Antitumor effects of photodynamic therapy are potentiated by 2-methoxyestradiol – a superoxide dismutase inhibitor

Jakub Gołąb[1]*, Dominika Nowis[1], Michał Skrzycki[2], Hanna Czeczot[2], Anna Barańczyk-Kuźma[2], Grzegorz M. Wilczyński[3], Marcin Makowski[1], Paweł Mróz[1], Katarzyna Kozar[1], Rafał Kamiński[1], Ahmad Jalili[1], Maciej Kopeć[1], Tomasz Grzela[4,5], Marek Jakóbisiak[1]

[1]Department of Immunology, Center of Biostructure Research, The Medical University of Warsaw;
[2]Department of Biochemistry, The Medical University of Warsaw;
[3]Department of Pathology, Center of Biostructure Research, The Medical University of Warsaw;
[4]Department of Histology and Embryology, Center of Biostructure Research; The Medical University of Warsaw
[5]Department of General and Vascular Surgery and Transplantation, The Medical University of Warsaw

***Correspondence to:** Jakub Gołąb, M.D., Ph.D., Department of Immunology, Center of Biostructure Research, The Medical University of Warsaw, Chałubińskiego 5, 02-004 Warsaw, Poland. Tel/fax: (0048 22) 622 6306 (e-mail: jgolab@ib.amwaw.edu.pl)

Key words: Photofrin®, photodynamic therapy, superoxide dismutase, 2-methoxyestradiol

Running title: 2-MeOE$_2$ potentiates antitumor effects of PDT

Summary

Photodynamic therapy (PDT), a promising therapeutic modality for the management of solid tumors, is a two-phase treatment consisting of a photosensitizer and visible light. Increasing evidence indicates that tumor cells in regions exposed to sublethal doses of PDT can respond by rescue responses that lead to insufficient cell death. We decided to examine the role of superoxide dismutases (SODs) in the effectiveness of PDT and to investigate whether 2-methoxyestradiol (2-$MeOE_2$), an inhibitor of SODs, is capable of potentiating the antitumor effects of this treatment regimen. In the initial experiment we observed that PDT induced the expression of MnSOD but not Cu,Zn-SOD in cancer cells. Pretreatment of cancer cells with a cell-permeable SOD mimetic - MnTBAP and transient transfection with MnSOD gene resulted in a decreased effectiveness of PDT. Inhibition of SOD activity in tumor cells by preincubation with 2-$MeOE_2$ produced synergistic antitumor effects when combined with PDT in 3 murine and 5 human tumor cell lines. The combination treatment was also effective *in vivo* producing retardation of the tumor growth and prolongation of the survival of tumor-bearing mice. We conclude that inhibition of MnSOD activity by 2-$MeOE_2$ is an effective treatment modality capable of potentiating the antitumor effectiveness of PDT.

Introduction

Photodynamic therapy (PDT) is an effective treatment modality that has been approved for the palliation or treatment of esophageal, lung and bladder cancers in at least 10 countries (1). Moreover, numerous clinical trials investigate the effectiveness of PDT in the treatment of gastric, colon, bile duct, pancreatic, breast and other cancers (2-9). The treatment consists of a systemic administration of a photosensitizer followed by an illumination with a laser light. Neither of the PDT components alone can induce antitumor effects but when combined with oxygen they produce lethal cytotoxic agents that can either directly kill tumor cells or destroy blood vessels within the tumor (1). The photochemical reaction produces singlet oxygen ($^1O_2$) as well as numerous forms of reactive oxygen, such as superoxide ion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\bullet}$) (10). Increasing evidence indicates that tumor cells can respond to photodynamic damage by either initiating a rescue response or by undergoing cell death by apoptosis or necrosis (11). Rescue response to sublethal changes allows tumor cells to cope with the damage induced by the physicochemical stress. This effect is particularly important in deeper layers of the tumor exposed to laser illumination. Since the light penetration is limited by the absorption, scattering and reflection characteristics of the tissues the effective dose of energy reaching the deeper layers of the tumor might cause insufficient damage allowing the initiation of the rescue response (12). The surviving cells might be the cause of relapse rendering the treatment less effective. Therefore, elucidation of molecular changes in the treated cells as well as identification of drugs that might interfere with rescue responses becomes an important area of investigation.

Although singlet oxygen has been demonstrated to play a dominant role in the cytotoxic effects invoked by the photodynamic therapy (13) there is also a number of observations implicating superoxide anion in phototoxicity. Superoxide generation increases several-fold after illumination of Photofrin®-bearing cells (14) and $O_2^{\bullet-}$ exerts multiple deleterious effects such as lipid peroxidation, DNA cross-linking, and formation of disulphide bonds in proteins (15). Intracellular $O_2^{\bullet-}$ production correlates with cell death in 5-aminolevulinic acid- and zinc(II) phthalocyanine-mediated PDT (16,17). Cells scavenge $O_2^{\bullet-}$ with the help of a constitutive Cu,Zn-SOD (SOD-1) as well as an inducible MnSOD (SOD-2)(18). The latter is associated with the mitochondrial matrix and participates in dismutating superoxide anion from the site of Photofrin®-mediated generation of reactive oxygen species (ROS) during photodynamic therapy (19). Mice treated with a SOD mimetic, β-carotene had considerably less ear swelling following Photofrin®-mediated PDT (20), and intravenous administration of bacterial SOD decreased, to a significant extent, the antitumor efficacy of Photofrin®-mediated PDT in three different murine tumor models (21). Accordingly, sodium diethyldithiocarbamate, a SOD inhibitor augments cutaneous photosensitization (20). Intuitively, combinations of SOD inhibitor with treatment modalities that produce free radicals might result in synergistic anticancer activity.

All these observations prompted us to evaluate the role of superoxide dismutases in the antitumor effectiveness of PDT and to investigate whether 2-$MeOE_2$, an estrogen metabolite which has recently been shown to inhibit SOD activity (22), would be capable of potentiating the antitumor effects of this treatment regimen.

Experimental procedures

Mice. (C57BL/6 x DBA/2)F_1 mice, hereafter called B6D2F_1, and Balb/c mice, 8-12 weeks of age, were used in the experiments. Breeding pairs were obtained from the Inbred Mice Breeding Center of the Institute of Immunology and Experimental Medicine (Wrocław, Poland) and from the Institute of Oncology (Warsaw, Poland). All experiments with animals were performed in accordance with the guidelines approved by the Ethical Committee of the Medical University of Warsaw.

Reagents. Photofrin® was a generous gift of QLT PhotoTherapeutics, Inc. (Vancouver, BC, Canada). Manganese-containing SOD from *E. coli*, 2-MeOE_1 and 2-MeOE_2 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Mn(II)tetrakis(4-Benzoic acid)porphyrin Chloride (MnTBAP) was purchased from Calbiochem (San Diego, CA, USA).

Tumors. Human pancreatic cancer cell lines (Panc-1, HPAC-I and HPAF-II), breast cancer cell line (T47-D) and bladder cancer cell line (T24) were purchased from ATCC. Murine Colon-26 (C-26), a poorly differentiated colon adenocarcinoma cell line, and Lewis lung carcinoma (LLC) cells were obtained from prof. C. Radzikowski (Institute of Immunology and Experimental Medicine, Wrocław, Poland) and prof. W. W. Jędrzejczak (Department of Immunology, Central Clinical Hospital, Military School of Medicine, Warsaw, Poland), respectively. Cells were cultured in RPMI-1640 medium (Gibco BRL, Paisley, UK) supplemented with 10% heat-inactivated fetal calf serum (FCS),

antibiotics, 2-mercaptoethanol (50 μM) and L-glutamine (2 mM) (all from Gibco BRL), hereafter referred to as culture medium.

