

Cytosolic superoxide dismutase activity after photodynamic therapy, intracellular distribution of Photofrin II and hypericin, and P-glycoprotein localization in human colon adenocarcinoma

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Abstract: In photodynamic therapy (PDT), a tumor-selective photosensitizer is administered and then activated by exposure to a light source of applicable wavelength. Multidrug resistance (MDR) is largely caused by the efflux of therapeutics from the tumor cell by means of P-glycoprotein (P-gp), resulting in reduced efficacy of the anticancer therapy. This study deals with photodynamic therapy with Photofrin II (Ph II) and hypericin (Hyp) on sensitive and doxorubicin-resistant colon cancer cell lines. Changes in cytosolic superoxide dismutase (SOD1) activity after PDT and the intracellular accumulation of photosensitizers in sensitive and resistant colon cancer cell lines were examined. The photosensitizers' distributions indicate that Ph II could be a potential substrate for P-gp, in contrast to Hyp. We observed an increase in SOD1 activity after PDT for both photosensitizing agents. The changes in SOD1 activity show that photodynamic action generates oxidative stress in the treated cells. P-gp appears to play a role in the intracellular accumulation of Ph II. Therefore the efficacy of PDT on multidrug-resistant cells depends on the affinity of P-gp to the photosensitizer used. The weaker accumulation of photosensitizing agents enhances the antioxidant response, and this could influence the efficacy of PDT.

Key words: Photodynamic therapy - Colon adenocarcinoma - Superoxide dismutase - P-glycoprotein - Photofrin II - Hypericin

Introduction

The major drawback of cancer therapy is the emergence of multidrug-resistant (MDR) tumor cells, which are cross-resistant to a broad range of structurally and functionally unrelated agents, making it difficult to treat these tumors. Therefore, one of the most important points is to develop new strategies in the treatment of multidrug-resistant carcinomas. Photosensitizers preferentially accumulate in the region of a tumor and provide the possibility of killing MDR cells or modulating the MDR phenotype [2]. PDT can thus be promoted as an alternative treatment for MDR tumors. In the last decade, investigations of photodynamic effects in MDR cells in culture have

given promising results [4,5,12]. This relatively new therapeutic modality for both neoplastic and non-neoplastic diseases involves a dye (photosensitizer) that has been selectively taken up by the target tissue, the presence of molecular oxygen, and light activation. This combined treatment leads to the generation of reactive oxygen species (ROS), such as singlet molecular oxygen, hydroxyl radicals, and/or superoxide anions [18]. Cells are endowed with enzymatic antioxidant defense systems to prevent cell death caused by reactive oxygen species. One of the most important antioxidant enzymes is superoxide dismutase, which catalyzes the dismutation of O_2^- to O_2 and H_2O_2 [9].

In this study, two human-origin colon adenocarcinoma cell lines, LoVo (sensitive) and LoVo DX (doxorubicin-resistant), were treated with two photosensitizers, Photofrin II and hypericin, and irradiated with laser light. The intracellular accumulation of both

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photosensitizers and the influence of the photodynamic therapy on cytosolic superoxide dismutase activity, one of the crucial antioxidant enzymes, were examined.

Materials and methods

Cell lines. Two human colon adenocarcinoma cell lines were used (doxorubicin-sensitive LoVo and doxorubicin-resistant LoVo DX). The cell lines were purchased from Institute of Immunology and Experimental Therapy, Polish Academy of Sciences. The cell lines were grown in F-12 Ham medium nutrient mixture (Sigma) with addition of 10% fetal bovine serum in 25 cm³ Falcon flasks. The cells were maintained in a humidified atmosphere at 37°C and 5% CO₂. For the experiments the cells were removed by trypsinizing and washed with phosphate-buffered saline.

Photodynamic treatment. The cells were treated with 30 µg/ml Photofrin II and 2.5 µg/ml hypericin in complete media for 4 h in the dark. Then they were irradiated with a light dose of 2.5 J/cm² using red laser light (λ =632.8 nm). After irradiation, the cells were incubated in a humidified atmosphere at 37°C and 5% CO₂ for 30 min.

