Original Research

Oxidative alterations induced *in vitro* by the photodynamic reaction in doxorubicin-sensitive (LoVo) and -resistant (LoVoDX) colon adenocarcinoma cells

Julita Kulbacka[1], Agnieszka Chwiłkowska[1], Julia Bar[2], Andrzej Poła[3], Teresa Banaś[1], Andrzej Gamian[1,4] and Jolanta Saczko[1]

[1]Department of Medical Biochemistry, Medical University, Chalubinskiego 10; [2]Department of Clinical Immunology, Medical University, Mikulicza-Radeckiego 7; [3]Department of Biophysics, Medical University, Chalubinskiego 10, 50-368 Wroclaw; [4]Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Weigla 12, 53-114 Wroclaw, Poland
Corresponding author: Julita Kulbacka. Email: jkulbacka@gmail.com

Abstract

In photodynamic therapy (PDT) a tumor-selective photosensitizer is administered and then activated by exposure to a light source of appropriate wavelength. Multidrug resistance (MDR) is largely caused by the drug efflux from the tumor cell by means of P-glycoprotein, resulting in reduced efficacy of the anticancer therapy. This study deals with photodynamic therapy with Photofrin® (Ph) on colon cancer cell lines (doxorubicin-sensitive and -resistant). The cells were treated with 15 and 30 μg/mL Ph and then irradiated by a light dose of 3 or 6 J/cm^2 (632.8 nm). After irradiation the cells were incubated for 0, 3 or 18 h. Crucial factors of oxidative stress (thiobarbituric acid reactive substances [TBARS], protein damage, thiazolyl blue tetrazolium bromide [MTT] assay), changes in cytosolic superoxide dismutase (SOD1) activity after photodynamic reaction (PDR), and the intracellular accumulation of photosensitizers in the cells were examined. Moreover, the expressions of glutathione *S*-transferase (GST)-pi, a marker protein for photochemical toxicity, and secretory phospholipase A_2, a prognostic and diagnostic marker for colon cancers, were determined. After PDR, increases in SOD1 activity and the level of TBARS were observed in both cell lines. The level of protein-associated –SH groups decreased after PDR. Both cell lines demonstrated stronger GST-pi and PLA_2 expression after PDR, especially after 18 h of incubation. The increasing level of reactive oxygen species following the oxidation of sulfhydryl cell groups and lipid peroxidation influence the activity of many transporters and enzymes. The changes in SOD1 activity show that photodynamic action generates oxidative stress in treated cells. Our study presents that PDR caused oxidative alterations in both examined colon adenocarcinoma cell lines. However, the MDR cells reacted more slowly and all oxidative changes occurred in the delay.

Keywords: photodynamic reaction, oxidative stress, colon adenocarcinoma, multidrug resistance

Experimental Biology and Medicine 2010; **235**: 98–110. DOI: 10.1258/ebm.2009.009162

Introduction

Photodynamic therapy (PDT) is a cancer treatment that involves systemic administration of a tumor-localizing photosensitizer which, when activated by the appropriate wavelength of light, interacts with molecular oxygen to form primarily a toxic short-lived species known as singlet oxygen and its reactive forms (ROS). ROS are believed to be responsible for the cytotoxic action of PDT. The photosensitizer localizes to the malignant tissue and the light is also focused on the lesion.[1] The cellular mechanisms of PDT are still under investigation. Mitochondria are considered to be the site where photosensitizers are best localized for efficient cell killing with PDT.[2] However, porphyrin-mediated PDT has been shown to cause the inactivation of numerous mitochondrial enzymes, inhibition of adenosine triphosphatase, and uncoupling of oxidative phosphorylation.[3]

The vast majority of colorectal cancers are adenocarcinomas, which arise from pre-existing adenomatous polyps that develop in the normal colonic mucosa. Most colorectal cancers show resistance to anticancer drugs clinically and experimentally.[4] The clinical use of chemotherapy in cancer treatment is limited by the occurrence of multidrug resistance (MDR) associated with the overexpression of membrane transporters, one of the best known being P-glycoprotein (Pgp), which actively expels drugs from tumor cells.[5,6] MDR-1/Pgp decreases the intracellular drug concentration by the active extrusion of natural products, anticancer drugs, and other

hydrophobic compounds from tumor cells, thereby causing drug resistance.[7] Multiple detoxification mechanisms are probably involved. Glutathione *S*-transferases (GSTs) have recently attracted interest in diagnosing and monitoring malignancy.[8,9] They have a considerably important role in the detoxification of carcinogens. GSTs are present in many species and tissues and also in relatively large amounts in the epithelial tissues of the human gastrointestinal tract.[4] In cancer cells with resistance to doxorubicin (DOX), overexpression of GST-pi is also suggested to influence cellular redox status through the suppression of DOX conversion to semiquinone free radical and the subsequent production of superoxide anion radicals and peroxides. Thus the overexpression of GST-pi is related to the development of drug resistance in cancer cells not only by increased detoxification of anticancer agents, but also by suppression of cellular ROS which induce cell death.[9]

The present study compares the early response to the photodynamic reaction (PDR) of sensitive colon adenocarcinoma cells and cells with the MDR-1 phenotype of expression. We applied the conventional photosensitizer Photofrin®, which is the most used in clinical practice. This photosensitizer has the ability to bind to low-density lipoproteins whose receptors are expressed on tumor cells.[10] To investigate the PDR we localized the photosensitizer and determined cytotoxicity in the cells. We determined the levels of lipid peroxidation (TBARS – thiobarbituric acid reactive substances) and protein damage (the level of protein-associated thiol groups) as the main marker of oxidative stress. To study the primary inflammation response we measured the expression of secretory phospholipase A_2 ($SPLA_2$), which directly mediates the release of arachidonic acid and is involved in many acute and chronic diseases. $SPLA_2$ exhibits features similar to C-reactive protein (CRP) as a marker of plaque inflammation.[11,12] To monitor malignancy and detoxification we measured the expression of GST, which is found to be expressed in most tumors.[8] Superoxide dismutase (SOD1) activity was determined as an antioxidant defense. Superoxide dismutases (SOD) are essential enzymes that eliminate superoxide radical (O_2^-) and thus protect cells from the destruction induced by free radicals.[13]

Materials and methods

Cell lines

Two human colon adenocarcinoma cell lines were used: doxorubicin-sensitive (LoVo) and doxorubicin-resistant (LoVoDX). The cell lines were purchased from the Institute of Immunology and Experimental Therapy, Polish Academy of Sciences. The cells were grown in F-12 Ham medium nutrient mixture (Sigma) with addition of 10% fetal bovine serum (FBS), 2 mmol/L glutamine, 1% of antibiotic solution (containing 10,000 U/mL penicillin, and 10 mg/mL streptomycin [Sigma]) in 25 cm^2 Falcon flasks. The cells were maintained in a humidified atmosphere at 37°C and 5% CO_2. For the tests cells were used at passage numbers >20. For the experiments the cells were removed by trypsinizing and washed with phosphate-buffered saline (PBS).

Photodynamic treatment

The cells were treated with 15 and 30 μg/mL Photofrin® (Ph; QLT PhotoTherapeutics, Inc, Vancouver, Canada) in complete media for four hours in the dark. Then they were irradiated by a light dose of 3 or 6 J/cm^2 using a lamp (OPTEL, Opole, Fibre Illuminator, Poland) with polarized light (fluence rate at the level of the cell monolayer: 10 mW/cm^2) and a red filter ($\lambda_{max} = 632.8$ nm). All irradiations were performed at room temperature. After irradiation the cells were incubated in a humidified atmosphere at 37°C and 5% CO_2 for 0, 3, or 18 h.

Photosensitizer distribution

Microcultures were derived from the culture dishes and harvested in concentration ca. 1000 cells on cover glasses (24 × 24 mm, Thermo scientific) in Petri dishes for 24 h. Then the cells were incubated with Ph at a concentration of 30 μg/mL for one and four hours. After incubation the cells were washed in PBS and fixed in 4% buffered formalin, then washed in PBS and water (Aqua pro injections; Polpharma). For cytoplasm identification, cells were incubated with 5 μmol/L CellTracker™ Green CMFDA (5-chloromethylfluorescein; Molecular Probes, CA, USA). For nuclei visualization, cells were incubated for 20 min in the presence of 300 nM DAPI (4′,6-diamidino-2-phenylindole; Molecular Probes). Then the samples were examined under a confocal microscope with an epi-fluorescence system (Nikon Eclipse TE 2000-E). For Ph localization a filter was used with an excitation wavelength of 528–553 nm and emission wavelength of 578–633 nm. For cytoplasm visualization the filter had an excitation wavelength of 465–495 nm and an emission wavelength of 515–555 nm and for nuclei filters the excitation wavelength was 340–380 nm and the emission wavelength 435–485 nm.