Measurements of SOD activity. The SOD activity was measured using a Ransod assay kit purchased from Randox Laboratories LTD (Antrim, United Kingdom) according to the manufacturers' protocol. The SOD inhibitory activity of 2-$MeOE_1$ or 2-$MeOE_2$ was measured using a manganese-containing SOD. For the measurements of SOD activity in tumor cells extracts, Colon-26 cells were scraped using a rubber policeman, washed twice in cold PBS and resuspended in the 0.01 M sodium-phosphate buffer with 1% Triton X-100. After a one freeze-thaw cycle the cells were homogenized, the extracts were preincubated with DMSO (controls) or 2-$MeOE_2$ for 15 min and the enzymatic activity of SOD was measured using a Ransod assay kit. In some experiments Colon-26 cells were preincubated with either DMSO (controls) or 2-$MeOE_2$ added to the cell cultures for 3, 6, 12, 24 and 48 hours. The SOD activity was measured directly from the cell homogenates.

Western blotting. For western blotting C-26 cells were cultured with 2.5 μg/mL Photofrin® for 24 hours before illumination. After washing with PBS, the cells were illuminated using a 50-watt sodium lamp (Philips) with a light filtered through a red filter to a final dose of 4.5 kJ/m^2. After 1, 2, 4, 12 or 24 hours of culture in the fresh medium the cells were washed with PBS and lysed in a sample buffer containing 2% SDS with protease inhibitors. Protein concentration was measured with the use of BCA protein assay (Pierce, Rockford, IL, USA). Equal amounts of proteins were separated on 15%

SDS-polyacrylamide gel, transfered onto PVDF membranes, blocked with TBST (Tris buffered saline pH 7.4, 0.05% Tween-20) with 5% non-fat milk and 5% FBS. The following primary anibodies were used for the 6-hour incubation: mouse monoclonal anti-β-tubulin at 1:10000 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), rabbit polyclonal anti-SOD-1 at 1:1000 (Santa Cruz) and- rabbit polyclonal anti-MnSOD at 1:1000 (Research and Diagnostics, Flanders, NJ, USA). After extensive washing the membranes were incubated for 45 minutes with the corresponding alkaline phosphatase-coupled secondary antibodies (Jackson Immuno Research Inc. West Grove, PA, USA). The color reaction was developed using NBT (p-nitro-blue tetrazolium chloride) and BCIP (5-bromo-4-chloro-3-indolyl phosphate) (Sigma).

Immunocytochemistry. Four hours after *in vitro* PDT (2.5 μg/mL Photofrin® and 4.5 J/m^2 light), Colon-26 cells were fixed *in situ* in culture dishes, in a mixture of 4% paraformaldehyde plus 0.1% glutaraldehyde for 1 hour at room temperature, scraped, suspended in 10% liquified gelatin, and pelleted at 1000 rpm at 4^{O}C. The pellets were cut into smaller fragments, washed in PBS, cryo-protected in 2.3 M sucrose (overnight at 4^{O}C), snap frozen in liquid nitrogen and stored at -70^{0}C until use. Cryo-sections (0.5 μm-thick) were cut in a cryo-ultramicrotome (MT-X model with CR-X cryosectioning system, RMC-Boeckeler Instruments, Tucson, AZ, USA) at -60^{O}C, collected on glass slides using a drop-of-sucrose technique (according to Liou *et al*., 1996), washed in distilled water, equilibtrated in PBS, and processed immediately for immunocytochemistry. The immunocytochemical procedure included overnight incubation (at 4^{0}C) in a solution of an anti-MnSOD primary antibody (Research and

Diagnostics) diluted 1:200 in 5% normal goat serum, followed by a standard avidin-biotin immunoperoxidase reaction, with DAB (diaminobenzidine, Sigma) as a chromogen. Omission of the primary antibody or its replacement by an irrelevant antibody served as controls of antibody-specificity. Phase-contrast images of the specimens were recorded using Axioplan microscope (Zeiss, Oberkochen, Germany) coupled to CCD camera, and image-analysis software (Analysis, Soft Imaging System, Münster, Germany).

Transient transfection. For the transfection experiments the pcDNA3 (Invitrogen, San Diego, CA, USA) plasmids containing Cu,Zn-SOD [from Dr. L.W. Oberley (Department of Radiation Oncology, University of Iowa, USA)] (23) and MnSOD [from Dr. K. Scharffetter-Kochanek (Department of Dermatology, University of Cologne, Germany)] (24) were used.

T24 cells were seeded at 8 x 10^5 cells/35-mm dish and incubated overnight at 37°C in a 5% CO_2 incubator. Subconfluent proliferating cultures were incubated overnight with 5 μg of each vector in serum-free DMEM containing LipofectAMINE™ 2000 (Life Technologies). Cultures were washed with PBS to remove the excess vector and LipofectAMINE™ and then incubated again for 24 hours in fresh complete culture medium containing Photofrin® (2.5 μg/mL). Immediately after illumination with 4.5 kJ/m^2 light the cells were trypsinized and dispensed into the wells of the 96-well flat-bottomed microtiter plate (Nunc) at concentrations of 8, 16 or 32 x 10^4 cells/200 μL/well. Similarly, T24 cells transiently transfected with each of the plasmid vectors (Cu,Zn-SOD, MnSOD or pcDNA3) and not exposed to PDT were trypsinized and dispensed into the wells at

the same concentrations as PDT-treated cells. Following 24 h incubation the cultures were rinsed with PBS and stained with 0.5% crystal violet in 30% ethanol for 10 min at room temperature. Plates were washed four times with tap water. Cells were lysed with 1% SDS solution, and dye uptake was measured at 550 nm using an ELISA reader (SLT Labinstrument GmbH, Salzburg, Austria), equipped with a 550 nm filter. Cytotoxicity was expressed as relative viability of tumor cells (% of control cultures transiently transfected with corresponding plasmid vector) and was calculated as follows: Cytotoxicity = $(A_{PDT} - A_b) \times 100 / (A_c - A_b)$, where A_b is background absorbance, A_{PDT} is absorbance of cells exposed to PDT and A_c is the absorbance of transfected controls.

Cytotoxicity assays. The cytostatic and/or cytotoxic effects of treatment on tumor cells were measured using a crystal violet staining as described above or a standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as described previously (25). Briefly, cells were dispensed into a 96-well flat-bottomed microtiter plate (Nunc) at a concentration of 5 to 10 x 10^4 cells/100 μL/well. Cells were treated with serial dilutions of 2-$MeOE_1$ or 2-$MeOE_2$ or with a control DMSO-containing medium (to a final volume of 200 μL) 2 hours after plating. After 24 hours the medium was completely removed and replaced with fresh 2-$MeOE_1$ or 2-$MeOE_2$ or a DMSO-containing medium in a volume of 100 μL. Then, 100 μl of culture medium with Photofrin® (2.5 μg/mL final concentration) was added to each well. Following 24 hours of incubation with the photosensitizer the cells in each well were exposed to a laser light delivered through the fiberoptic light delivery system. The illumination area was exactly matching the size of

the wells. In some experiments the cells were preincubated with MnTBAP (100 μM) for 2 hours before laser illumination. After another 24 hours 25 μl of MTT was added to each well for the last 4 hours of incubation. The plates were read in an ELISA reader (SLT Labinstrument GmbH, Salzburg, Austria), using a 450 nm filter. In all experiments a cytostatic/cytotoxic effect was expressed as relative viability of tumor cells (% of control cultures incubated with medium only) and was calculated as follows: Relative viability = $(A_e - A_b) \times 100 / (A_c - A_b)$, where A_b is background absorbance, A_e is experimental absorbance and A_c is the absorbance of untreated controls.

Interaction analysis. For the examination of the interactions between PDT and 2-$MeOE_2$, an isobologram analysis was used as described in details elsewhere (26). Briefly, inhibition of cell proliferation was determined as described in the above section. For the analysis of the interactions the equi-effective doses were used. These were the doses of either treatment alone or used in the combination that produced equivalent inhibition of cell growth in comparison with controls ($P < 0.005$; Student's *t*-test). Combinations of the treatments had to induce growth inhibition higher than those of the same doses used alone ($P < 0.001$; Student's *t*-test). The interaction index for two treatment combinations was computed according to the following formula: interaction index = $(P_c/P_a) + (M_c/M_a)$, where P_a and M_a are doses of PDT and 2-$MeOE_2$, respectively, that produce specified effect when used alone; P_c and M_c are doses of PDT and 2-$MeOE_2$, respectively, that produce the same effect when used in combination. Synergy is claimed when the interaction index is < 1.0.