Photosensitizer distribution. Microcultures were derived from the culture dishes and harvested on cover glasses for 24 hours. The cells were subsequently incubated with two photosensitizers: Photofrin II (Ph II) (QLT Phototherapeutics, Inc., Vancouver, Canada), at a concentration of 30 µg/ml, and hypericin (Sigma), at a concentration of 2.5 µg/ml, for 4 hours. After incubation, the cells were fixed in 4% formalin buffer, washed in PBS, and examined under a fluorescence microscope (Nikon Eclipse TE 2000-E) using a filter with an excitation wavelength of 528-553 nm and emission wavelength of 578-633 nm.

Immunofluorescence - P-gp expression. The cells were plated in growth medium into 8-dip glass (Nunc) and grown for two days. Then the cells were fixed in 4% formalin buffer and washed in PBS. The intracellular localization of P-gp (1:40, Novoacstra) was labeled with FITC (1:60, Sigma, excitation wavelength: 470 ± 20 nm and emission wavelength: λ =525 ± 20 nm). DAPI (0.2 µl/ml, Molecular Probes, excitation wavelength: 541 nm and emission wavelength: 445 ± 50 nm) was used for nuclei staining. Fluorescence was monitored using a microscope with LED illumination (Zeiss Axio Imager.A1).

SOD1 activity. After photodynamic treatment *in vitro*, the cells were removed by trypsinizing and washed twice in PBS. Then the cells were suspended in 50 mM phosphate buffer with a protease inhibitor mixture (Complete Mini EDTA-free, Roche). SOD1 activity was measured using the method describe by Segura-Aguilar [16]. The substrate for cytosolic superoxide dismutase is provided by the reduction of oxygen during the autoxidation of riboflavin in the presence of UV light. Hydrogen peroxide is quantitated using a coupled reaction where horseradish peroxidase catalyzes the formation of a fluorescent product, 6,6'-diOH-(1,1'-biphenyl)-3,3'-diacetic acid, from 4-OH-phenylacetic acid and hydrogen peroxide. SOD1 activity was measured after incubation with Ph II and Hyp without irradiation immediately following irradiation and 30 min after irradiation.

Statistical analysis. The significance of the differences between mean values of different groups of cells was assessed by Student's *t*-test with values of $p \leq 0.05$ taken to imply statistical significance.

Results

Distribution of photosensitizers

The intracellular distribution of Ph II and Hyp in the LoVo and LoVo DX cell lines was monitored after 4 hours of incubation with the photosensitizers. We observed different distributions of these photosensitizers in the two investigated cell lines. The fluorescence emitted by Photofrin II in LoVo was stronger than in LoVo DX (Fig. 1C and D). In contrast, the accumulation of Hyp was stronger in LoVo DX than in LoVo cells (Fig. 1A and B). The present study shows that both photosensitizers are diffused after 4 hours in the cytoplasm predominantly in intracellular membranes (Fig. 1A, B, C and D).

P-gp expression

The P-gp expression allowed evaluation of the level of multidrug resistance in LoVo and LoVoDX cells (Fig. 2). In LoVo cells, P-glycoprotein is mainly accumulated in the cytoplasm and around the nuclear envelope (Fig. 2A). Cytoplasmatic localization was also identified in LoVoDX cells, but we observed very intensive fluorescence in the nuclear envelope (Fig. 2B).

SOD1 activity

SOD1 activity was seen to increase after incubation with hypericin with and without irradiation compared with all control cells without PDT (Fig. 3A). However, in the cells treated with Ph II without irradiation, the level of SOD1 activity was not significantly decreased. An increase in enzyme activity was also observed in the cells incubated with Ph II after irradiation. (Fig. 3B). The activity was lower in both cell lines treated with Ph II than in cells treated with hypericin. SOD1 activity was observed to increase more in LoVo cells treated hypericin compared with the LoVo DX cell line. On the other hand, the level of SOD1 activity in the cells after Ph II PDT was not significantly higher in LoVoDX cells compared with LoVo cells.

Discussion

The resistance to chemotherapeutic drugs is a frequent phenomenon in which tumor cells develop cross-resistance to chemically unrelated cytotoxic agents. The overexpression of multidrug-resistant transporter proteins in cell membranes is one of the reasons for the resistance and can affect the efficacy of photodynamic therapy by reducing the intracellular accumulation of the photosensitizer [4,13].