MTT assay

Cell viability was determined by the thiazolyl blue tetrazolium bromide (MTT) assay (Sigma, Germany; In Vitro Toxicology Assay). The cells were seeded onto 96-well microculture plates at 1×10^4 cells/well and allowed to attach overnight. The medium was removed and replaced with fresh medium with or without Ph. The cells were incubated for four hours and then irradiated. After PDT the cells were washed twice in PBS and treated according to the manufacturer's protocol. The absorbance was determined using a multiwell scanning spectrophotometer at 570 nm (Labsystem Multiscan MS type 352). Mitochondrial function was expressed as the percentage of viable treated cells relative to untreated control cells (without light and Ph). The experiments were repeated three times.

Protocol for cells preparation to SOD, TBARS and –SH groups

Cells were harvested in 25 cm^2 flasks (Falcon, Becton Dickinson France SAS BD, France) to obtain full monolayer (one week harvesting enables obtaining 5×10^6 cells). After PDR *in vitro*, cells were removed by trypsinization (0.25%

Trypsin-EDTA, Sigma) and put in 15 mL centrifuge probes (Falcon). Trypsin and medium was removed by centrifugation for 4 min at 537*g*. Then each sample was washed twice in PBS.

Determination of SOD activity

Each sample of the cells were suspended in 50 mM phosphate buffer, pH 7, with a mixture of protease inhibitors (Complete Mini EDTA-free; Roche). Total intracellular SOD activity was measured spectophotometrically using a Ransod kit purchased from Randox Laboratories Ltd (Antrim, UK) according to the manufacturer's protocol.

Lipid peroxidation (TBARS)

Cell samples (ca. 5×10^6) were suspended in 200 μL of PBS. Then 200 μL of 15% TCA (trichloric acid; Roth, Germany) in 0.25 mol/L HCl and 200 μL of 0.37% TBA (2-thiobarbituric acid; Fluka, Germany) in 0.25 mol/L HCl were added. The control sample contained 200 μL of deionized water instead of the cell suspension. Then the samples were incubated for 20 min at 90°C (Termoblock TB-941 U). After incubation, the samples were centrifuged for 5 min at 5000 rpm. Malondialdehyde (MDA), the final product of fatty-acid peroxidation, reacts with TBA to form a colored complex. The level of TBARS was measured by the absorbance at a wavelength of 535 nm (Jasco V-530). The concentration of MDA was quantified spectrophotometrically based on a set of MDA standards of known concentration.

Protein damage: the level of thiol groups

Cells (ca. 5×10^6) were suspended in 200 μL of PBS. Then 200 μL of 10% SDS (sodium dodecyl sulfate; Biochemical) in 10 mmol/L phosphate buffer, pH 8.0, (buffer A) and 200 μL of 0.04% DTNB (5,5′-dithiobis-2-nitrobenzoic acid 3,3′-6; Fluka) in buffer A were added. The control sample contained 200 μL of buffer A instead of the cell suspension. Then all the samples were refilled with 1.6 mL buffer A. Next the samples were incubated for one hour at 37°C in a water bath. The determination of protein damage was based on Ellman's method. This method uses the reaction of 5,5′-dithiobis-2-nitrobenzoic acid (DTNB acid) with the thiol groups (–SH) of proteins.[14]

Immunocytochemistry: the expressions of GST-pi and group II sPLA$_2$

Immunocytochemistry was performed using the ABC method. Briefly, cells were plated onto eight-well glass slides (100–200 cells/well; Thermo Scientific, Germany) and then PDT was performed. The cultures were then fixed and dehydrated using 4% paraformaldehyde and an ethanol gradient, respectively. The samples were then permeabilized and blocked by incubation with 0.1% Triton X-100 in PBS. Inducible nitric oxide synthase was visualized with rabbit polyclonal antibodies (GST-pi, 1:100, Novoacstra; group II sPLA$_2$, 1:100, Santa Cruz, CA, USA). For conventional bright-field microscopy (peroxidase-ABC labeling, DAKO LSAB 2 kit), the samples were incubated with a diaminobenzidine-H_2O_2 mixture to visualize the peroxidase label and counterstained with hematoxylin for 30 s. The samples were examined on an Olympus microscope (Japan). The stained cells were determined by counting 100–200 cells in three randomly selected fields. Each slide was counted by two independent investigators. Cells were judged positive if the color reaction was observed in more than 5% of the cells. The intensity of immunocytochemical staining was evaluated as (−) negative (no stained reaction), (+) weak, (++) moderate and (+++) strong. Positive and negative controls were performed. All experiments were repeated twice.

Statistical analysis

Statistical analysis was performed using commercial Statistica version 6.0 software. The Shapiro–Wilk test of normality was performed. The significance of differences between the mean values of substantive different groups of cells was assessed by Student's *t*-test. In the statistical evaluations a level $P \leq 0.05$ for every data group was regarded as statistically significant.

Results

Photosensitizer distribution

In Figures 1A–D the intracellular location of Ph after one and four hours is presented. After one hour of incubation the fluorescence intensity in the LoVoDX cells was strong and the photosensitizer was mainly distributed in the cell membranes and partially in the cytoplasm (Figure 1B). After four hours the fluorescence was weaker, dispersed throughout the whole cytoplasm (Figure 1D). However, the LoVo cells showed the same level of fluorescence after one and four hours (Figures 1A and C). There were no significant differences. Ph was distributed throughout the whole cytoplasm and cell membrane.

MTT assay

The MTT test was performed immediately after PDT and after 3 and 18 h. Obtained results are presented in Figures 2a and b. Because of nonsignificant differences of control values after 0, 3 and 18 h, only basic controls taken immediately after irradiation are presented on the plots. A significant decrease in oxidoreductive mitochondrial activity was observed directly after the PDR in the LoVo cells and after three hours of incubation in the cells resistant to DOX. Post three hours IC_{50} was achieved for all Ph and light doses in the case of LoVoDX cells. However, 18 h of incubation after PDR with 3 J/cm^2 irradiation induced an increase in mitochondrial activity in the LoVoDX cells by about 20%. This study revealed statistically significant attenuation of mitochondrial activity in LoVoDX cells for all the applied PDT doses ($P \leq 0.004$–0.01) except for the weakest dose of 15 μg/mL and 3 J/cm^2. Incubation with Ph (without light) or cell irradiation only did not affect mitochondrial activity.

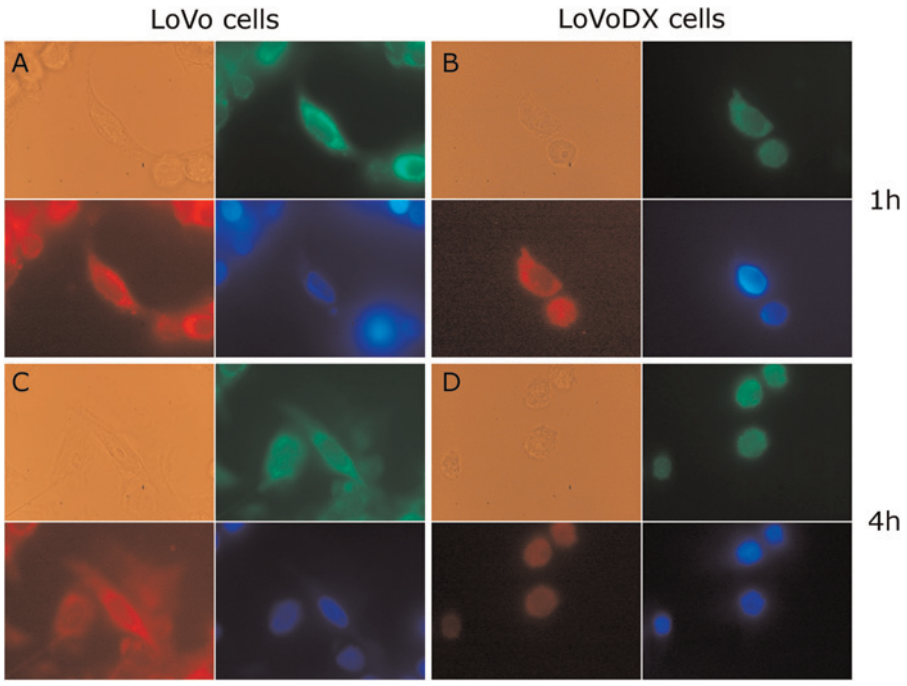

Figure 1 Photofrin distribution in LoVo (A and C) and LoVoDX (B and D) cells. (A) and (B): After one hour incubation with photosensitizer ($c_{ph} = 30\ \mu g/mL$); (C) and (D): after four hours incubation with photosensitizer. All figures contain control view of cells (upper left side of A–D); cytoplasm stained CytoTracker Green (upper right side of A–D); photofrin localization (lower left side of A–D); nuclei stained DAPI (lower right side of A–D) (×1000)

The LoVo cells demonstrated sensitivity directly after PDR and after 18 h of incubation. Statistically significant decreases in cell viability were observed at 6 J/cm^2 and at both Ph concentrations (15 and 30 μg/mL) ($P = 0.0342$ and $P = 0.0199$).