Tumor treatment and monitoring. For *in vivo* experiments exponentially growing tumour cells were harvested, resuspended in PBS medium to appropriate concentration of cells and injected (1×10^5 C-26 cells or 5×10^5 3LL cells in 20 μl PBS) into the footpad of the right hind limb of experimental mice. Tumour cell viability measured by trypan exclusion was always above 95%.

Tumor-bearing mice were treated orally with 2-MeOE$_2$ at a dose of 100 mg/kg dissolved in DMSO and suspended in olive oil. The treatment with 2-MeOE$_2$ started on day 3 following inoculation of tumor cells and continued for 6 consecutive days. Mice in the control groups received oral DMSO in olive oil in the same regimen as the treated mice.

Photofrin® was administered intraperitoneally (i.p.) at a dose of 10 mg/kg 24h before illumination with 630-nm light (day 5 of 2-MeOE$_2$ administration, day 7 of the experiment). Control mice received 5% dextrose. The light source was a He-Ne ion laser (Amber, Warsaw, Poland). The light was delivered on day 8 of the experiment using a fiberoptic light delivery system. The power density at the illumination area, which encompassed the tumour and 1-1.5 mm of the surrounding skin, was approximately 80 mW/cm^2 (40 mW laser output). The total light dose delivered to the tumors was 150 J/cm^2. During the light treatment mice were anesthetized with ketamine (87 mg/kg) and xylazine (13 mg/kg) and restrained in a specially designed holder. Local tumor growth was determined as described (27) by the formula:

$$\text{Tumor volume (mm}^3\text{)} = \text{(longer diameter)} \times \text{(shorter diameter)}^2$$

Relative tumor volume was calculated as follows:

Relative tumor volume = [(tumor volume) ÷ (initial tumor volume)] × 100%.

Statistical analysis. Data were calculated using Microsoft© Excell 98. Differences in *in vitro* cytotoxicity assays and in tumor volume were analyzed for significance by Student's *t*-test. Additionally, data were analyzed with the nonparametric Mann-Whitney *U* test (Instat™, GraphPad Software, San Diego, CA, USA). Kaplan-Meier plots were generated using days of animal death (after inoculation of tumor cells) as a criterion, and survival time of animals was analyzed for significance by log-rank survival analysis. Significance was defined as a two-sided $P<0.05$.

RESULTS

Photodynamic therapy induces the expression of MnSOD

It has previously been demonstrated using Northern blotting that PDT induces the expression of SOD gene (28). However, it was not investigated whether increased RNA levels correlate with the expression of SOD at the protein level. Therefore, we have initially investigated whether and to what extent PDT influences the level of Cu,Zn-SOD and MnSOD proteins using Western blotting. C-26 cells were exposed to 2.5 μg/mL Photofrin® and after 24 hours of incubation with the photosensitizer were washed with PBS and illuminated with a sublethal light dose of 4.5 kJ/m^2. Although, there was no influence of PDT on the expression of Cu,Zn-SOD we have observed a time-dependent increase in the level of MnSOD (Fig. 1A). Immunocytochemical staining of tumor cells exposed to PDT revealed that MnSOD expression was detectable in surviving but not in lethally damaged cells (Fig. 1B1 and 1B2).

The influence of MnSOD overexpression on the antitumor effects of PDT

To determine the role of SOD in the antitumor effectiveness of PDT, C-26 and T24 cells were preincubated with MnTBAP, a cell permeable SOD mimetic, and then exposed to laser illumination. Remarkably, the antitumor efficacy of the PDT was significantly reduced (Fig. 2A and 2B). Since these studies did not reveal which of the SOD isoforms might be responsible for the protective effects, T24 cells have been transiently transfected with Cu,Zn-SOD, MnSOD or an empty control (pcDNA3) vector. Exposure of pcDNA3- or Cu,Zn-SOD-transfected cells to PDT resulted in a comparable cytotoxicity (Fig. 2C). However, T24 cells transiently transfected with a MnSOD containing plasmid were significantly less susceptible to the antitumor effects of PDT (Fig. 2C).

Inhibition of SOD activity by 2-MeOE$_2$

We have observed that 2-MeoE$_2$, but not 2-MeOE$_1$, another estrogen metabolite, significantly suppresses the activity of manganese-containing SOD from *E. coli* (Table 1). Additional experiments were done to determine the potential suppressive effects of 2-MeOE$_2$ on the SOD activity in Colon-26 cells. These studies demonstrated that 2-MeOE$_2$ indeed effectively inhibits in a dose-dependent manner the SOD activity in extracts from Colon-26 cells (Table 2). Moreover, preincubation of Colon-26 cells with 2-MeOE$_2$ revealed a time-dependent inhibition of SOD activity in tumor cells (Table 3). These studies confirmed that 2-MeOE$_2$ can effectively enter the tumor cells and inhibit SOD activity.

Inhibition of SOD activity potentiates the cytotoxic effects of PDT

Since 2-$MeOE_2$ has been shown to inhibit SOD activity in tumor cells we decided to examine the influence of this agent on the antitumor activity of PDT *in vitro*. The experiments performed in three murine cell lines (C-26, LLC and MDC) revealed that 2-$MeOE_2$ potentiates the cytotoxic effects of sublethal doses of PDT (Fig. 3). According to Berenbaum analysis the interactions between 2-$MeOE_2$ and PDT were in all cell lines synergistic. Moreover, the antitumor effects of the 2-$MeOE_2$ + PDT combination were examined in 5 human tumor cell lines of breast (T47-D), pancreatic (PANC-1, HPAF-II and HPAC), and bladder (T24) origin. In all these cell lines there was also a synergistic potentiation of the antitumor effects of PDT by 2-$MeOE_2$ (Fig. 3). Of note, 2-$MeOE_1$ another estrogen derivative devoid of SOD inhibitory activity did not influence the antitumor effects of PDT in two murine (C-26 and LLC) and one human (T47-D) cancer cell line (Fig. 4).

Antitumor effects of PDT in combination with 2-$MeOE_2$ in mice

Next, we decided to evaluate the antitumor activity of the combination treatment *in vivo* in two murine models of syngeneic tumors, namely in C-26 adenocarcinoma syngeneic with Balb/c mice and Lewis lung carcinoma (LLC) growing in $B6D2F_1$ mice. Treatment with 2-$MeOE_2$ was started 5 days prior to laser illumination of the tumors. Although 2-$MeOE_2$ initially inhibited tumor growth, it did not show significant antitumor effects as measured by tumor volume or mouse survival time. PDT caused a typical edema that disappeared 24-36 hours after illumination and was followed by a significant retardation

of tumor growth as compared with controls. Importantly, administration of 2-MeOE$_2$ significantly potentiated the antitumor effects of PDT in both tumor models, leading not only to the retardation of tumor growth but also to the prolongation of mouse survival time (Fig. 5). Remarkably, 60% of Colon-26-bearing mice were completely cured (no tumor for 150 days of observation).

DISCUSSION

The mechanisms of the tumoricidal effects of PDT have not yet been completely elucidated. It has been well established that the presence of molecular oxygen is an absolute requirement for the effective cells killing during PDT. Hypoxic or anoxic conditions almost completely reduce the antitumor effectiveness of PDT *in vitro* (29). Moreover, PDT was shown to be ineffective in a poorly vascularized xenograft model (30) and chemotherapy-induced anemia leads to decreased effectiveness of PDT in mice (31). During photodynamic therapy, a photosensitizer absorbs light and crosses from its excited singlet state to the reactive triplet state. It then either transfers its energy to triplet-state molecular oxygen generating singlet oxygen (type II photochemical reactions) or by hydrogen atom extraction or electron transfer reaction it generates radicals and radical ions (type I reactions) (10). The latter, which include reactive oxygen species such as superoxide anion, are molecules with a higher reactivity than ground-state molecular oxygen. For the effective photodamage both type I and type II reactions seem to be necessary and inhibition of the enzymes scavenging reactive oxygen species might offer an attractive way of potentiating the cytotoxic action of PDT.

