (0, 1, 5, and 10 J/cm^2) were measured using a Bio-Plex Pro Assay kit. The effects of light, HY, and HY-PDT on IL concentrations in individual cell lines were analyzed by descriptive statistics. The descriptive statistics for IL-8 andIL-10 concentrations are presented in Tables 1 to 4. We found that the concentration of IL-8 in the SW480 cell line was affected by the intensity of light. Fluences of 1, 5, and 10 J/cm^2 caused a statistically

significant decreases in IL-8 during HY-PDT in the SW480 cell line (at 1 J/cm^2: $P = .05$, 5 J/cm^2: $P = .035$, and 10 J/cm^2: $P = .035$) in comparison to control (0 J/cm^2; Table 1). In contrast to IL-8 release in SW480 cells, higher concentrations of IL-8 were released in cells of the SW620 line after treatment with HY-PDT (at 1 J/cm^2: $P < 0.01$, 5 J/cm^2: $P = .002$, and 10 J/cm^2: $P = .025$; Table 2). No statistical significant differences in IL-10 concentration were found following HY-PDT in the SW480 cell line (at 1 J/cm^2: $P > .4$, 5 J/cm^2: $P = .1$, and 10 J/cm^2: $P = .075$; Table 3) or in the SW620 cell line (at 1 J/cm^2: $P > .4$, 5 J/cm^2: $P > .4$, and 10 J/cm^2: $P > .4$; Table 4). The concentration of interleukin (IL)-8 in SW480

Figure 6. Cytotoxic effect of hypericin on SW480 and SW620 cell lines using light at an energy dose of 10 J/cm^2.

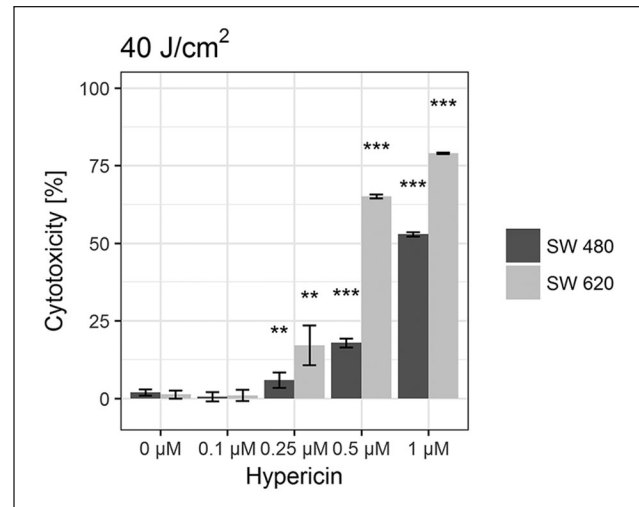**Figure 8.** Cytotoxic effects of hypericin on SW480 and SW620 cell lines using light at an energy dose of 40 J/cm^2.