Determination of SOD activity

The highest SOD activity in the LoVo cells was observed directly after PDR (5 U/mg of protein for 30 μg/mL and 3 J/cm^2) (Figure 3a). After three hours of incubation, we found enzyme activity decreased to 2 U/mg of protein for the same dose of PDT and to 0.5 U/mg of protein for the other doses. In Figure 3a, a slight but statistically significant increase in SOD activity after 18 h of incubation post-PDR ($P = 0.0317$) is shown.

In the LoVoDX cells the highest SOD activity was detected three hours after PDR (Figure 3b). At the dose of 6 J/cm^2 and 30 μg/mL it even reached 48 U/mg of protein and for the remaining doses it was about 20 U/mg of protein. Directly after PDR and after 18 h of incubation the levels of SOD activity were similar and reached about 1 U/mg of protein (results comparable to controls cells without PDR application). There was no statistically significant increase in SOD activity related to PDT doses (Ph concentration and light dose). However, the results are statistically significant for the LoVoDX cells if we consider the time of incubation post-PDR ($P \leq 0.027$–0.043).

Lipid peroxidation

The TBARS levels in the LoVo cells were similar directly after PDR and after three hours of incubation and reached 0.7 μmol/L. This was not a significant deviation from the TBARS level of the control cells. Only after 18 h of incubation after PDR was lipid peroxidation more intense and TBARS increased by about 100% (Figure 4a). In the LoVoDX cells the TBARS level increased directly following PDR (0.9 μmol/L). After 3 and 18 h of incubation, we could observe no large decrease in TBARS level by 0.2 μmol/L (Figure 4b). In the LoVo cells, lipid peroxidation increased with the time of incubation, while in the LoVoDX cells it reached comparable levels for all doses of PDT. For both Ph concentrations and for both light doses we obtained statistically significant increases in TBARS level ($P \leq 0.02$). The results were statistically analyzed according to PDT dose and time of incubation.

Protein damage: the level of the thiol groups

The strongest protein-associated –SH group degradation (Figures 5a and b) was observed in both cell lines after three hours of incubation post-PDR. Directly after PDR the cells showed only a slight decrease in –SH concentration compared with the control cells (for LoVo by 5 nmol/L and for LoVoDX by 10 nmol/L) (Figure 5). The figure shows that the level of –SH was lowest for LoVo cells after 3 and 18 h post-PDR (about 20 nmol/L). In the LoVoDX cells the level of thiols was lower than 10 nmol/L and increased slightly to over 15 nmol/L after 18 h. The resistant cells responded only after 3 h post-PDR. These experiments showed a statistically significant decrease in the thiol groups ($P \leq 0.004$–0.01). In the LoVo cells, statistically significant protein degradation was obtained after three hours of incubation ($P \leq 0.045$).

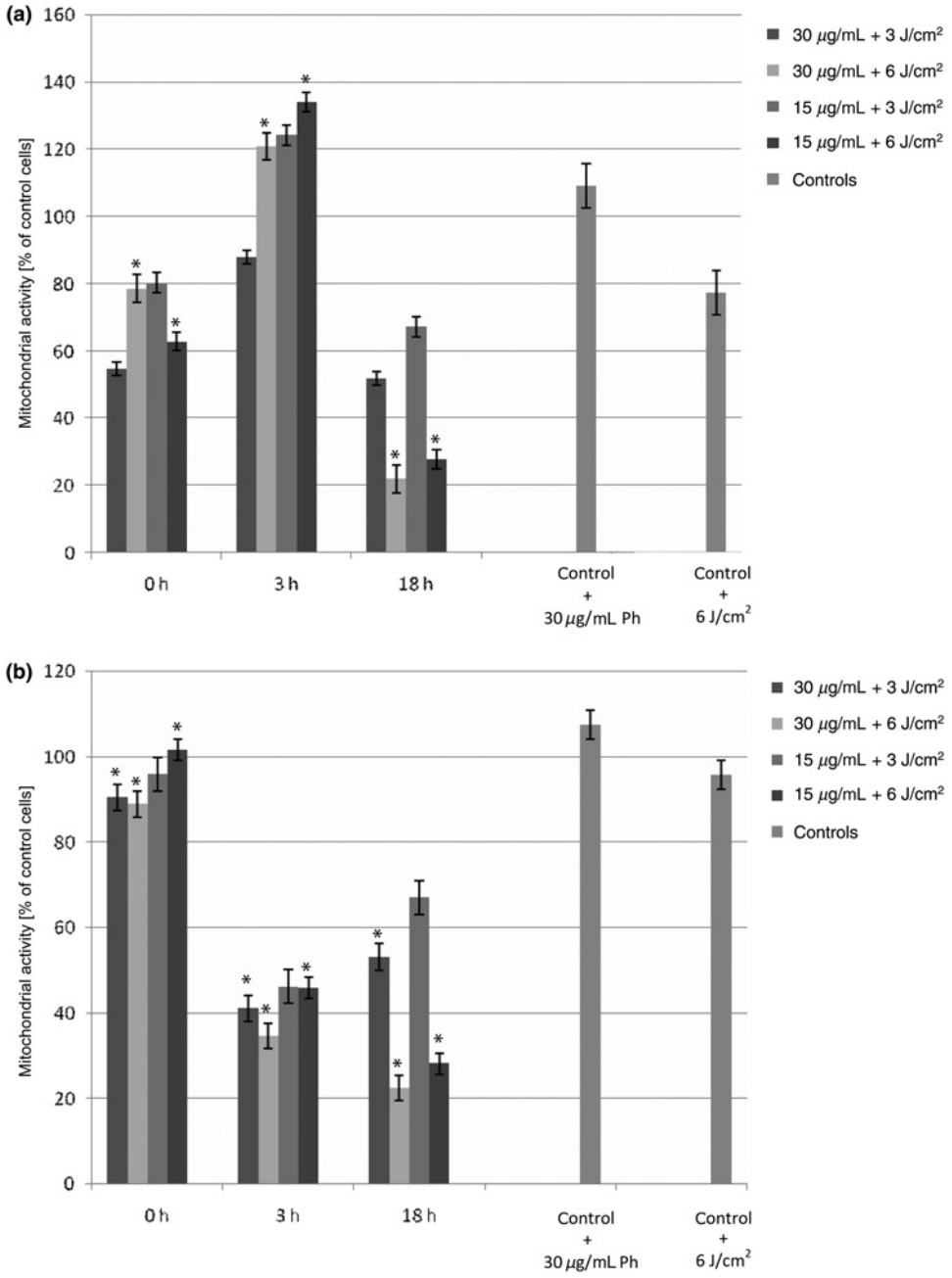

Figure 2 Determination of oxidoreductive mitochondrial activity (MTT assay) in LoVo (a) and LoVoDX (b) cells after photodynamic reaction with photofrin treatment in concentrations 15 and 30 μg/mL, and light dose 3 and 6 J/cm^2, for 0, 3 and 18 h incubation post-irradiation. Controls: cells + 30 μg/mL Ph – incubated 4 h with photosensitizer prior assay; cells + 6 J/cm^2 – cells incubated 0 h after irradiation before assay. Error bars shown are means ± SD for $n = 4$; $^*P < 0.05$

Expression of GST-pi and group II sPLA$_2$

The expressions of GST-pi and sPLA$_2$ in the LoVo and LoVoDX cell lines are presented in Figures 6 and 7. Results of the immunocytochemical studies are presented in Tables 1–4. Slightly positive GST-pi and sPLA$_2$ expressions are detectable in the control cells without therapy. A positive GST-pi reaction was observed at all the PDT doses and times of incubation. In the LoVo cells the intensity of the reaction increased directly after PDR and increased proportionally to incubation time (Figures 6A, C and E). Very characteristic for LoVo cells is the expression of GST-pi in the nuclei observed directly after PDR (Figure 6A). The strongest GST-pi expression was observed after 18 h. Moreover, morphological features of cell disintegration were observed after this time.