Numerous previous studies demonstrated an increased generation of $O_2^{\bullet-}$ in cancer cells exposed to PDT (14,16,17). Since normal as well as malignant cells have the capacity to scavenge this reactive oxygen species we decided to investigate the role of superoxide dismutases in the antitumor effectiveness of PDT. We observed that following PDT there is an induction of MnSOD expression in tumor cells. Interestingly, there was no influence of PDT on the expression of Cu,Zn-SOD in tumor cells. These observations can be interpreted through the intracellular localization of the Photofrin®. This photosensitizer localizes initially in the plasma membrane and after 24 hour incubation it can be found mainly in the mitochondria (32). Although both short- and long-term incubations with Photofrin® are used experimentally in *in vitro* studies, we chose a 24-hour preincubation since this is concordant with clinical situation, where laser illumination is performed 24-48 hours following Photofrin® injection. The total light dose delivered to tumor cells in our experiments was 4.5 kJ/m^2 since previous studies with Photofrin® revealed that at this dose superoxide generation increases by a factor of approximately 2.5 (14).

SODs appear to be important antioxidative enzymes that regulate the sensitivity of cancer cells to various treatment modalities. Indeed, the expression of SODs negatively correlates with the sensitivity of cancer cells to anticancer drugs and radiation therapy (33,34). Overexpression of MnSOD suppresses apoptosis (35) and transfection of tumor cells with antisense oligonucleotides that block SOD activity are more susceptible to apoptosis induced by chemotherapeutics, hyperthermia and γ-radiation (36,37). Remarkably, pretreatment of tumor cells with a cell-permeable SOD mimetic – MnTBAP as well as transient transfection of tumor cells with plasmids

encoding MnSOD renders tumor cells less susceptible to the cytotoxic effects of PDT (Fig. 2A and 2B).

Although several small molecule compounds, such as cyanide ion (CN^-), hydroxyl ion (OH^-) and azide ion (N_3^-) were previously shown to inhibit SOD at the catalytic site (38), these agents are highly toxic and therefore of limited potential for cancer therapy. Recently, 2-$MeOE_2$, an endogenous estrogen metabolite with antitumor activity, was shown to selectively inhibits the activity of superoxide dismutases (22). Although this finding was questioned in one study (39), our observations (Tables 1-3) as well as a recent report (40) confirmed that 2-$MeOE_2$ can effectively block superoxide dismutase activity. In direct, cell-free inhibition assays 50 μM concentration of 2-$MeOE_2$ was required to inhibit SOD activity by less that 50% (Tables 1 and 2). However, in cell cultures the effective inhibition of SOD was obtained at much lower concentrations and in a time dependent manner (Table 3). One speculative explanation for this apparent discrepancy might be that intracellular 2-$MeOE_2$ undergoes a metabolic activation that converts this mediator to a more potent SOD inhibitor.

Additionally, 2-$MeOE_2$ demonstates a number of antitumor effects including antiproliferative and apoptotic effects (41), induction of p53 (42), tubulin depolymerization (43) and inhibition of angiogenesis (44). This drug has already proved effective in potentiating the antitumor effects of radiotherapy (45,46). Moreover, 2-$MeOE_2$ has proved effective in the treatment of experimental tumors (43,46,47) and two clinical trials with 2-$MeOE_2$ in patients with advanced solid tumors have recently been initiated.

To verify whether cancer cells use MnSOD in order to avoid the action of $O_2^{\bullet-}$, we have evaluated the antitumor effectiveness of PDT in cells treated with a SOD inhibitor - 2-$MeOE_2$. In all investigated cell lines (3 murine and 5 human) there was a significant potentiation of the antitumor effects of PDT (Fig. 3). Berenbaum analysis of the results revealed that in all instances the cytotoxic effects of the combination treatment are synergistic. These observations prompted the *in vivo* studies evaluating the antitumor efficacy of the combination treatment. Our studies revealed that 2-$MeOE_2$ can sensitize tumors in two murine models to more effective PDT (Fig. 5). The combined treatment led not only to retardation of tumor growth, but also to prolonged survival and in C-26 colon adenocarcinoma bearing mice to complete cures in 60% of animals.

Altogether, these studies indicate that interference with the mechanisms used by tumor cells to resist PDT is a rational approach of potentiating the antitumor effectiveness of this promising treatment modality. Moreover, our studies suggest that treatment regimens designed to selectively enhance free radical generation and suppress anti-oxidant defenses in the tumor cells may form an effective approach in the treatment of cancer although toxic side effects of increased activity of reactive oxygen species should be taken into consideration in such therapeutic modalities.

Acknowledgements

We would like to thank eng. Adam Gołąb (Erco Leuchten. GmBH, Warsaw) for the construction of the sodium lamp for *in vitro* experiments and to Anna Czerepińska and Elżbieta Gutowska for excellent technical assistance. This work was supported by grant 1M19/M2/2000 from the Medical University of Warsaw, Poland, by grant 4 P05A 025 18

from the State Committee for Scientific Research (K.B.N.) and by grant from the Foundation for Polish Science (F.N.P.).

REFERENCES

1. Dougherty, T. J., Gomer, C. J., Henderson, B. W., Jori, G., Kessel, D., Korbelik, M., Moan, J., and Peng, Q. (1998) *J Natl Cancer Inst* **90**, 889-905

2. Bown, S. G., Rogowska, A. Z., Whitelaw, D. E., Lees, W. R., Lovat, L. B., Ripley, P., Jones, L., Wyld, P., Gillams, A., and Hatfield, A. W. (2002) *Gut* **50**, 549-557

3. Wyss, P., Schwarz, V., Dobler-Girdziunaite, D., Hornung, R., Walt, H., Degen, A., and Fehr, M. (2001) *Int J Cancer* **93**, 720-724

4. Kashtan, H., Konikoff, F., Haddad, R., and Skornick, Y. (1999) *Gastrointest Endosc* **49**, 760-764

5. Mimura, S., Ito, Y., Nagayo, T., Ichii, M., Kato, H., Sakai, H., Goto, K., Noguchi, Y., Tanimura, H., Nagai, Y., Suzuki, S., Hiki, Y., and Hayata, Y. (1996) *Lasers Surg Med* **19**, 168-172

6. Berr, F., Wiedmann, M., Tannapfel, A., Halm, U., Kohlhaw, K. R., Schmidt, F., Wittekind, C., Hauss, J., and Mossner, J. (2000) *Hepatology* **31**, 291-298

7. Allardice, J. T., Abulafi, A. M., Grahn, M. F., and Williams, N. S. (1994) *Surg Oncol* **3**, 1-10

8. Allison, R., Mang, T., Hewson, G., Snider, W., and Dougherty, D. (2001) *Cancer* **91**, 1-8

9. Goodell, T. T., and Muller, P. J. (2001) *J Neurosci Nurs* **33**, 296-300

10. Sharman, W. M., Allen, C. M., and van Lier, J. E. (2000) *Methods Enzymol* **319**, 376-400

11. Moor, A. C. (2000) *J Photochem Photobiol B* **57**, 1-13

12. MacDonald, I. J., and Dougherty, T. J. (2001) *J Porphyrins Phthalocyanines* **5**, 105-129

13. Weishaupt, K. R., Gomer, C. J., and Dougherty, T. J. (1976) *Cancer Res* **36**, 2326-2329

14. Salet, C., Moreno, G., and Ricchelli, F. (1997) *Free Radic Res* **26**, 201-208

15. Martinez-Cayuela, M. (1995) *Biochimie* **77**, 147-161

16. Gilaberte, Y., Pereboom, D., Carapeto, F. J., and Alda, J. O. (1997) *Photodermatol Photoimmunol Photomed* **13**, 43-49