Figure 7. Cytotoxic effects of hypericin on SW480 and SW620 cell lines using light at an energy dose of 20 J/cm^2.
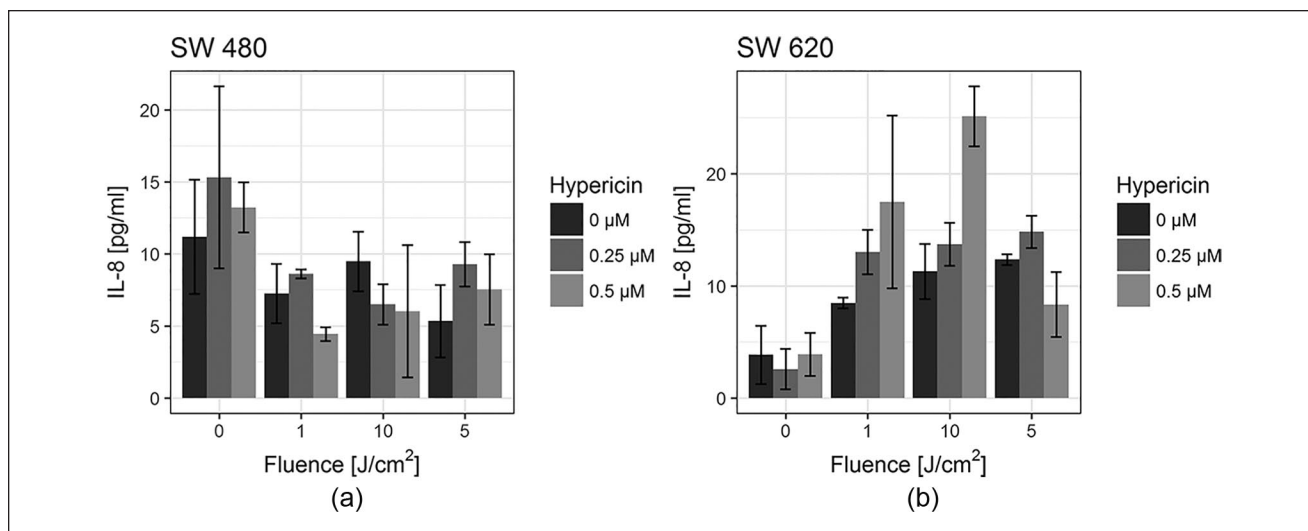
was measured in the SW480 cell line, and no change in IL-10 release was detected in SW 620 cells. Untreated SW480 cells secreted higher levels of IL-8 than in SW620 cells.

Most ILs are produced by cells of the immune system. Here, IL-8 and IL-10 were selected since were found in the supernatants of the cancer cells tested. IL-8 is a chemotactic factor, which in vivo may be a factor leading to the local accumulation of immune cells, which may subsequently initiate inflammation. IL-10 is considered to be one of the immunosuppressive ILs. In addition, secreted cytokines are at relatively low concentrations in relation to immune cells, which also increase the measurement error. Photodynamic effects of HY have been studied in other types of colon cancer cells.[38,39] Other differences between our work and previous studies are lower HY concentrations and longer incubation times (24 hours vs 1 hour). Shorter incubation times in our study may have caused larger differences in results.

Discussion

Aggressive treatment of cancer has significant side effects such as chemotherapeutic toxicity, complications due to radiation, and often mutilating surgical procedures. As an alternative modality of cancer treatment, PDT is comparatively mild but effective without serious side effects, albeit confirmation of the effectiveness requires clinical trials. Often, we can gain an understanding of the mechanisms of PDT when conducted in vitro in cell lines and in in vivo animal studies.

As colon cancer progresses, an immune-tolerant microenvironment seems to be enhanced with balanced, homogeneous cytotoxic immune and cellular responses especially apparent during the late stages of cancer. Conventional cancer treatments can rarely control metastases, and host

as a function of hypericin concentration and light dose (a) and concentration of IL-8 in SW620 as a function of hypericin concentration and light dose (b) are presented in Figures 9. The concentration of IL-10 in SW480 as a function of hypericin concentration and light dose (a) and concentration of IL-10 in SW620 as a function of hypericin concentration and light dose (b) are presented in Figure 10. Untreated SW480 cells secreted significantly higher levels of IL-8 ($P = .015$) than SW620 cells; there was no significant difference in IL-10 secretion between untreated SW480 and SW620 cells ($P = .15$). In summary, HY-PDT was found to increase release of IL-8 in the SW620 cell line and decrease release of IL-8 in the SW480 cell line. An increase in secretion of IL-10