In the case of the LoVoDX cells an increase in reaction intensity was observed directly after PDR, but after three hours of incubation the GST-pi expression decreased (Figures 6B, D and F). Single cells with positive reaction were observed. The strongest immunocytochemical reaction was observed after 18 h of incubation post-PDR and, similar to LoVo cells, cell disintegration was observed (Figure 6G). After this time, we could also detect strong GST-pi expression in the cell membrane. It is significant that 100%

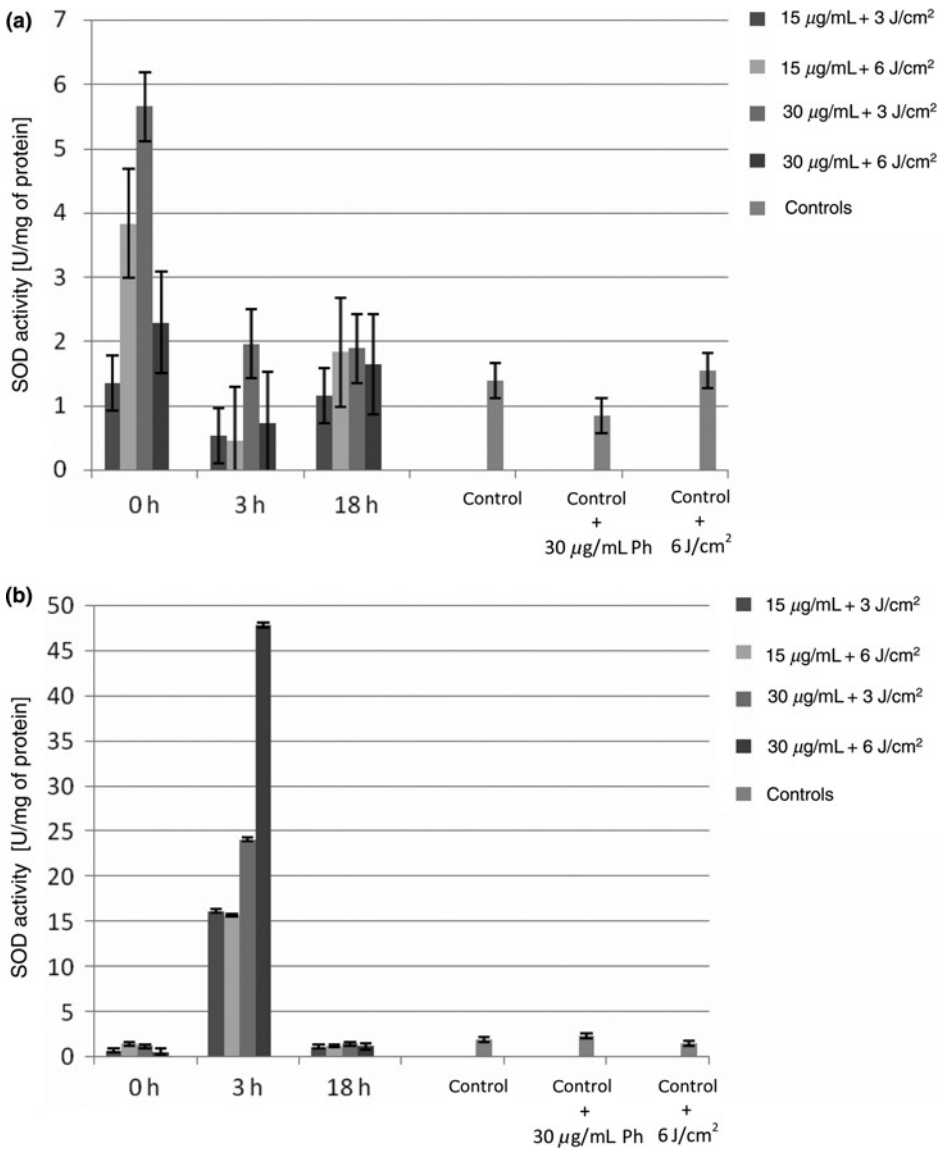

Figure 3 Total superoxide dismutase activity (SOD) after photodynamic reaction in (a) LoVo cells and (b) LoVoDX cells. Controls: control cells – untreated cells without PDR; cells + 30 μg/mL Ph – incubated four hours with photosensitizer prior assay; cells + 6 J/cm^2 – cells incubated 0 h after irradiation before assay. Error bars shown are means ± SD for $n = 3$

of the cells underwent reaction; only 50% of the LoVoDX cells initially did. After 3 and 18 h we observed reaction in 100% of the cells.

sPLA$_2$ expression increased in the cytoplasm of the cells incubated with the photosensitizer and was stronger in the LoVo cells (Figure 7B). In the LoVo cells the intensity of the immunocytochemical reaction increased proportionally to the time of incubation post-PDR (Figures 7A and C). Only for the longest time (18 h) was the reaction observed in all cells in the cytoplasm, cell membranes, and nuclei (Figure 7C). Directly after PDR and after three hours of incubation, the reaction was visible in 50% of the LoVo cells. The LoVoDX cells showed intensive reaction in the first phase of PDT (Figure 7E). Then, after three hours (Figure 7D), over 70% of the cells underwent reaction and after 18 h almost 100%.

Discussion

The location of the photosensitizer used in cancer cells seems to be the decisive aspect of the efficiency of PDT. It was observed that hematoporphyrin derivatives can selectively accumulate in malignant cells.[15–17] Afterwards, these photosensitizers enter the cytoplasm, where they can sensitize enzymes such as pyruvate kinase and make contact with the external mitochondrial membrane for a short time, affecting monoamine oxidase.[18,19] In the present study it was demonstrated that Ph localizes to the cytoplasmic area and the cell membrane in the LoVoDX drug-resistant cells after one hour. After four hours the intensity of Ph fluorescence was weaker. Similarly to the work of Saczko *et al.*,[2] these results might suggest the influence of the overexpression of Pgp in the cell membrane on