17. Hadjur, C., Wagnieres, G., Ihringer, F., Monnier, P., and van den Bergh, H. (1997) *J Photochem Photobiol B* **38**, 196-202

18. McCord, J. M. (2002) *Methods Enzymol* **349**, 331-341

19. Morgan, J., Potter, W. R., and Oseroff, A. R. (2000) *Photochem Photobiol* **71**, 747-757

20. Athar, M., Mukhtar, H., Elmets, C. A., Zaim, M. T., Lloyd, J. R., and Bickers, D. R. (1988) *Biochem Biophys Res Commun* **151**, 1054-1059

21. Korbelik, M., Parkins, C. S., Shibuya, H., Cecic, I., Stratford, M. R., and Chaplin, D. J. (2000) *Br J Cancer* **82**, 1835-1843

22. Huang, P., Feng, L., Oldham, E. A., Keating, M. J., and Plunkett, W. (2000) *Nature* **407**, 390-395

23. Zhang, Y., Zhao, W., Zhang, H. J., Domann, F. E., and Oberley, L. W. (2002) *Cancer Res* **62**, 1205-1212

24. Wenk, J., Brenneisen, P., Wlaschek, M., Poswig, A., Briviba, K., Oberley, T. D., and Scharffetter-Kochanek, K. (1999) *J Biol Chem* **274**, 25869-25876

25. Golab, J., Zagozdzon, R., Kaminski, R., Kozar, K., Gryska, K., Izycki, D., Mackiewicz, A., Stoklosa, T., Giermasz, A., Lasek, W., and Jakobisiak, M. (2001) *Leukemia* **15**, 613-620

26. Berenbaum, M. C. (1981) *Adv Cancer Res* **35**, 269-335

27. Golab, J., Wilczynski, G., Zagozdzon, R., Stoklosa, T., Dabrowska, A., Rybczynska, J., Wasik, M., Machaj, E., Olda, T., Kozar, K., Kaminski, R., Giermasz, A., Czajka, A., Lasek, W., Feleszko, W., and Jakobisiak, M. (2000) *Br J Cancer* **82**, 1485-1491

28. Das, H., Koizumi, T., Sugimoto, T., Yamaguchi, S., Hasegawa, K., Tenjin, Y., and Nishimura, R. (2000) *Int J Clin Oncol* **5**, 97-103

29. Henderson, B. W., and Fingar, V. H. (1987) *Cancer Res* **47**, 3110-3114

30. White, L., Gomer, C. J., Doiron, D. R., and Szirth, B. C. (1988) *Br J Cancer* **57**, 455-458

31. Golab, J., Olszewska, D., Mroz, P., Kozar, K., Kaminski, R., Jalili, A., and Jakobisiak, M. (2002) *Clin Cancer Res* **8**, 1265-1270

32. Wilson, B. C., Olivo, M., and Singh, G. (1997) *Photochem Photobiol* **65**, 166-176

33. Nakano, T., Oka, K., and Taniguchi, N. (1996) *Cancer Res* **56**, 2771-2775

34. Ueta, E., Yoneda, K., Yamamoto, T., and Osaki, T. (1999) *Jpn J Cancer Res* **90**, 555-564

35. Kuroda, M., Himei, K., St Clair, D. K., Urano, M., Yoshino, T., Akagi, T., Asaumi, J., Akaki, S., Takeda, Y., Kanazawa, S., and Hiraki, Y. (2000) *Anticancer Res* **20**, 7-10

36. Ueta, E., Yoneda, K., Kimura, T., Tatemoto, Y., Doi, S., Yamamoto, T., and Osaki, T. (2001) *Int J Cancer* **94**, 545-550

37. Kuninaka, S., Ichinose, Y., Koja, K., and Toh, Y. (2000) *Br J Cancer* **83**, 928-934

38. Rigo, A., Viglino, P., and Rotilio, G. (1975) *Biochem Biophys Res Commun* **63**, 1013-1018

39. Kachadourian, R., Liochev, S. I., Cabelli, D. E., Patel, M. N., Fridovich, I., and Day, B. J. (2001) *Arch Biochem Biophys* **392**, 349-353

40. Wood, L., Leese, M. P., Leblond, B., Woo, L. W. L., Ganeshapillai, D., Purohit, A., Reed, M. J., Potter, B. V. L., and Packham, G. (2002) *Anti-Cancer Drug Design* **16**, 209-215

41. Schumacher, G., Kataoka, M., Roth, J. A., and Mukhopadhyay, T. (1999) *Clin Cancer Res* **5**, 493-499

42. Seegers, J. C., Lottering, M. L., Grobler, C. J., van Papendorp, D. H., Habbersett, R. C., Shou, Y., and Lehnert, B. E. (1997) *J Steroid Biochem Mol Biol* **62**, 253-267

43. D'Amato, R. J., Lin, C. M., Flynn, E., Folkman, J., and Hamel, E. (1994) *Proc Natl Acad Sci U S A* **91**, 3964-3968

44. Fotsis, T., Zhang, Y., Pepper, M. S., Adlercreutz, H., Montesano, R., Nawroth, P. P., and Schweigerer, L. (1994) *Nature* **368**, 237-239

45. Amorino, G. P., Freeman, M. L., and Choy, H. (2000) *Radiat Res* **153**, 384-391

46. Kinuya, S., Kawashima, A., Yokoyama, K., Kudo, M., Kasahara, Y., Watanabe, N., Shuke, N., Bunko, H., Michigishi, T., and Tonami, N. (2001) *Eur J Nucl Med* **28**, 1306-1312

47. Klauber, N., Parangi, S., Flynn, E., Hamel, E., and D'Amato, R. J. (1997) *Cancer Res* **57**, 81-86

Tables

Table 1. The influence of 2-MeOE$_2$ or 2-MeOE$_1$ on the activity of manganese containing SOD.

Manganese containing SOD from *E. coli* (100 U/mL or 200 U/mL) was preincubated with either 2-MeOE$_1$ or 2-MeOE$_2$ (at concentrations of 1, 10 and 50 μM) for 15 minutes at 37^{O}C. Controls (SOD at 100 U/mL or 200 U/mL) were incubated for 15 minutes with a diluent (DMSO). Then the SOD activity was measured as described in Materials and Methods.

		measured SOD activity [U/mL]			
	2-MeOE$_1$	0	1 μM	10 μM	50 μM
SOD [U/mL]					
100		137,0 ± 2,0	138,0 ± 2,8	133,0 ± 3,8	140,0 ± 0,0
200		205,2 ±13,1	210,9 ± 27,1	194,0 ± 8,5	202,0 ± 2,8
	2-MeOE$_2$	0	1 μM	10 μM	50 μM
SOD [U/mL]					
100		98,0 ± 8,5	110,0 ± 0,0	92,0 ± 0,0	66,0 ± 5,7
200		200,0 ± 0,0	184,0 ± 22,6	152,0 ± 0,0	148,0 ± 5,6

Table 2. The influence of 2-MeOE$_2$ on the activity of SOD in extracts from C-26 cells.

C-26 cells (50×10^6) were rinsed with PBS, scraped, pelleted and resuspended in a sodium-phosphate buffer with 1% Triton X-100. After a one freeze-thaw cycle the cells were homogenized and the SOD activity was measured in samples incubated for 15 minutes at 37^{O}C with 2-MeOE$_2$ (at 5, 10 or 50 μM) or with a diluent (DMSO).

2-MeOE$_2$	SOD activity [U/mL]
Controls	213,0 ± 9,5
5 μM	183,0 ± 6,8
10 μM	178,0 ± 16,5
50 μM	144,0 ± 17,3

Table 3. The influence of preincubation of C-26 cells with 2-MeOE$_2$ on the activity of SOD.