Figure 9. (a) Concentration of interleukin (IL)-8 in SW480 as a function of hypericin concentration and light dose. (b) Concentration of IL-8 in SW620 as a function of hypericin concentration and light dose.
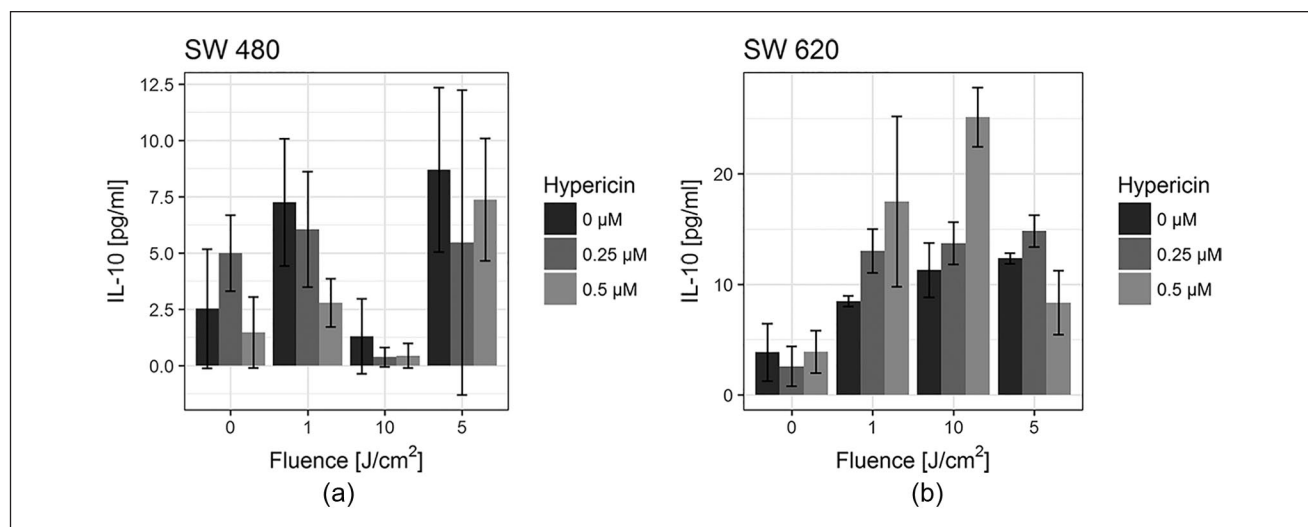
Figure 10. (a) Concentration of interleukin (IL)-10 in SW480 as a function of hypericin concentration and light dose. (b) Concentration of IL-10 in SW620 as a function of hypericin concentration and light dose.

immune system stimulating therapeutic agents are playing an increasing role in the treatment of colon cancer. PDT allows for selective destruction of cancer cells due to release of ROS resulting in apoptosis and necrosis that stimulate release of inflammatory mediator cytokines.

Hypericin is a hydrophobic PS and, as such, must be delivered to tissue via a drug carrier, and it seems to be comparable in many aspects to clinically approved PS with such advantages as high localization in diseased tissue.[40-42] The problem of HY solubility has been overcome by with various methods: potassium salts,[42] binding to serum albumin,[43] and lipid nanoparticle enclosure.[44] HY-PDT is known to have an influence on cell signaling pathways and one of the most important influences is HY-PDT suppression of the MAPK pathway in tumor cells that is necessary for proliferation and differentiation process.[45] HY also has been reported to promote the mitochondrial mechanism of apoptosis (Bax oncoprotein release of cytochrome c, procaspase-9, and procaspase-9 DNA repair proteins).[37,46-48]

The crucial aim in investigations is to preserve the unique phenotype of cells obtained from tissues and to replicate their innate environments. Currently, the 2-dimensional (2D) cell model is used routinely as an experimental gauge. Although there are limitations in the use of this model, especially connected with the huge role of the microenvironmental conditions, cell-extracellular matrix signaling and also

Table 1. Descriptive Statistics for the Concentration of Interleukin (IL)-8 (pg/mL) in SW480 Cells as a Function of Hypericin Concentration and Light Dose.