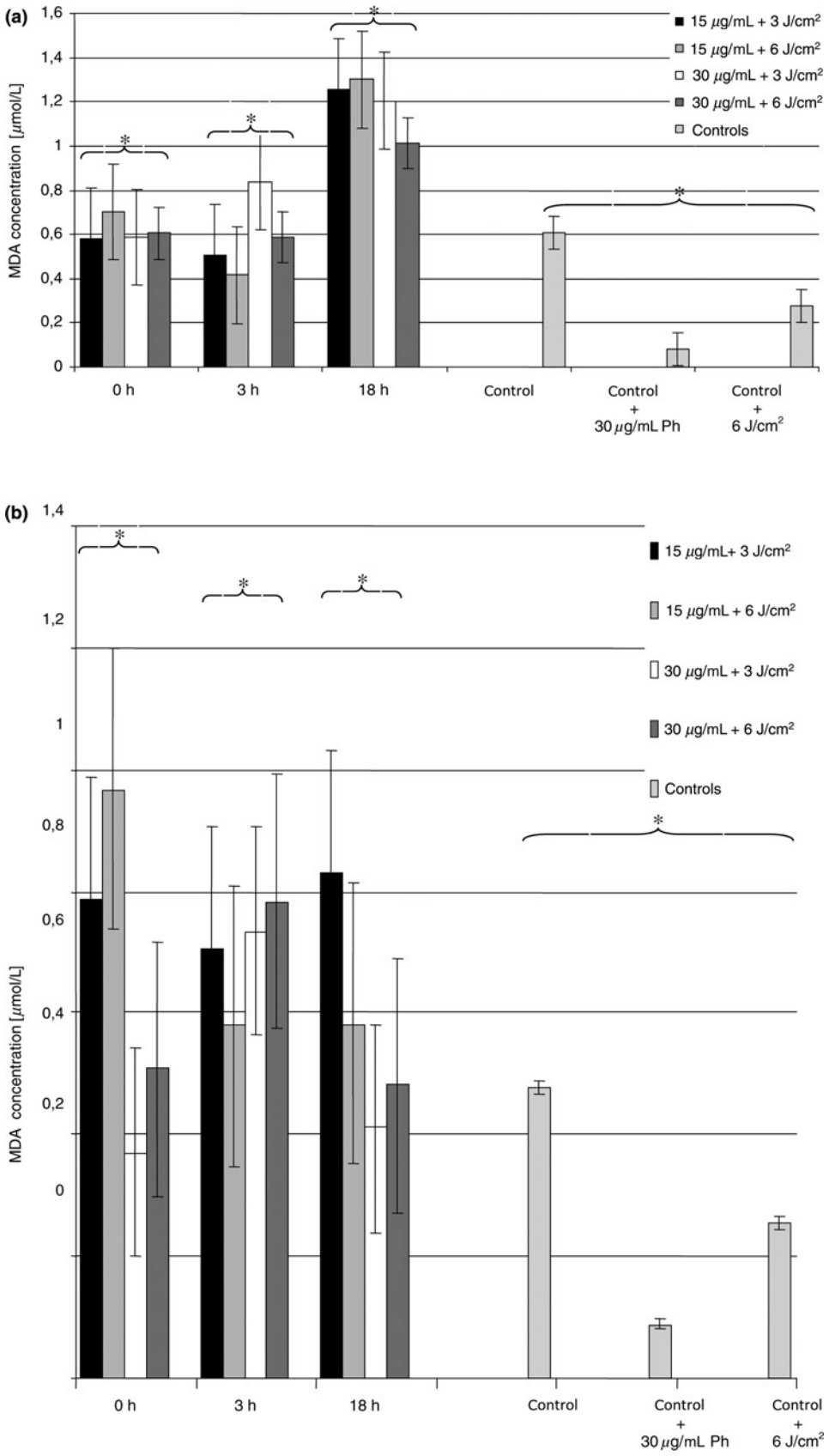

Figure 4 The level of malondialdehyde in (a) LoVo cells; (b) LoVoDX cells after photodynamic reaction. Controls: control cells – untreated cells without PDR; cells $+30\,\mu g/mL$ Ph – incubated 4 h with photosensitizer prior assay; cells $+\,6\,J/cm^2$ – cells incubated zero hours after irradiation before assay. Error bars shown are means $\pm$ SD for $n = 3$; $^*P < 0.05$

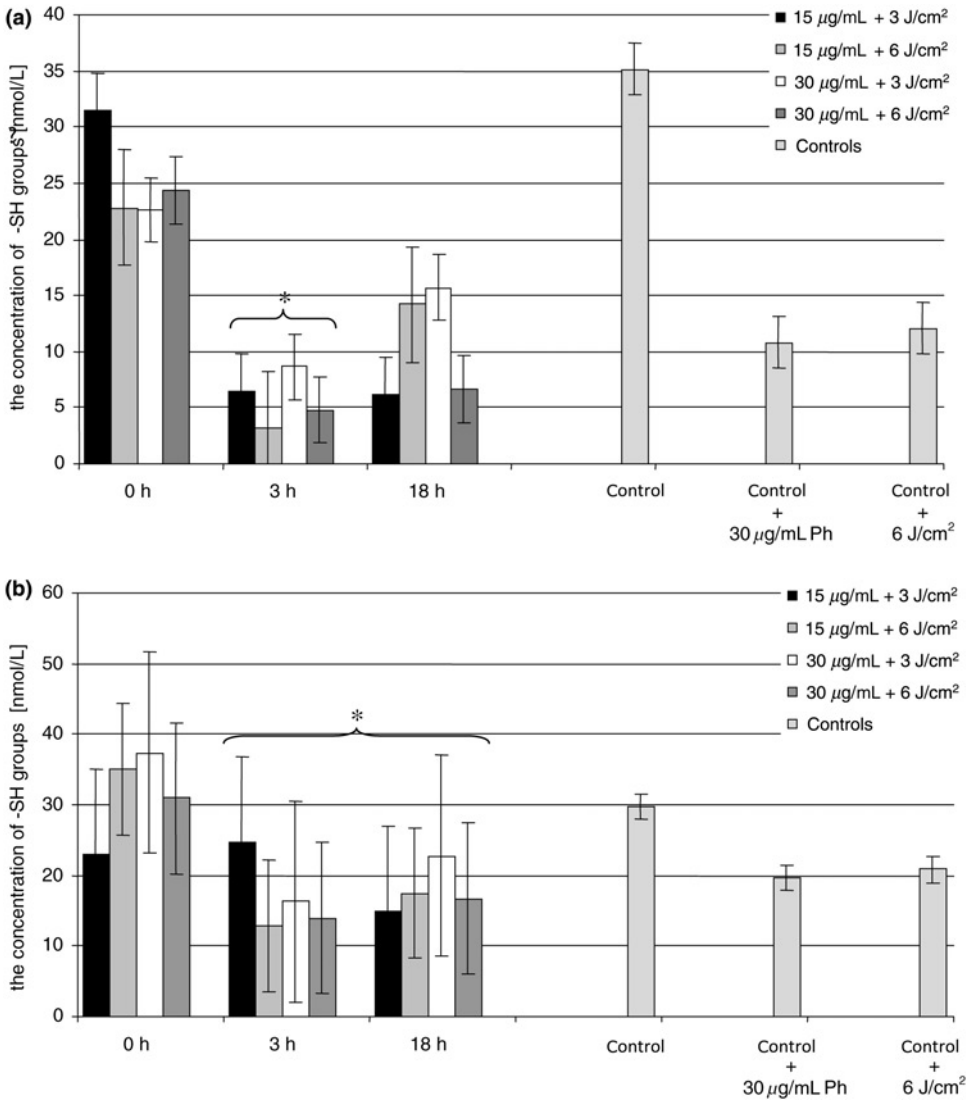

Figure 5 The level of thiol groups (–SH) in (a) LoVo cells; (b) LoVoDX cells after photodynamic reaction. Controls: control cells – untreated cells without PDR; cells + 30 μg/mL Ph – incubated four hours with photosensitizer prior assay; cells + 6 J/cm^2 – cells incubated zero hours after irradiation before assay. Error bars shown are means ± SD for $n = 3$; $^*P < 0.05$

the reduced concentration of the photosensitizer in cells during incubation. Our results suggested that Ph-PDT would be more efficient in sensitive cells because photosensitizer located in the cell membrane would be not pumped out by Pgp. Many studies have demonstrated that the resistance phenotype may be involved in PDT effectiveness.[20] Some studies have revealed several proteins directly involved in MDR, these being metallothioneins (MT), GST, Pgp, multidrug-resistance proteins (MRP), and type II topoisomerases.[4] Aliya *et al.* confirm that a high level of GST-pi expression can be a prognostic factor in malignant diseases. They observed that each organ has an individual level of GST-pi, which is a key factor involved in chemosensitivity.[9] Guo *et al.* indicate that GST-pi could detoxify chemical mutagenesis, cancer promoters, lipids, and DNA hyperoxidase and protect normal cells from the influence of carcinogenic materials. GST-pi might play an important role in the antimutation process. The authors suggest that the characteristic nuclear expression of GST-pi can be a significant marker in colon and rectum cancers.[21] Our results also demonstrate GST-pi overexpression in the nuclei of LoVo cells immediately after PDR. After longer incubation times the nuclear enzyme expression was not detectable. This can be evidence for the effectiveness of the applied therapy and the implicated GST-pi mechanism.

Volm *et al.* indicated significant correlation between high levels of Pgp and GST-pi with decreased level of topoisomerase II, and the expressions of c-fos, epidermal growth factor receptor (EGFR) and c-neu proteins. The oncoproteins c-fos and c-jun create a transcription factor, AP1, which activates the transcription of specific genes encoding Pgp and GST-pi. Thus c-fos activity can involve two resistance mechanisms based on MDR-1 and GST-pi.[22,23]

Decreased mitochondrial membrane potential, disturbance of the electron transport chain, and excessive release of ATP can be observed as consequences of oxidative stress.[19,24,25] The determination of mitochondrial oxidoreductive activity provides essential information about the

