THE ROLE OF LIPID PEROXIDATION AND PROTEIN DEGRADATION IN THE PHOTODESTRUCTION OF EHRLICH ASCITES CARCINOMA CELLS SENSITIZED BY HEMATOPORPHYRIN DERIVATIVE

I.N. Shevchuk, V.A. Chekulayev, L.V. Chekulayeva*
Institute of Chemistry of Tallinn Technical University, Tallinn 12618, Estonia

РОЛЬ ПЕРЕКИСНОГО ОКИСЛЕНИЯ ЛИПИДОВ И ДЕГРАДАЦИИ БЕЛКОВ В ФОТОДЕСТРУКЦИИ КЛЕТОК АСЦИТНОЙ КАРЦИНОМЫ ЭРЛИХА, СЕНСИБИЛИЗИРОВАННЫХ ПРОИЗВОДНЫМ ГЕМАТОПОРФИРИНА

И.Н. Шевчук*, В.А. Чекулаев, Л.В. Чекулаева
Институт химии Таллиннского Технического Университета, Таллинн, Эстония

In the work, a comparative study on the role of peroxidation of lipids and degradation of proteins in inactivation of tumor cells by photodynamic therapy (PDT) with hematoporphyrin derivative (HPD) was performed. Ehrlich ascites carcinoma (EAC) cells were pre-incubated with HPD in a serum-free medium and then irradiated with red light at 630 nm. The rate of lipid peroxidation was estimated by measuring of accumulations of conjugated dienes (CD) and malondialdehyde (MDA). It was found that irradiation of EAC cells led to a powerful inhibition of their glycolytic and respiratory activity, and notably lowered the content of adenosine triphosphate. Studies pointed to the very low probability that the HPD-PDT induced impairment of mitochondria and, as a consequence, cell death were mediated by the peroxidation of membrane lipids. At a light dose causing a strong (> 2-fold) decrease in the respiratory activity of EAC cells as well as inactivation of ~ 98% of the cells, as estimated by MTT-test, only very small amounts of CD and MDA were detected. However, we found that cell proteins were substantially more sensitive to the damaging influence of HPD-PDT than lipid components; a clearly expressed fall in the content of protein-bound SH groups and, especially, histidine was registered. Our data suggest that cell proteins, but not lipids, are the primary target of PDT with HPD in EAC cells.
***Key Words*: photodynamic therapy, Ehrlich ascites carcinoma, malondialdehyde, conjugated dienes, protein.**

В работе было проведено сравнительное изучение роли перекисного окисления липидов (ПОЛ) и деградации белков в инактивации опухолевых клеток посредством фотодинамической терапии (ФДТ) с производным гематопорфирина (ПГП). В этом исследовании клетки асцитной карциномы Эрлиха (АКЭ) инкубировали с ПГП в бессывороточной среде, а затем облучали красным светом при 630 нм. Скорость ПОЛ оценивали по накоплению конъюгированных диенов (КД) и малонового диальдегида (МДА). Обнаружено, что облучение клеток АКЭ приводило к сильному ингибированию их гликолитической и дыхательной активности, а также к существенному снижению содержания аденозинтрифосфата. Полученные результаты свидетельствуют об очень низкой вероятности того, что фотодинамическое повреждение митохондрий и, вследствие этого, гибель клеток были индуцированы ПОЛ мембран. Так, лишь следовые количества КД и МДА были обнаружены при световой дозе, вызывающей сильное (> 2 раз) снижение дыхательной активности клеток АКЭ, а также инактивацию около 98% этих клеток (в МТТ-тесте). Установлено, что в клетках АКЭ белки более чувствительны к повреждающему воздействию ФДТ с ПГП, чем их липидные компоненты; наблюдалось значительное снижение содержания белок-ассоциированных SH-групп и особенно гистидина. Полученные данные указывают на то, что клеточные белки, но не липиды, являются первичной мишенью ФДТ с ПГП в клетках АКЭ.
***Ключевые слова*: фотодинамическая терапия, карцинома Эрлиха, малоновый диальдегид, конъюгированные диены, белок.**

Received: July 22, 2002.
*Correspondence: Tel/Fax: 372 620 4378;
E–mail: igor@chemnet.ee

Abbreviations used: ATP — adenosine triphosphate; CD — conjugated dienes; Cys — cysteine; DMSO — dimethyl sulphoxide; EAC — Ehrlich ascites carcinoma; GSH — reduced glutathione; His — histidine; HPD — hematoporphyrin derivative; LND — lonidamine; MTT — 3–(4,5–dimethylthiazol–2–yl)–2,5–diphenyltetrazolium bromide; MDA — malondialdehyde; $^1O_2$ — singlet oxygen; PS — photosensitizer; PBS — phosphate–buffered saline; PDT — photodynamic therapy; PrSH — protein–associated sulfhydryl groups; PUFA — polyunsaturated fatty acids; TCA — trichloroacetic acid; TB — trypan blue; Trp — tryptophan; UV — ultraviolet.