Fluence (J/cm^2)	Hypericin (μM)	Number of trials	Average concentration of IL-8 (pg/mL)	SD
0	0.00	5	11.19	3.97
	0.25	3	15.32	6.31
	0.50	3	13.23	1.74
1	0.00	5	7.26	2.06
	0.25	3	8.62	0.32
	0.50	3	4.44	0.47
5	0.00	5	5.34	2.50
	0.25	3	9.28	1.54
	0.50	3	7.53	2.45
10	0.00	5	9.49	2.07
	0.25	3	6.50	1.40
	0.50	3	6.03	4.59

Table 3. Descriptive Statistics for pg/mL Interleukin (IL)-10 in SW480 Cells as a Function of Hypericin Concentration and Light Dose.

Fluence (J/cm^2)	Hypericin (μM)	Number of trials	Average concentration of IL-10 (pg/mL)	SD
0	0.00	5	2.54	2.64
	0.25	3	5.00	1.69
	0.50	3	1.48	1.57
1	0.00	5	7.26	2.82
	0.25	3	6.06	2.57
	0.50	3	2.80	1.07
5	0,00	5	8.70	3.65
	0.25	3	5.48	6.77
	0.50	3	7.38	2.72
10	0.00	5	1.31	1.66
	0.25	3	0.39	0.43
	0.50	3	0.45	0.54

Table 2. Descriptive Statistics for the Concentration of Interleukin (IL)-8 (pg/mL) in SW620 Cells as a Function of Hypericin Concentration and Light Dose.

Fluence (J/cm^2)	Hypericin (μM)	Number of trials	Average concentration of IL-8 (pg/mL)	SD
0	0.00	5	3.86	2.60
	0.25	3	2.59	1.81
	0.50	3	3.91	1.92
1	0.00	3	8.48	0.47
	0.25	3	13.04	1.98
	0.50	3	17.49	7.71
5	0.00	3	12.36	0.48
	0.25	3	14.85	1.44
	0.50	3	8.35	2.90
10	0.00	3	11.30	2.47
	0.25	3	13.73	1.91

Table 4. Descriptive Statistics for the Concentration of Interleukin (IL)-10 (pg/mL) in SW620 Cells as a Function of Hypericin Concentration and Light Dose.

Fluence (J/cm^2)	Hypericin (μM)	Number of trials	Average concentration of IL-10 (pg/mL)	SD
0	0.00	5	0.4	0.11
	0.25	3	0.09	0.01
	0.50	3	0.10	0.01
1	0.00	3	0.09	0.01
	0.25	3	0.08	0.01
	0.50	3	0.10	0.01
5	0.00	3	0.10	0.01
	0.25	3	0.10	0.00
	0.50	3	0.09	0.00
10	0.00	3	0.09	0.01
	0.25	3	0.10	0.01
	0.50	3	0.09	0.01

the fact that 2D model creates 1 homogenous layer. Also, extracellular conditions could affect cellular phenotypes, especially hypoxic conditions. These facts can disturb the objective results of therapeutic effect of HY-PDT, assessed on the 2D model. It was also shown that cells cultured in 3D cell models are more resistant to treatment than the cells cultured in 2D cell models.[49] Some authors suggest experiments on 3D spheroid models or chorioallantoic membrane (CAM) models. To examine the effect of HY-PDT, we chose the 2D model, which is extremely useful for assessing the effect of this form of therapy at the level of the colorectal cancer cell initially before clinical trials to assess the effectiveness of HY-PDT and also to assess the possible adverse effect of this form of therapy.[50]