The mechanism of cell response to PDT was investigated through the effects of two different photosensi-

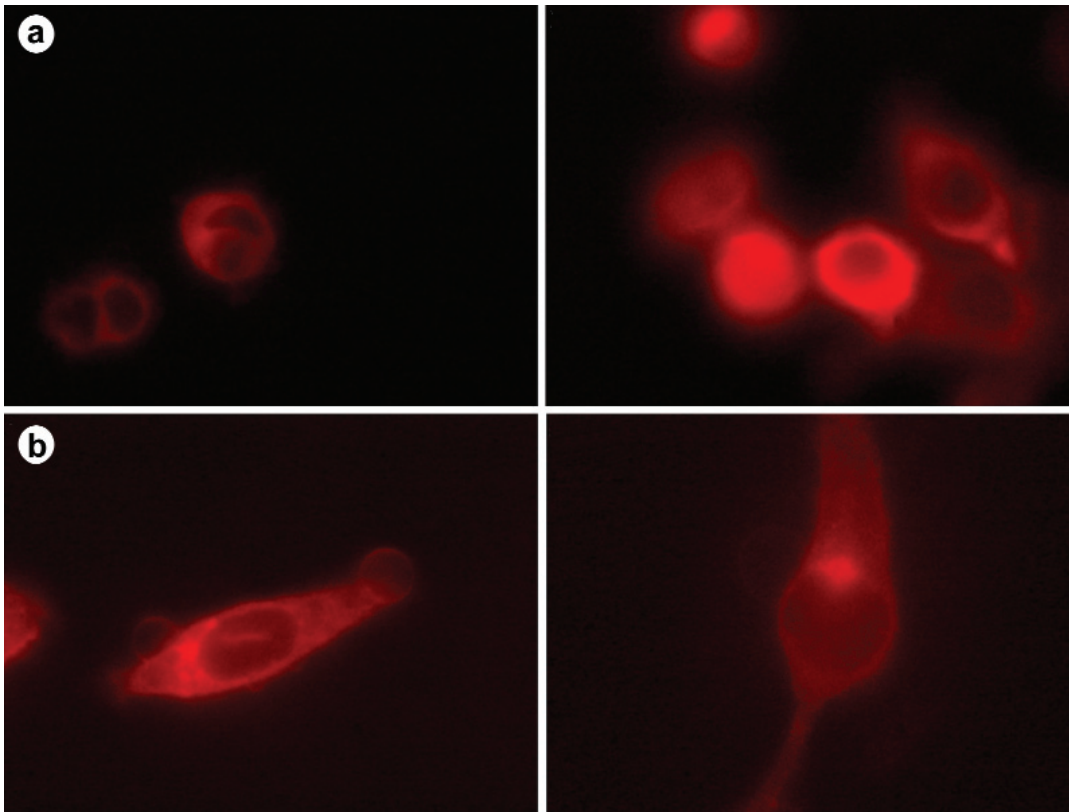


Fig. 1. Fluorescence distribution of (a) hypericin (cHyp=2.5 µg/ml) and (b) Photofrin II (cPhII=30 µg/ml) in LoVo and LoVoDX cell lines after 4-h incubation; left: in LoVo, right: in LoVoDX cells.