C-26 cells (5×10^6) were incubated (for 3 to 48 h) with 2-MeOE$_2$ (at 0.5 or 1.0 μM) or with DMSO (controls). The cells were then rinsed with PBS, scraped, pelleted and resuspended in a sodium-phosphate buffer with 1% Triton X-100. After a one freeze-thaw cycle the cells were homogenized and the SOD activity was measured in samples.

	measured SOD activity [U/mL]	
Incubation time	2-MeOE$_2$ [0,5 μM]	2-MeOE$_2$ [1,0 μM]
Controls	69,6 ± 10,3	69,6 ± 10,3
3h	58,1 ± 7,2	52,8 ± 6,2
6h	38,9 ± 12,4*	31,0 ± 0,0*
12h	31,2 ± 0,0*	18,0 ± 0,0*
24h	20,4 ± 11,5*	10,4 ± 0,0*
48h	15,6 ± 6,2*	8,8 ± 3,4*

*P < 0,01, Student's t-test, two-sided, as compared with controls

Legends to Figures

Fig. 1. PDT induces MnSOD in tumor cells.

(A) C-26 cells were exposed to 2.5 μg/mL Photofrin® for 24 hours and then to 4.5 kJ/m^2 light and incubated for the indicated times. Total cell lysates were prepared, and Western blot analysis was performed using anti-Cu,Zn-SOD, anti-MnSOD or anti-β-tubulin antibodies.

(B) Immunocytochemical detection of MnSOD in control (B1) C-26 cells and in cells exposed to PDT (B2). In B2, the characteristic granular MnSOD immunoreactivity is present exclusively in cells that appear to survive the treatment, whereas it is virtually absent in severly damaged, presumably necrotic cells. The images were recorded under the phase-contrast optics. Bar indicates 10 μm.

Fig. 2. Superoxide dismutase protects tumor cells from lethal damage induced by PDT.

(A) C-26 and (B) T24 cells were incubated with 2.5 μg/mL Photofrin® for 24 hours and then preincubated for 1 hour prior to light exposure with 100 μM MnTBAP, a cell-permeable SOD mimetic. Prior to light treatment the cells were rinsed with PBS. Immediately after illumination the cells were re-fed with fresh MnTBAP (100 μM). Following 24 hour incubation the cytotoxic effects were measured by crystal violet staining. The bars represent percent cytotoxicity vs non-treated controls. Black bars represent cultures of MnTBAP-treated cells exposed to light illumination without Photofrin®. Data refer to means ± SD. * P<0.01 vs PDT alone group (Student's t-test).

(B)(C) T24 cells were transiently transfected with a control plasmid (pcDNA3) or plasmids containing Cu,Zn-SOD or MnSOD as described under Materials and Methods. After an overnight incubation with plasmids complexed with LipofectAMINE™ 2000 the cultures were postincubated for 24 h in fresh complete culture medium with Photofrin® (2.5 μg/mL). Immediately after illumination with 4.5 kJ/m^2 light the cells were trypsinized and dispensed into the wells of the 96-well flat-bottomed microtiter plate at concentrations of 8, 16 or 32 x 10^4 cells/200 μL/well. Similarly, T24 cells transiently transfected with each of the plasmid vectors (Cu,Zn-SOD, MnSOD or pcDNA3) were trypsinized and dispensed into the wells at the indicated concentrations. The bars represent the percent cytotoxicity of PDT-exposed cells to vector-transfected cells seeded at the same concentration. Data refer to means ± SD. * P<0.01 vs all other groups (Student's *t*-test).

(D) Efficiency of transfection was verified by Western blotting. Total lysates of T24 cells were prepared, and Western blot analysis was performed using anti-Cu,Zn-SOD, anti-MnSOD or anti-tubulin antibodies.

Fig. 3. Potentiation of *in vitro* cytotoxic effects of PDT by 2-MeOE$_2$.

Tumor cells of murine (C-26, LLC and MDC) and human (T47-D, PANC-1, HPAF-II, HPAC and T24) origin were dispensed into a 96-well flat-bottomed microtiter plate at a concentration of 5 to 10 x 10^4 cells/100 μL/well. Cells were pretreated with serial dilutions of 2-MeOE$_2$ or with a control DMSO-containing medium. After 24 hours the medium was completely removed and replaced with fresh 2-MeOE$_2$ or a control medium. Then, Photofrin® (2.5 μg/mL final concentration) was added to each well and

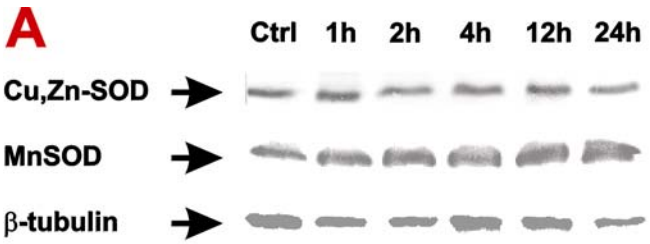
after 24 hours of incubation with the photosensitizer the cells in each well were exposed to a laser light delivered through the fiberoptic light delivery system. Cytotoxic effects were tested in a MTT assay and are expressed as means ± SD. An isobologram analysis presents an interaction between PDT and 2-$MeOE_2$ at inhibiting the growth of tumor cells cells. Solid line represents concentrations of both drugs required to inhibit cell growth to 50% (IC_{50}). Broken lines represent doses of each treatment required to produce the same growth inhibition as if the interactions were additive. The numbers in boxes represent interaction indexes.

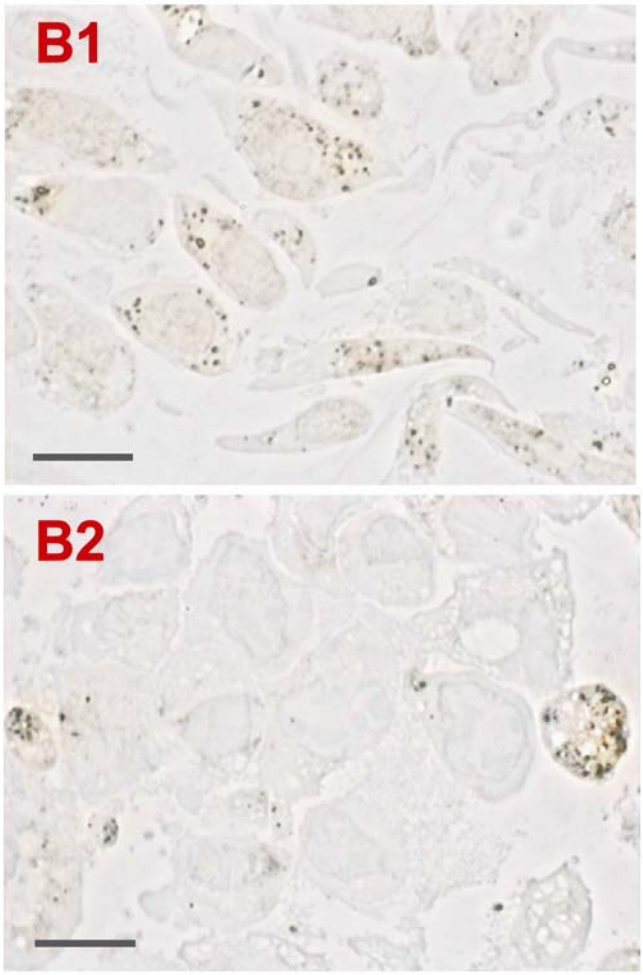
Fig. 4. The antitumor effects of PDT are not potentiated by 2-$MeOE_1$.

Tumor cells of murine (C-26, LLC) and human (T47-D) origin were dispensed into a 96-well flat-bottomed microtiter plate at a concentration of 5 to 10 x 10^4 cells/100 μL/well. Cells were pretreated with serial dilutions of 2-$MeOE_1$ or with a control DMSO-containing medium. After 24 hours the medium was completely removed and replaced with fresh 2-$MeOE_1$ or a control medium. Then, Photofrin® (2.5 μg/mL final concentration) was added to each well and after 24 hours of incubation with the photosensitizer the cells in each well were exposed to a laser light delivered through the fiberoptic light delivery system. Cytotoxic effects were tested in a MTT assay and are expressed as means ± SD.