We have found that reduction in cell viability in HY-treated cells requires light, and the cytotoxic effect of HY-PDT on SW480 and SW620 depends on HY concentration, the dose of light energy, and the degree of malignancy. Yonar et al

investigated HY-PDT–induced biophysical changes in colon cancer cells, which agree with our observation of HY accumulation in colon cancer cells.[51] In their study, HT-29 colon adenocarcinoma cells were subjected to HY-PDT at HY concentrations of 0.04, 0.08, and 0.15 μM. Electron paramagnetic resonance (EPR) spectra of spin-labeled adenocarcinoma cells were evaluated post HY-PDT with the EPRSIMC program demonstrating concentration-dependent changes in the domain parameters that indicated an increase in HY concentration. Siboni et al found that HY can internalize to colon cancer cells via free diffusion, pinocytosis, and endocytosis, and consequently subcellular sites are specifically targeted.[52] Furthermore, experiments performed by Mühleisen et al[53] agree with our results indicating that the cytotoxic effect of HY-PDT depends on the concentration of the PS and the dose of energy. We found a significant dose-dependent increase of intracellular HY during the incubation time. Mühleisen et al[53]

characterized toxicity and HY-mediated effects on cancer cells in vitro and confirmed that the cytotoxicity depends on concentration and illumination time. Additionally, a double-targeting strategy consisting of magnetic accumulation and laser-induced photoactivation by loading HY on superparamagnetic iron oxide nanoparticles (SPIONs) and guiding them to the desired place using an external magnetic field (referred to as magnetic drug targeting) was demonstrated, which improved HY-PDT effectiveness, specificity, with a reduction in side effects.[53]

Research by Mikeš et al[54] indicated that the main mechanism of HY cytotoxicity effect is accumulation of ROS and not higher HY concentration. Kleban et al[55] provided confirmation of this effect and demonstrated that HY-PDT induced cell cycle arrest and apoptosis presumably through the mitochondrial pathway. A study by Ali et al[56] supports the idea that HY-PDT induces apoptosis. The authors detected cellular fluorescence localization of HY predominantly in mitochondria and lysosomes. Plasma membrane damage was also found by electron microscopy at large HY dose ($>5\ \mu M$), resulting in necrosis as opposed to apoptosis at sublethal lower dose ($<2.5\ \mu M$).[56] Sanovic et al[21] found that low-dose HY-PDT has additional immunologic effects. BALB/c mice with CT26 colon carcinoma were intravenously given low-dose HY followed by irradiation after 30 to 240 minutes. Light applied after 30 minutes of HY administration eradicated the colon cancer cells; rechallenge of these mice with CT26 mouse colon carcinoma cells prevented new tumor growth. The advantages of low-dose PDT are less drug and light exposure, a nondestructive effect on the skin, earlier lesion healing, and induction of an antitumor immune response.[21]

Our study supports prior observations that HY-PDT facilitates not only tumor cell death but also inhibition of tumor cell proliferation in a dose-dependent manner with sublethal dose IL release. Our results in sublethal HY-PDT confirm the immunomodulatory effects of HY-PDT reported by Sanovic et al.[21] This effect results from a decrease in IL-8 concentration in SW480 cells and an increase of this cytokine concentration in SW620 cells, which may result in increased chemotaxis and stimulation of immune mechanisms in the case of higher grade malignant cells. In lower grade cells, the opposite effect is observed. Exposure of colorectal cancer cells to HY-PDT did not affect the progression of tumor cells dependent on the release of IL-10, which is related to the lack of differences in IL concentration for both the SW480 and SW620 cell lines after PDT, compared with controls. Tumor cells of lower grade SW480 secrete higher concentrations of IL-8 and IL-10 than higher grade cells SW620. The concentration of IL-10 in SW620 cells in the presence of both HY and light exposure alone is also lower than in cells of the SW480 line. Exposure of colorectal cancer cells to HY-PDT has an immunomodulatory effect leading to the release of factors that stimulate IL-8-dependent immune mechanisms and does not affect

the progression of cancer cells conditioned by IL-10. Experimental results may have clinical justification in the application of PDT in the treatment of colorectal cancer.