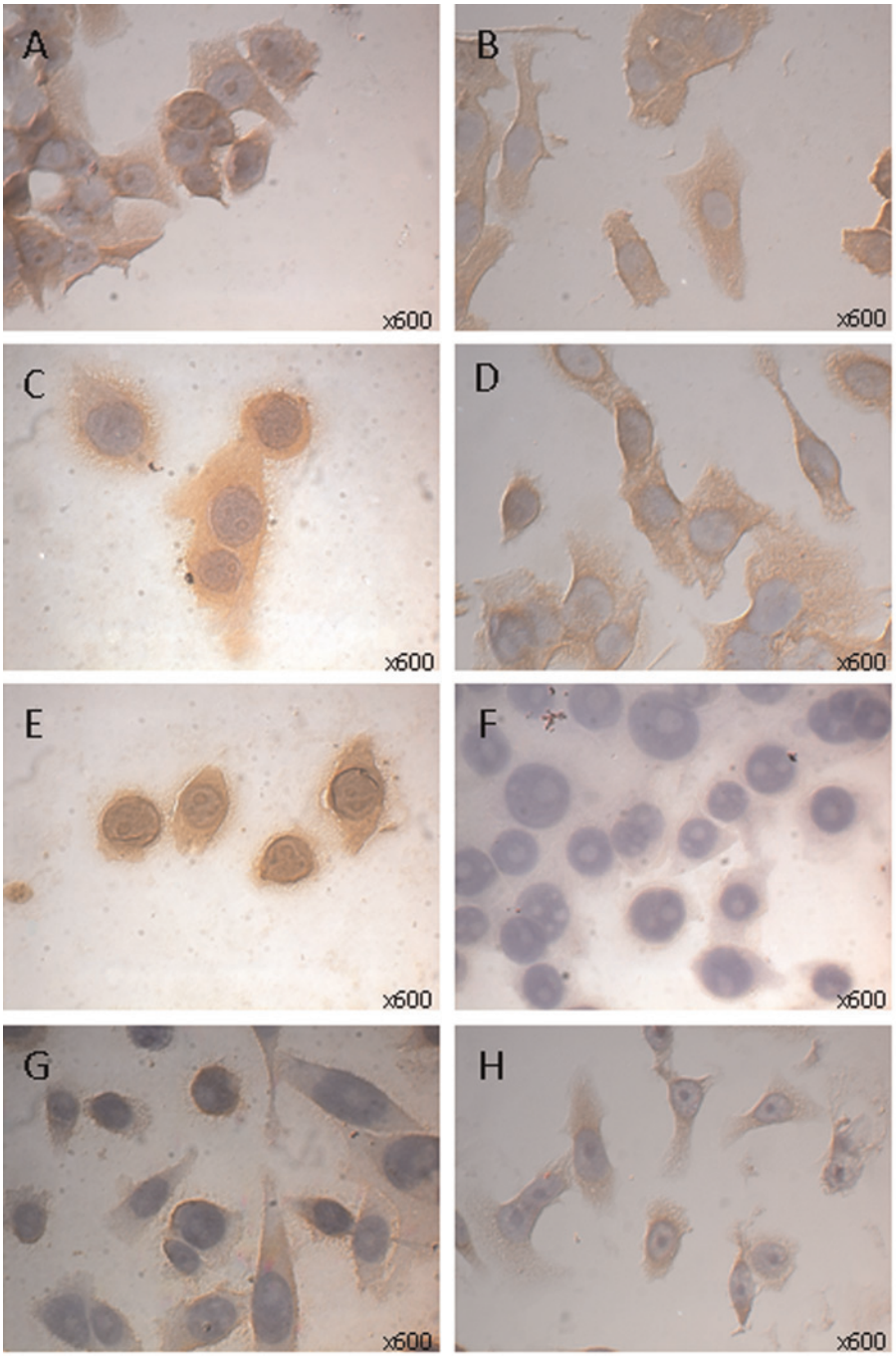

Figure 6 Expression of *S*-glutathione transferase pi in (A) LoVo cells immediately post-PDR (light dose = 3 J/cm^2, c_{Ph} = 15 μg/mL); (B) in LoVoDX cells immediately post-PDR (light dose = 3 J/cm^2, c_{Ph} = 15 μg/mL); (C) in LoVo cells after 3 h incubation post-PDR (light dose = 6 J/cm^2, c_{Ph} = 30 μg/mL); (D) in LoVoDX cells immediately post-PDR (light dose = 6 J/cm^2, c_{Ph} = 30 μg/mL); (E) in LoVo cells after three hours incubation post-PDR (light dose = 3 J/cm^2, c_{Ph} = 15 μg/mL); (F) in LoVoDX cells after three hours incubation post-PDR (light dose = 3 J/cm^2, c_{Ph} = 15 μg/mL); (G) in LoVoDX cells after 18 h incubation post-PDR (light dose = 6 J/cm^2, c_{Ph} = 15 μg/mL); (H) in LoVoDX cells after four hours incubation post-PDR without irradiation (c_{Ph} = 30 μg/mL). ($\times$600; in all figures except (F) Nomarski contrast was applied) (A color version of this figure is available in the online journal)

energetic potential of the cell. Many studies have demonstrated that mitochondria are the main target of PDT. Mitochondrial breakage or activity disturbance is the main result of phototoxicity.[26] The results of the present study showed that Ph showed the strongest decrease in mitochondrial activity after 18 h of incubation post-PDR in both types of colon cancer cells (sensitive and resistant), but the LoVoDX cells demonstrated a little increased mitochondrial response after 18 h post-PDR, while the viability of the LoVo cell line decreased. Thus we conclude the the optimal time to achieve decreased mitochondrial dehydrogenase activity for LoVo cells is 18 h and for LoVoDX cells three hours after applying the therapy. Such a weakness of cells could be a turning point for the application of the next PDR dose or other equivalent therapy. Leunig's experiments also proved that Ph is not toxic. Only after irradiation did cell

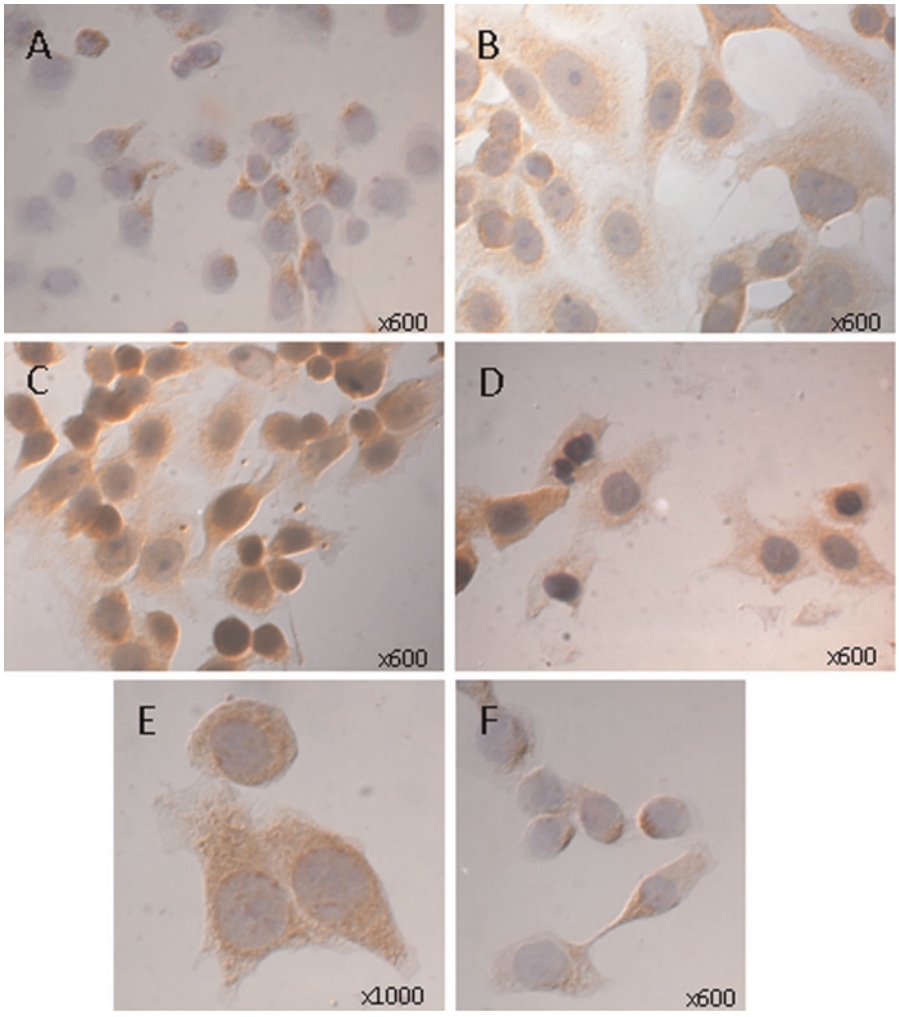

Figure 7 Expression of secretory phospholipase A_2 in (A) LoVo cells immediately post-PDR (light dose = 3 J/cm^2, c_{Ph} = 30 μg/mL); (B) in LoVo cells after 4 h incubation post-PDR without irradiation (c_{Ph} = 30 μg/mL); (C) in LoVo cells after 18 h incubation post-PDR (light dose = 3 J/cm^2, c_{Ph} = 15 μg/mL); (D) in LoVoDX cells after three hours incubation post-PDR (light dose = 3 J/cm^2, c_{Ph} = 15 μg/mL); (E) in LoVoDX cells immediately post-PDR (light dose = 3 J/cm^2, c_{Ph} = 30 μg/mL); (F) in LoVoDX cells after four hours incubation post-PDR without irradiation (c_{Ph} = 30 μg/mL). (In figures Nomarski contrast was applied) (A color version of this figure is available in the online journal)