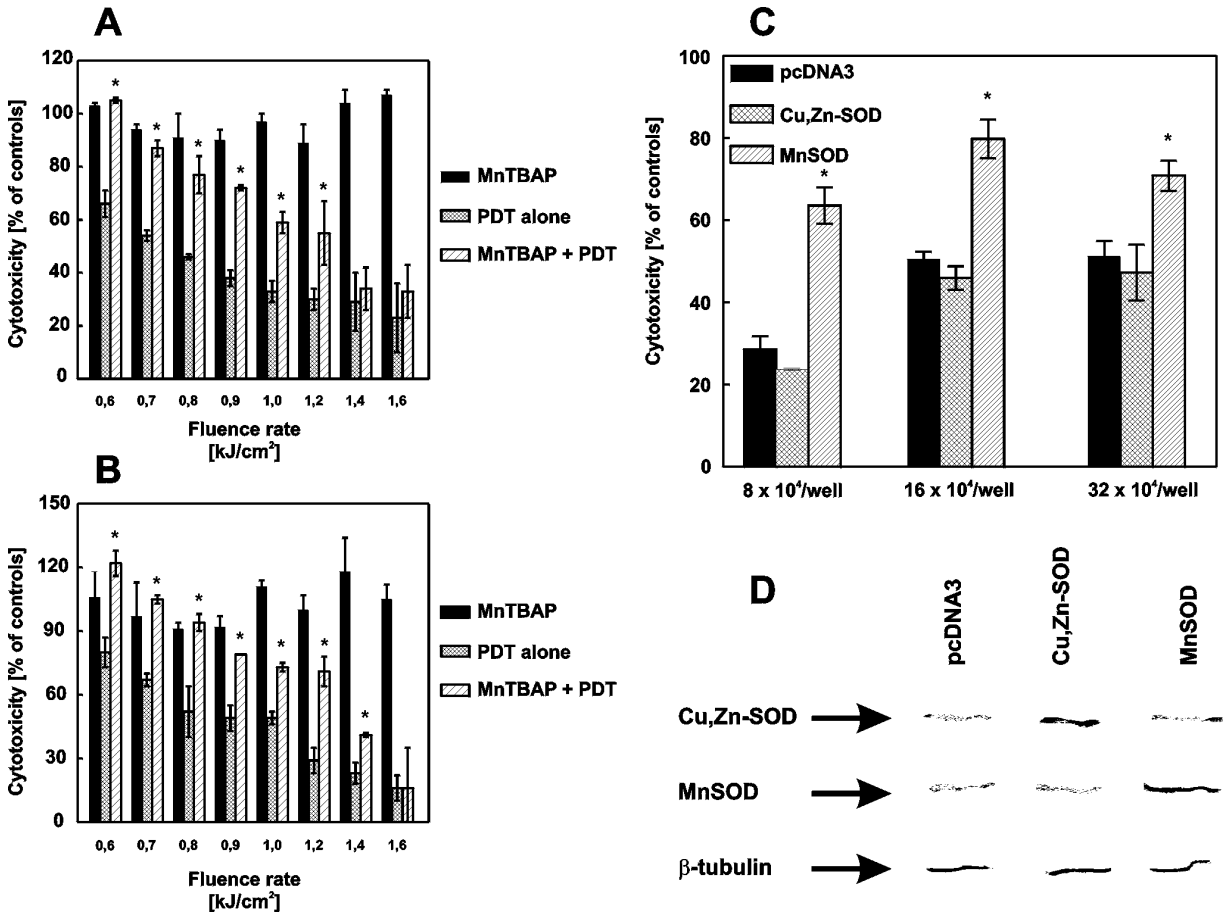
Fig. 5. Antitumor effects of the combined treatment with Photofrin®-based PDT and 2-MeOE$_2$.

Oral administration of 2-MeOE$_2$ (100 mg/kg) started on day 3 following inoculation of tumor cells and continued for six consecutive days. Photofrin® was administered i.p. at a dose of 10 mg/kg, 24 hours before laser illumination (150 J/cm^2 on day 7 after inoculation of tumour cells). Measurements of tumor diameter started on day 5 after inoculation of tumor cells. (A) The influence of the combined treatment on the growth of LLC tumors in B6D2F$_1$ mice (n=8). (B) Kaplan-Mayer plot of the survival of B6D2F$_1$ mice bearing LLC tumors. (C) The influence of the combined treatment on the growth of C-26 tumors in Balb/c mice (n=6-8). (D) Kaplan-Mayer plot of the survival of Balb/c mice bearing C-26 tumors. $^{\bullet}P<0.03$ (Mann-Whitney *U* test) in comparison with all other groups. $^{\#}P=0.038$; $^{\#\#}P=0.003$ (log-rank survival analysis) in comparison with all other groups.