At present, the porphyrin–photosensitized damage of mammalian cells is the subject of active research. This is largely motivated by new applications of these compounds in photodynamic therapy (PDT) of cancer and other diseases. PDT is a rapidly expanding approach to the treatment of malignant tumors involving the administration of a tumor–localizing photosensitizer (PS) and its subsequent activation by visible light. Hematoporphyrin derivative (HPD) and its improved version, called Photofrin II, are the most widely used PSs for PDT of cancer. Photoexcitation of HPD leads to the formation of singlet molecular oxygen ($^1O_2$), a highly reactive oxidant, which is re–

ported to be the main agent responsible for induction of tumor necrosis [1]. A variety of different types of tumors in many locations have been treated with excellent results by PDT, e.g. [2]. However in spite of the large amount of experimental data, the damaging processes directly responsible for cell death are studied insufficiently. At the same time, the information on the mechanism of HPD–sensitized photokilling of neoplastic cells is needed for the further development of more effective PDT protocols.

Being hydrophobic, HPD tends to localize in plasma and subcellular membranes, making these structures especially sensitive to the photooxidative damage. It was reported that photodynamic treatment of tumor cells with HPD induced extensive alterations in the cell morphology [3], inhibited various membrane transport systems [4], destructed cellular membranes and mitochondria [5]. Moreover, among the subcellular organelles mitochondria are considered to be critical targets for the cell killing induced by photoactivated HPD [6, 7]. As known, both of the two main constituents of cellular membranes, lipids and proteins, may be damaged by the photodynamic action of porphyrins. Studies from several laboratories suggest that lipids in the membranes are the most critical targets upon photoexcitation of HPD. It was demonstrated that photodynamic treatment of tumor cells may cause the peroxidative degradation of unsaturated fatty acyl groups and cholesterol (a process commonly referred to as lipid peroxidation) that correlated with alterations in membrane permeability, loss of its fluidity, and finally with cell death [8–11]. However, in these publications it has not been proven that photodamage to lipids is the main cause of cell inactivation, since the effects of PDT with HPD on the intactness of cellular proteins were not examined in these *in vitro* experiments. Furthermore, the analysis of the literature data showed that in the majority of cases the investigations on HPD–sensitized photooxidation of membrane lipids were performed using leukaemia cells. At the same time, it is well known that the polyunsaturated fatty acids (PUFA) content of different cell lines can vary greatly. Therefore, it is of great interest to examine the role of lipid peroxidation in HPD–PDT induced cytotoxicity using neoplastic cells of another histological types. As it is generally accepted, unsaturated lipids are the target for $^1O_2$; although protein molecules may be oxygenation targets, too. There are some indications that cellular proteins are very sensitive to the photodynamic action of HPD. It was reported that photosensitization of tumor cells by HPD may cross–link membrane proteins [12], decrease the activity of various enzymes [13,14], and affect the Na^+/K^+–ATPase pump [4]. Hence, one may suppose that at HPD–PDT the photooxidative degradation of proteins, but not lipids, may play a key role in the initiation of cell death.

The main goal of this study was, therefore, to determine the importance of damage to lipids compared to proteins in deterioration of the plasma membrane integrity, the mitochondrial function, and in cell killing by photodynamic therapy with PHD as a PS.

MATERIALS AND METHODS

Chemicals. HPD was prepared from hematoporphyrin IX dihydrochloride (Aldrich, USA) by the original method of Lipson et al. [15] modified by Kessel et al. [16]. The obtained product was diluted with 0.9% NaCl solution (pH 7.4) to the final porphyrin concentration of 5 mg/ml, and stored in the dark at –70 °C. Working solutions of HPD (0.5 mg/ml) were prepared immediately before use by further diluting of the stock solution with the medium (see below). Lonidamine (LND), kindly supplied by Dr. Aristide Floride from F. Angelini Research Institute (Rome, Italy) (Lot 41A), was dissolved in dimethyl sulphoxide (DMSO) up to 100 mM and was used on the same day as prepared. Other chemicals (of analytical grade or better) were purchased from Sigma St. Louis (USA) unless noted otherwise. In the work, all solutions, including HPD preparations, were sterilized by filtration through 0.22 μm filter units.