Garg et al reported that HY-PDT induces autophagy, necrosis, apoptosis, and induction of dendritic cell maturation with IL-6 production and proliferation of CD4(+) or CD8(+) T-cells.[57] Bhuvaneswari et al[58] revealed that a combination of HY-PDT and Avastin (bevacizumab) improves tumor response by downregulating angiogenic proteins improving tumor response to treatment. Experiments demonstrated that the targeted therapy by Avastin in conjunction with HY-PDT can improve tumor responsiveness in bladder tumor xenografts.[58] Estimation of IL-6 and IL-8 showed, as in our study, a decrease of these cancer progression markers in groups treated with HY-PDT.[58] Majerník et al,[18] in experiments performed on colon cancer cell models (HT-29, HCT 116 and CT26.WT) 3D spheroid and CAM models, reported that despite the cytotoxic effect of HY-PDT, an amplified appearance of some chosen pro-angiogenic factors was revealed.

Furthermore, Du et al[59] revealed that the type of cell, degree of histological differentiation, and basal expression of cytokines influence cytokine response following HY-PDT.[59] IL-6 expression was found in 2 different types of NPC post HY-PDT. IL-6 transcription was increased in PDT-treated CNE-2 poorly differentiated cells, but not in HK1 well-differentiated cells. In vivo, IL-6 mRNA expression increased in PDT-treated CNE-2 tumors but not in HK1 tumors.[59] Also, in 2 Epstein-Barr virus (EBV)–positive NPC cell lines, HK1 and CNE-2, endogenous production of IL-8 and IL-10 in vitro was observed following HY-PDT, although the level of IL-8 was 2-fold higher in supernatant from HK1 cells.[60] The authors revealed that HY increased IL-8 significantly only in HK1 cells, although HY-PDT did not alter the expression of IL-8 levels. The authors confirmed our observations that IL-10 is not induced by HY-PDT. Although it is known that PDT can upregulate IL-8 transcription via ROS and activates the IL-10 promoter, it was observed that IL-8 levels in 2 EBV-positive NPC cell lines were not altered, nor was production of IL-10 observed.[60]

In this work, the cytotoxic effect of PDT with HY was found to be dependent on the concentration of the PS, the dose of light energy, and the degree of malignancy of colon cancer cells. Control experiments showed that HY at a concentration of $1\ \mu M$ are absorbed by the SW480 and SW620 cell lines, and these concentrations do not affect cell viability. PDT with HY showed immunomodulatory effects resulting from a decrease in IL-8 concentration in SW480 cell culture and an increase of this cytokine concentration in SW620 cells, which may result in increased chemotaxis and stimulate immune mechanisms in the case of higher malignant cells. In lower grade cells, the opposite effect was observed. Exposure of colorectal cancer cells to HY-PDT did not affect the progression of tumor cells dependent on

the release of IL-10, which is related to the lack of differences in IL concentration for both the SW480 and SW620 cell lines after PDT, compared with controls.

Tumor cells of lower grade SW480 secrete higher concentrations of IL-8 and IL-10 than higher grade cells SW620. The concentration of IL-10 in SW620 cells in the presence of both HY and light exposure alone is also lower than in cells of the SW480 line. Exposure of colorectal cancer cells to HY-PDT has an immunomodulatory effect leading to the release of factors that stimulate IL-8–dependent immune mechanisms and does not affect the progression of cancer cells conditioned by IL-10. Experimental results obtained in the experiment may have clinical justification in the application of PDT in the treatment of colorectal cancer.

Conclusion

Previous studies have demonstrated that HY-PDT can inhibit capillary development, disturb cancer cell progression by influencing cell migration, and stimulate release of cytokines responsible for the progression of cancer and metastasis. These outcomes are in agreement with our findings, and results focused on colon cancer cell activity demonstrated not only a cytotoxic effect of HY-PDT but also a sublethal dose influence on secretion of factors responsible for cancer progression. Based on these research findings, we suggest that HY-PDT, which can both eliminate and control a primary tumor via cytotoxic effects, can, at sublethal doses, affect IL release by colon cancer cells. In this experiment, this influence depended on the level of tumor cell metastatic activity. In our opinion, it is imperative to confirm this effect in clinical studies of HY-PDT.

Authors' Note

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Declaration of Conflicting Interests

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