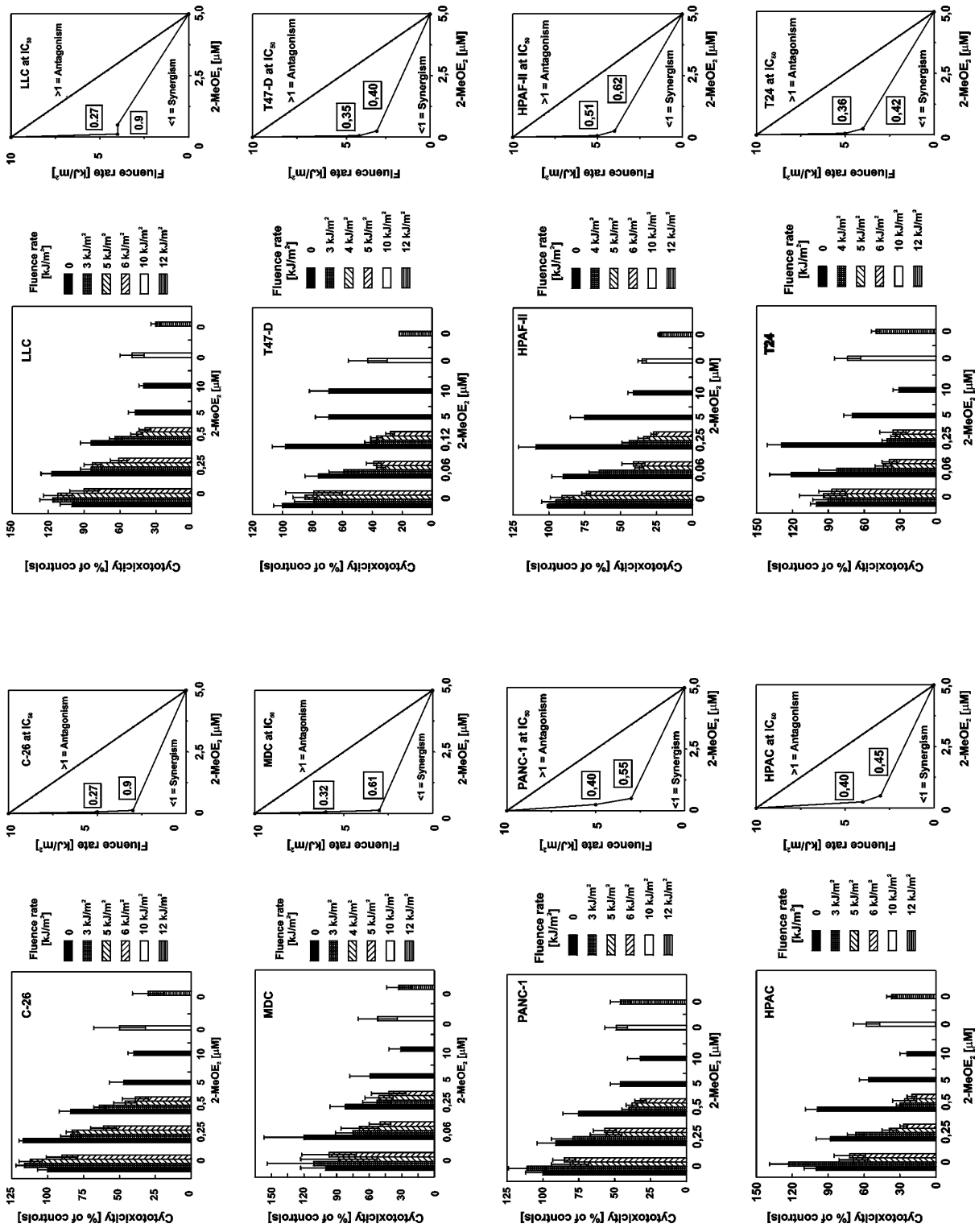




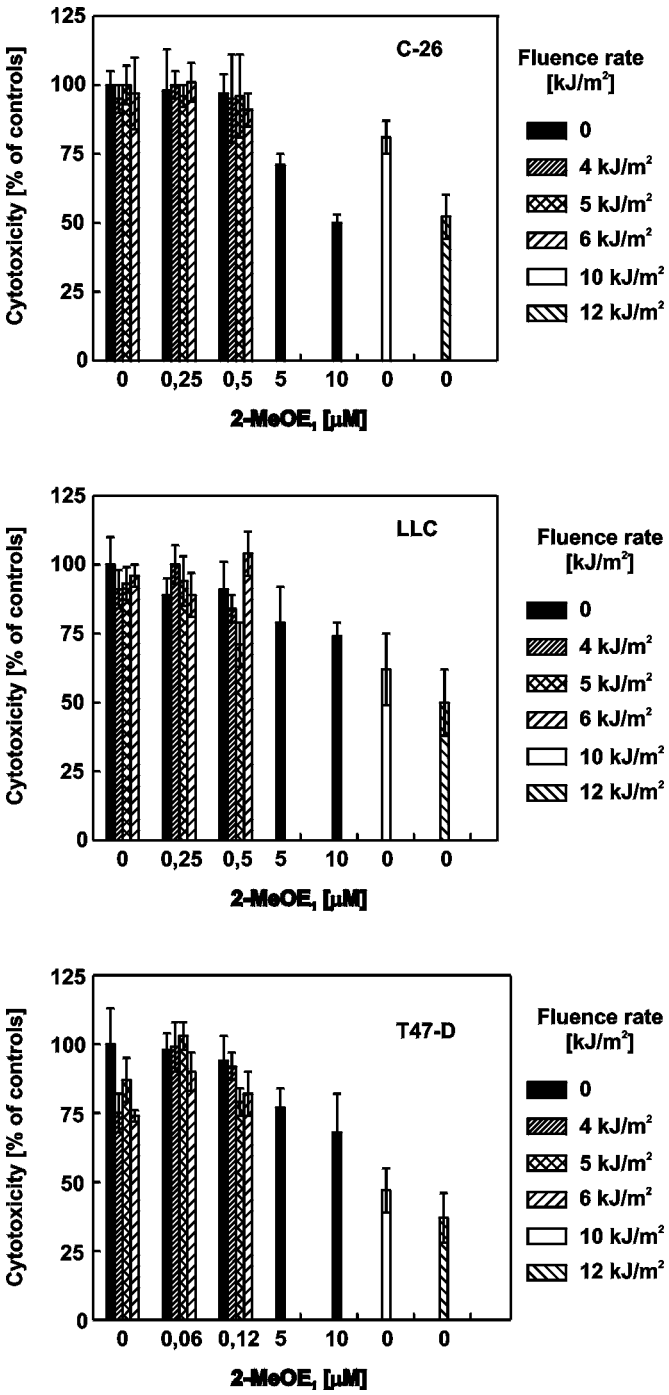
Gołąb et al., Fig. 1



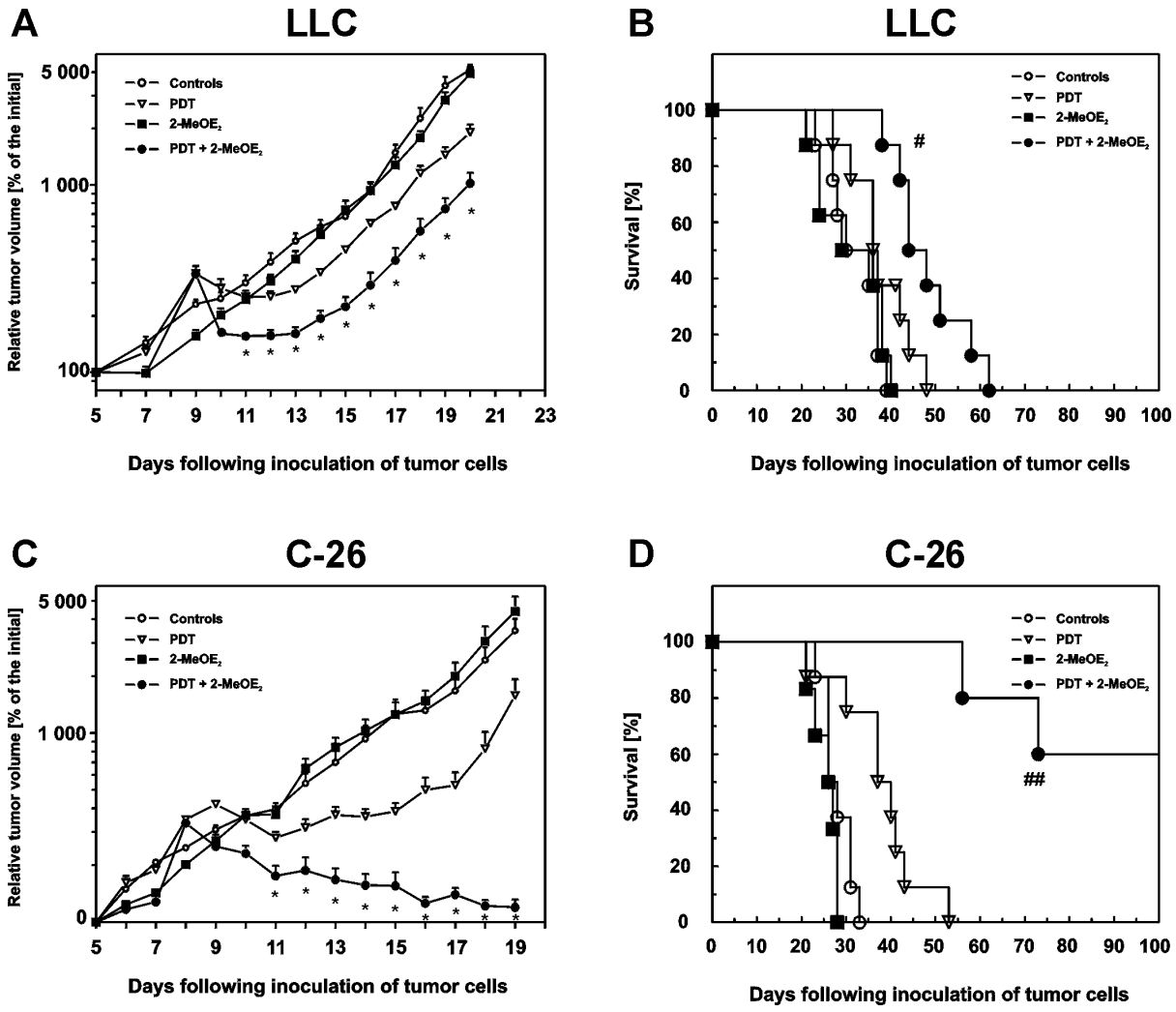
Gołąb et al., Fig. 2



Gołąb et al., Fig. 3



Gołąb et al., Fig. 4



Gołąb et al., Fig. 5