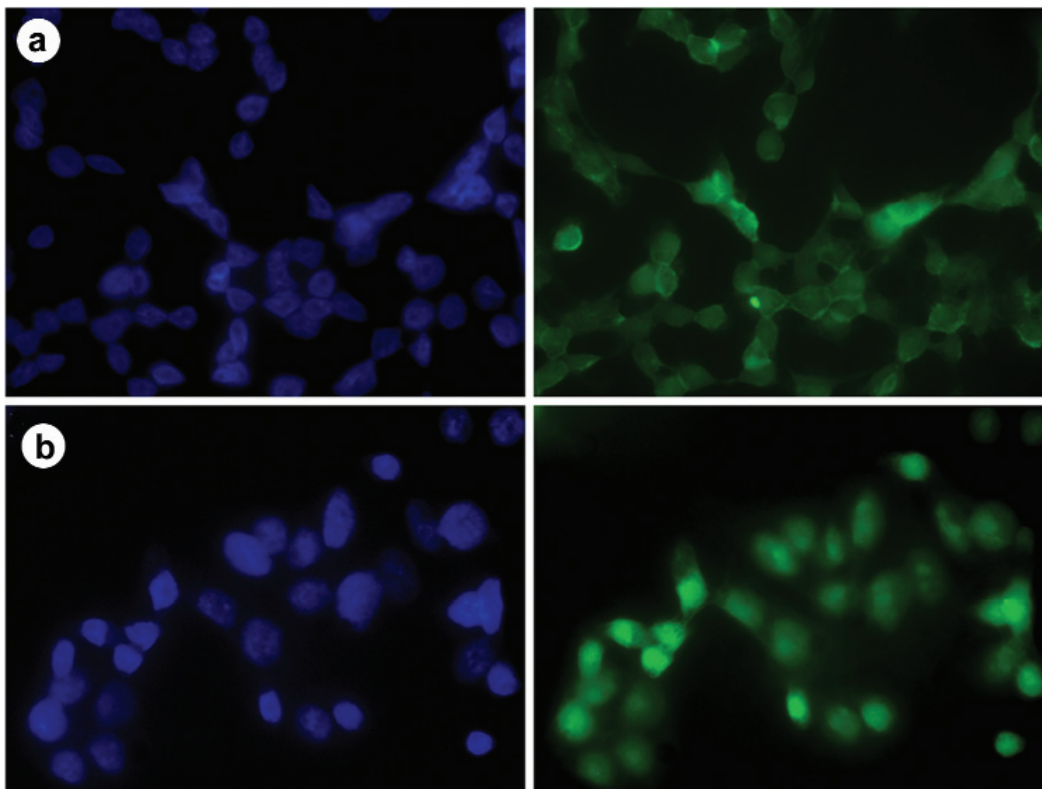


Fig. 2. LoVo (a) and LoVoDX (b) cells, right: DAPI fluorescence (blue) showing nuclear localization; left: P-glycoprotein labelled FITC (green).

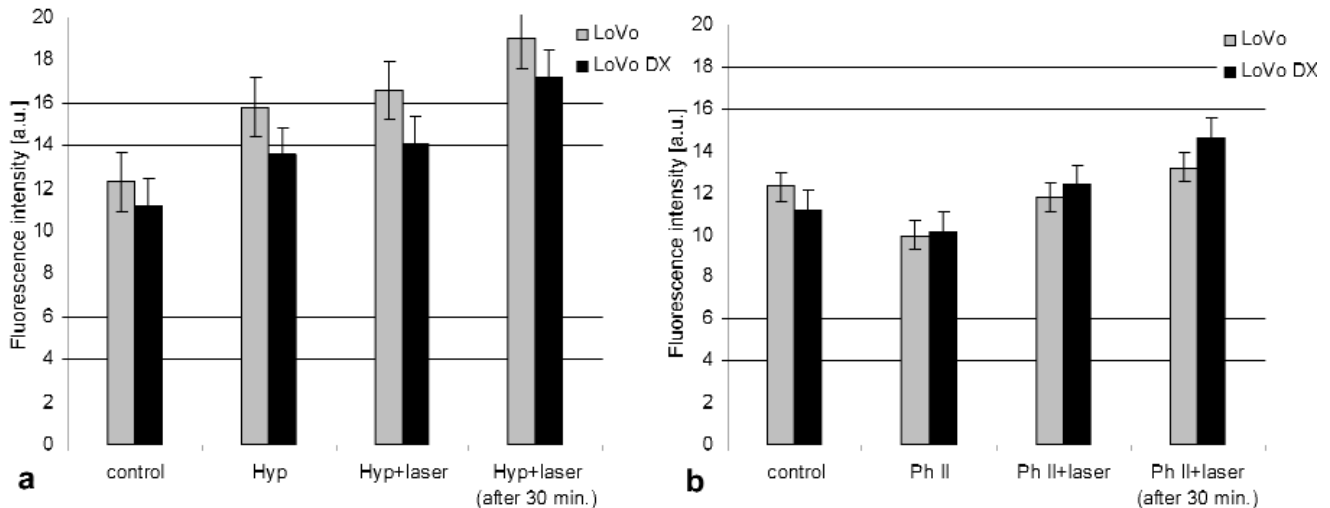


Fig. 3. The effect of hypericin (a) and Photofrin (b), and light on cytosolic superoxide dismutase activity (SOD1). The activity was measured control cells without PDT, after incubation with Ph II and Hyp without irradiation, directly after irradiation and after 30 min. $p \leq 0.05$ statistically significant compared with the control.

tizing agents in cell lines sensitive to (LoVo) and resistant to (LoVo DX) doxorubicin. Other investigations of MDR in PDT have focused primarily on P-gp-mediated resistance, and differing results for specific cells lines and photosensitizers have led to various conclusions [14]. Cells overexpressing P-gp display a reduced intracellular concentration of drugs that are substrates for this transporter. In our previous studies, some chemical features of the uptake of both photosensitizers, Ph II and Hyp, were examined [3,10]. The present study demonstrates the different sensitivities of LoVo and LoVoDX cells to the two photosensitizers.