Table 1 Expression of S-glutathione transferase pi in LoVo cells after photodynamic reaction

Incubation time post PDR	PDT dose		Intensity of immunocytochemical reaction*	% of immunopositive cells
	Light [J/cm^2]	Photofrin® [μg/mL]		
Control	0	0	+ (printed reaction)	8
Control with Ph	0	30	++	50
Immediately post PDR	3	15	++	100
		30	++	100
	6	15	++/+++	100
		30	+++	46.9
3 h	3	15	++	100
		30	++	100
	6	15	++	100
		30	++	100
18 h	3	15	++/+++	Single disintegrated cells indicating very strong reaction
		30	+++	
	6	15	+++	
		30	+++	

*The evaluation of stained reaction: (−) negative, no reaction, (+) weak, (++) moderate and (+++) strong

Table 2 Expression of S-glutathione transferase pi in LoVoDX cells after photodynamic reaction

Incubation time post PDR	PDT dose		Intensity of immunocytochemical reaction*	% of immunopositive cells
	Light [J/cm^2]	Photofrin® [μg/mL]		
Control	0	0	+ (printed reaction)	10
Control with Ph	0	30	++	23
Immediately post PDR	3	15	+++	43.2
		30	++	33.1
	6	15	++	27.1
		30	++	30.2
3 h	3	15	+	100
		30	+/++	100
	6	15	+	100
		30	+	90
18 h	3	15	++	70
		30	++	100
	6	15	+++	Collapsed cells
		30	+++	Collapsed cells

*The evaluation of stained reaction: (−) negative, no reaction, (+) weak, (++) moderate and (+++) strong

viability decrease, particularly at the highest dose of Ph (15 μg/mL).[27] Marchal *et al.* applied PDT with m-tetrahydroxyphenylchlorin (mTHPC) in a model of cultured HT29 adenocarcinoma cells. Early events of cell death were assessed by evaluating mitochondrial response to mTHPC-mediated PDT, cytochrome c release and membrane depolarization.[26] Studies with Photofrin, tin ethyl etiopurpurin (SnET2), and porphycene-monomer (PcM) demonstrated decreased intracellular ATP. Mitochondrial function has a key role in regulating the apoptotic pathways initiated by PDT.[28]

PDT causes damage to mitochondria and induces reactive oxygen species. In the current study we determined some factors of oxidative stress, i.e. lipid peroxidation, the level of protein degradation (thiol level), and SOD1 activity. During PDT, proteins undergo many modifications resulting from the direct reaction of amino acid residues with reactive oxygen species or other oxidants. In this study we observed the highest activity of SOD immediately after the PDR in LoVo cells; LoVoDX cells had increased activity only three hours after PDR. For the other times of incubation we observed decreased SOD activity level. These results could confirm the theory that the decrease in SOD activity can be induced by decreased synthesis velocity, oxidative modifications, and inactivation of the active site of the enzyme.[29,30] Similar results were obtained by Johnson *et al.* The authors applied PDT with hypericin on mouse EMT6 cells. The highest SOD activity was in the first phase of PDT for the first 30 min, then the activity suddenly fell to the control level. The rapid cell response to PDT suggests that mitochondria are the initial target of therapy.[30] Current studies showed that PDT caused increased antioxidant enzyme activity and expression.[2,31]

The results demonstrated in the present study on colon adenocarcinoma cells indicate a decreased level of thiol groups and increased TBARS with increasing incubation time post-PDR. These results suggested that LoVoDX cells reacted to PDR more slowly, and protein regeneration was observed after a long time of incubation. These studies demonstrate that MDR cells responded weaker to PDT with Photofrin. We obtained similar results in lung adenocarcinoma cells (A549).[32] Proteins undergo modifications during PDT by the direct reaction of amino acid residues with reactive oxygen forms or other free radicals. Some studies indicate that thiol groups are a better marker of oxidative stress than total antioxidant capacity (TAC).[33]

Table 3 Expression of secretory phospholipase A_2–IIA ($sPLA_2$) in LoVo cells after photodynamic reaction

Incubation time post PDR	PDT dose		Intensity of immunocytochemical reaction*	% of immunopositive cells
	Light dose [J/cm^2]	Photofrin® [μg/mL]		
Control	0	0	+ (printed reaction)	14.9
Control with Ph	0	30	++	34.1
Immediately post PDR	3	15	++/+	39.6
		30	++/+	39.3
	6	15	++	52.8
		30	++	54.2
3 h	3	15	++	25.7
		30	+++	100
	6	15	++	30
		30	+	90
18 h	3	15	+++	100
		30	+++	100
	6	15	+++	100
		30	+++	68.5

*The evaluation of stained reaction: (−) negative, no reaction, (+) weak, (++) moderate and (+++) strong

Table 4 Expression of secretory phospholipase A_2–IIA ($sPLA_2$) in LoVoDX cells after photodynamic reaction

Incubation time post PDR	PDT dose		Intensity of immunocytochemical reaction*	% of immunopositive cells
	Light dose [J/cm^2]	Photofrin® [μg/mL]		
Control	0	0	+	28.6
Control with Ph	0	30	+/++	70.8
Immediately post PDR	3	15	+/++	25.5
		30	+/++	62.2
	6	15	+	69.6
		30	+	65.4
3 h	3	15	++	26.7
		30	+	78.8
	6	15	+	21.2
		30	+	23
18 h	3	15	++	90
		30	++	
	6	15	+++	Single cells
		30	+++	

*The evaluation of stained reaction: (−) negative, no reaction, (+) weak, (++) moderate and (+++) strong

Consequent to oxidative modification, a change in protein activity can appear involving changes in structure and, finally, inactivation. Other protein modifications depend on lipids and carbohydrates. Oxidative reactions can cause weakened interactions between lipids and proteins, membrane protein modification and fragmentation, and the loss of membrane integrity and resulting cell death.[34]

Numerous internal transformations involve lipid metabolism and intracellular Ca^{2+} regulation. Several studies indicate that PDT leads to rapid changes in intracellular Ca^{2+} concentration. In many cases, a fast release of arachidonic acid metabolites was observed.[35] The release of arachidonic acid and prostaglandin synthesis E_2 was induced by phospholipase A_2.[36] The main role of phospholipase A_2 is mediating in the inflammatory state. There is an hypothesis that group II PLA_2 is a regulating factor in metabolic processes.[12,37,38] In the present study we demonstrated that the strongest $sPLA_2$ expression appeared directly after PDR and after 18 h. The reaction was observed in the cytoplasm and nuclear envelope. Characteristic was a significant decrease in the level of $sPLA_2$ after three hours of incubation post-PDR (20% cells with reaction). The control cells also indicated a permanent level of this enzyme and this was comparable to other studies performed by Laye *et al.* and Jensen *et al.* The authors indicated overexpression of $sPLA_2$-II gene and protein in human adenocarcinomas of the colon, rectum and ventriculus.[37,39] There are two potentially contrasting roles for $sPLA_2$ in colon cancer; one is protection against polyp formation and the other is the release of arachidonic acid for prostaglandin production and subsequent tumor promotion.[12,39] Measurement of tumor markers such as GSTs and $sPLA_2$ in plasma and tissue samples can be used as supportive evidence for colonoscopic observations and diagnosis and for other markers.

Our study suggests that multidrug resistance requires more intensive examinations to not be the eliminating factor in photodynamic treatment. Many specific transport proteins are involved in the mechanisms of MDR; it is only one of the reasons for the lower efficacy of anticancer therapies. A good solution could be a combination of PDT and MDR modulators, electroporation (in preparation), or other antitumor therapies (chemo- and radiotherapy). Such combinations could cause slow tumor progression.

ACKNOWLEDGMENT

All experiments were sponsored by Statutory Funds of the Wroclaw Medical University supported by the Polish Ministry of Science and Higher Education.