Several authors have claimed that there was no difference in Photofrin II accumulation and photosensitization between sensitive and MDR Friend leukemia cell lines. But the accumulation of the hematoporphyrin derivative was weaker in multicellular spheroids derived from a human colorectal carcinoma cell line, a kind of tumor that usually overexpresses P-gp [2,11]. Doxorubicin-resistant and parental cancer cells were treated with mTHPC (m-tetrahydroxyphenylchlorin)-mediated PDT by Teiten *et al.* [17]. They observed that the effect of photodynamic action was significantly greater in the resistant than in the sensitive, parental cell line. The highest applied concentration of 7.5 mM of mTHPC resulted in a significantly greater accumulation in MDR cells. We observed the same correlation for LoVoDX cells incubated with hypericin compared with the LoVo cell line. Interestingly, we obtained conflicting results after incubation with Photofrin II. These differences in the accumulation of the two photosensitizers in sensitive and resistant colon cancer cells suggest that Ph II could be a substrate for P-gp in MDR cells. However, Merlin *et*

al. [12] showed that the cellular accumulation of Ce6 in MCF-7 and MCF-7/DXR induced no significant alteration after 3 h of incubation. These data were consistent with the fact that Ce6 should not be a substrate for P-glycoprotein. The authors demonstrated the nuclear localization of P-gp in MCF-7/DXR. We also observed accumulation of P-gp in the nuclear environment and the cytoplasm, but in the sensitive cell line LoVo (Fig. 2B). P-gp accumulated in the nuclei can be involved in active cytotoxic drug removal [1,13]. In turn, the LoVoDX cell line revealed intensive P-gp accumulation in the cell membrane.

Calcabrini *et al.* [1] demonstrated that earlier and higher depolarization after treatment with BSAO and spermine was found in the membrane of LoVoDX than in the membrane of the drug-sensitive cells. In addition, basal ROS production was higher in LoVoDX cells. Other results comparing Rh 123 (Rhodamine 123) and Photofrin[®] showed that there was lower Photofrin[®] accumulation in resistant than in wild-type cells. This seems to be mainly related to intracellular drug turnover [4]. The results obtained in this study suggest a more effective PDT action in the sensitive human colon adenocarcinoma cell line.

Apart from the uptake of photosensitizer, this study shows the antioxidant response to PDT. The present experimental results suggest an influence of hypericin and Photofrin II in combination with irradiation on increased SOD1 activity caused by the generation of ROS and oxidative stress in both cell lines. Johnson and Pardini performed photodynamic treatment with hypericin on EMT6 cells. A significant increase in SOD activity was already noticed after 30 minutes [8]. Golab *et al.* [6] showed that Ph II-mediated PDT

induced the expression of MnSOD in murine colon-26 (C-26). The inhibition of SOD activity in tumor cells induced an increase in the cytotoxic effect of PDT. In contrast, transient transfection with MnSOD gene resulted in decreased effectiveness of PDT. These results suggest that MnSOD plays a leading role in the response to PDT and that mitochondria impairment may be a critical factor in phototoxicity. The SODs appear to be important antioxidative enzymes that regulate the sensitivity of cancer cells in some therapeutic methods, such as photodynamic therapy [7].

This study indicates a difference in the uptake of various photosensitizers by two colon adenocarcinoma cell lines. We suggest that the stronger accumulation of Ph II in LoVo and Hyp in LoVoDX cells causes the impairment of SOD1 activity after PDT. On the other hand, weaker fluorescence was observed in cells with higher SOD1 activity after PDT. Verification of the dependency between dyes applied in PDT and multidrug-resistant transporters could clarify the problem of the proper photosensitizer. The development and characterization of multidrug-resistant cells not only helps to understand the mechanism of PDT in the resistance of tumor cells, but also may contribute to the application of more effective treatment. The search for new antitumor drugs has been directed toward finding compounds having a novel mechanism of action, activity against resistant cancers, and a higher therapeutic index [2,4,15].

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