REFERENCES

1 Zawacka-Pankau J, Krachulec J, Grulkowski I, Bielawski KP, Selivanova G. The p53-mediated cytotoxicity of photodynamic therapy of cancer: recent advances. *Toxicol Appl Pharmacol* 2008;**232**:487–97
2 Saczko J, Kulbacka J, Chwilkowska A, Pola A, Lugowski M, Marcinkowska A, Malarska A, Banas T. Cytosolic superoxide dismutase activity after photodynamic therapy, intracellular distribution of photofrin II and hypericin, and P-glycoprotein localization in human colon adenocarcinoma. *Folia Histochem Cytobiol* 2007;**45**:93–8
3 Shen XY, Zacal N, Singh G, Rainbow AJ. Alterations in mitochondrial and apoptosis-regulating gene expression in photodynamic therapy-resistant variants of HT29 colon carcinoma cells. *Photochem Photobiol* 2005;**81**:306–13
4 Sutoh I, Kohno H, Nakashima Y, Hishikawa Y, Tabara H, Tachibana M, Kubota H, Nagasue N. Concurent expressions of matallothionein, glutathioneS-tansferase-π, and P-glycoprotein in colorectal cancers. *Dis Colon Rectum* 2000;**43**:221–32
5 Wang HP, Hanlon JG, Rainbow AJ, Espiritu M, Singh G. Up-regulation of Hsp27 plays a role in the resistance of human colon carcinoma HT29 cells to photooxidative stress. *Photochem Photobiol* 2002;**76**:98–104
6 Pajak B, Orzechowski A. Złożony charakter niewrażliwości immunologicznej ludzkiego raka jelita grubego na niektóre cytokiny (TNF-alfa, interferony) na przykładzie linii komórkowej COLO 205. Mechanizm niewrażliwości – z uwzględnieniem białek sygnałowych*. *Postepy Hig Med Dosw* (online) 2004;**58**:428–37
7 Filipitis M. Mechanisms of cancer: multidrug resistance. *Drug Discov Today: Disease Mech* 2004;**1**:229–34
8 Nomani H, Ghobadloo SM, Yaghmaei B, Rezvanie NA, Yaghmaei K. Glutathione S-transferases activity in patients with colorectal cancer. *Clin Biochem* 2005;**38**:621–4
9 Aliya S, Reddanna P, Thyagaraju K. Does glutathione *S*-transferase Pi (GST-Pi) a marker protein for cancer? *Mol Cell Biochem* 2003;**253**:319–27
10 Jalili A, Makowski M, Świtaj T, Nowis N, Wilczyński GM, Wilczek E, Chorąży-Massalska M, Radzikowska A, Maśliński W, Biały Ł, Sieńko J, Sieroń A, Adamek M, Basak G, Mróz P, Krasnodębski IW, Jakóbisiak M, Gołąb J. Effective photoimmunotherapy of murine colon carcinoma

induced by the combination of photodynamic therapy and dendritic cells. *Clin Cancer Res* 2004;**10**:4498–508
11 Piek JJ, de Winter RJ. Type II secretory phospholipase A2: The emerging role of biochemical markers of plaque inflammation. *Eur Heart J* 2003;**24**:1804–6
12 Mounier C, Wendum D, Greenspan E, Flejou J-F, Rosenberg DW, Lambeau G. Distinct expression pattern of the full set of secreted phospholipases A2 in human colorectal adenocarcinomas: sPLA2-III as a biomarker candidate. *Br J Cancer* 2008;**98**:1–9
13 Huang P, Feng L, Oldham EA, Keating MJ, Plunkett W. Superoxide dismutase as a target for the selective killing of cancer cells. *Nature* 2000;**407**:390–5
14 Bartosz G. Druga twarz tlenu, Wolne rodniki w przyrodzie. In: *Warszawa PWN*, 2003;Part 4:343–93
15 Croce AC, Supino R, Lanza KS, Locatelli D, Baglioni P, Bottiroli G. Photosensitizer accumulation in spontaneous multidrug resistant cells: a comparative study with Rhodamine 123, Rose Bengal acetate and Photofrin®. *Photochem Photobiol Sci* 2002;**1**:71–8
16 Du H-Y, Olivo M, Tan BK-H, Bay B-H. Hypericin-mediated photodynamic therapy induces lipid peroxidation and necrosis in nasopharyngeal cancer. *Int J Oncol* 2003;**23**:1401–5
17 Castano AP, Demidova TN, Hamblin MR. Mechanisms in photodynamic therapy: part two – cellular, cell metabolism and modes of cell death. *Photodiag Photodyn Ther* 2005;**2**:1–23
18 Don AS, Hogg PJ. Mitochondria as cancer drugs targets. *Trends Mol Med* 2004;**10**:372–8
19 Hilf R. Mitochondria are targets of photodynamic therapy. *J Bioenerg Biomembr* 2007;**39**:85–9
20 Capella MAM, Capella LS. A light in multidrug resistance: photodynamic treatment of multidrug-resistant tumors. *J Biomed Sci* 2003;**10**:361–6
21 Guo WJ, Zhou GD, Wu HJ, Liu YQ, Wu RG, Zhang WD. Ultrastructural localization of glutathione *S*-transferase-pi in human colorectal cancer cells. *World J Gastroenterol* 2000;**6**:454–5
22 Volm M, Rittgen W. Cellular predictive factors for the drug response of lung cancer. *Anticancer Res* 2000;**20**:3449–58
23 Cieslak A, Szejnach J. Molekularne mechanizmy chemoopornosci w raku nerki. *Wsp Onkol* 2005;**9**:123–8
24 Moor ACE. Signaling pathways in cell death and survival after photodynamic therapy. *J Photochem Photobiol B: Biol* 2000;**57**:1–13
25 Murphy MP. Investigating mitochondrial radical production using targeted probes. *Biochem Soc Transact* 2004;**32**:1011–4
26 Marchal S, Bezdetnaya L, Guillemin F. Modality of cell death induced by Foscan-based photodynamic treatment in human colon adenocarcinoma cell line HT29. *Biochemistry (Mosc)* 2004;**69**:45–9
27 Leunig A, Staub F, Peters J, Heiman A, Csapo Ch, Kempski O, Goetz AE. Relation of early Photofrin® uptake to photodynamically induced phototoxicity and changes of cell volume in different cell lines. *Eur J Cancer* 1994;**30A**:78–83
28 Kessel D, Luo Y. Mitochondrial photodamage and PDT-induced apoptosis. *J Photochem Photobiol B* 1998;**42**:89–95
29 Bors W, Czapski G, Saran M. An expanded function for superoxide dismutase. *Free Radic Res Commun* 1991;**12–13**:411–7
30 Johnson SA, Pardini RS. Antioxidant enzyme response to hypericin in EMT6 mouse mammary carcinoma cells. *Free Radic Biol Med* 1998;**24**:817–26
31 Das H, Koizumi T, Sugimoto T, Yamaguchi S, Hasegawa K, Tenjin Y, Nishimura R. Induction of apoptosis and manganese super-oxide dismutase gene by photodynamic therapy in cervical carcinoma cell lines. *Int J Clin Oncol* 2000;**5**:91–103
32 Saczko J, Kulbacka J, Chwiłkowska A, Ługowski M, Banaś T. Levels of lipid peroxidation in A549 cells after PDT in vitro. *Ann Acad Med Bialost* 2004;**49**:82–4
33 Balcerzyk A, Bartosz G. Thiols are main determinants of total antioxidant capacity of cellular homogenates. *Free Radic Res* 2003;**37**:537–41
34 Visconti R, Grieco D. New insights on oxidative stress in cancer. *Curr Opin Drug Discov Dev* 2009;**12**:240–5
35 Tajiri H, Hyakawa A, Matsumoto Y, Yokoyama I, Yoshida S. Changes in intracellular Ca^{2+} concentrations related to PDT-induced apoptosis in photosensitized human cancer cells. *Cancer Lett* 1998;**128**:205–10
36 Andreani M, Olivier JL, Berenbaum F, Raymondjean M, Bereziat G. Transcriptional regulation of inflammatory secreted phospholipases A2. *Biochim Biophys Act* 2000;**1488**:149–58
37 Laye JP, Gill JH. Phospholipase A2 expression in tumours: a target for therapeutic intervention? *Drug Discov Today* 2003;**8**:710–5
38 Giannattasio G, Lai Y, Granata F, Mounier CM, Nallan L, Oslund R, Leslie CC, Marone G, Lambeau G, Gelb MH, Triggiani M. Expression of phospholipases A(2) in primary human lung macrophages role of cytosolic phospholipase A(2)-alpha in arachidonic acid release and platelet activating factor synthesis. *Biochim Biophys Acta* 2009;**1791**:92–102
39 Jensen SS, Andresen TL, Davidsen J, Høyrup P, Shnyder SD, Bibby MC, Gill JH, Jørgensen K. Secretory phospholipase A2 as a tumor-specific trigger for targeted delivery of a novel class of liposomal prodrug anticancer etherlipids. *Mol Cancer Ther* 2004;**3**:1451–8

(Received May 11, 2009, Accepted September 1, 2009)