Animals and cells. White, mongrel, three month old female mice obtained from the Institute of Experimental and Clinical Medicine (Tallinn, Estonia) were used in the experiments. The animals were fed *ad libitum* on standard pellets and had permanent access to water. Ehrlich ascites carcinoma (EAC) cells obtained from the Institute of Chemical Physics and Biophysics (Tallinn, Estonia) were maintained by intraperitoneal transplantation of 0.2 ml ascites fluid (about $2.5 \cdot 10^7$ cells) from mouse to mouse every 7 days.

Preparation of cell suspensions and the irradiation conditions. Six to seven days old EAC cells were withdrawn from the sacrificed animals and resuspended in phosphate buffered saline (PBS) containing 154 mM NaCl, 6.2 mM KCl, 5.55 mM glucose and 10 mM sodium phosphate buffer (pH 7.4). The cells were centrifuged at 400 *g* for 5 min at 4 °C and washed 2 times with the same medium. The packed cells were resuspended in the PBS to get a concentration of $1.2 \cdot 10^8$ cells/ml and kept in an ice bath until use. The washed cells were then diluted with PBS to a final concentration of $4 \cdot 10^7$ cells/ml and incubated in plastic flasks (Nunclon, Denmark) with 5 μg/ml HPD for 25 min in the dark at 37 °C. In a previous work [17] we found that about 60% of the porphyrin initially added to the medium was associated with cells at the end of the incubation period. An 8 ml sample of the cell suspension was transferred into a 2 x 2 cm quartz cuvette and a microstirring magnet was added. Further the cells were irradiated in air with stirring at 20 ± 1 °C. In all the experiments a voltage regulated 1 kW xenon arc lamp equipped with a focusing optical system and glass filters (KS–10 together with SZS–25) to deliver the light at 630 nm (the range between 590–830 nm) served as the radiation source. The flux of light was focused as a spot (2.54 cm^2) and directed on the front face of a quartz cuvette containing EAC cell suspension. The intensity of the emitted light at 630 nm was always 150 mW with a power density of 59 mW/cm^2, as measured by an IMO–2N radiometer (Russian Federation). In some experiments (when LND was used as a PS), the cells were also exposed to ultraviolet (UV) radiation (λ_{max} = 330 nm, the range be–

tween 260–390 nm). In this study, the intensity of UV radiation was 170 mW (fluence rate, 67 mW/cm^2).

Cytotoxicity assay. Immediately after photoirradiation, 20 μl aliquots of the cell suspension ($4 \cdot 10^7$ cells/ml) were placed into separate wells of a 24–well plate (Nuclon, Denmark) containing 1.5 ml Dulbecco's modified Eagles medium (from Gibco Laboratories, Paisley, Scotland), supplemented with 10% fetal calf serum and 8 μg/ml tylosin. The cells were then incubated for 16 h at 37 °C in a humidified atmosphere of 5% CO_2 in air. After the incubation, cell viability was measured using the 3–(4,5–dimethylthiazol–2–yl)–2,5–diphenyltetrazolium bromide (MTT) method. This method is based on the reduction of MTT by the succinate dehydrogenase of the mitochondria [18]. For the determination of the dehydrogenase activity, the cells were transferred into test tubes and the culture medium was removed by centrifugation. After the addition of 0.5 ml of a 2 mg/ml MTT solution (in Hanks' balanced salt solution, without phenol red, supplemented with 10 mM Hepes buffer, pH 7.2), the cells were incubated for 3 h in a water bath at 37 °C. At the end of the incubation period, the tubes with samples were centrifuged at 3000 *g* for 10 min at 4 °C. The supernatant was carefully removed by aspiration and the pellet was dissolved in 3.0 ml DMSO. Absorbance of the samples was measured at 570 nm. To calculate the fraction of surviving cells, absorbance of the solution from treated cells was divided by absorbance of the solution from control (no treatment) cells.

Assessment of membrane permeability. Trypan blue (TB) dye exclusion was used to determine membrane permeability. During the whole irradiation procedure 10 μl aliquots of the cell suspension ($4 \cdot 10^7$ cells/ml) were removed every 5 min and mixed with 150 μl TB solution (0.1%, in PBS). 25 μl of the mixture was then transferred to the microscopic slide, covered with a cover slip and observed microscopically. Hundred and fifty tumor cells were then observed at random, and the proportion of TB–stained cells was determined.

Assessment of mitochondrial function and glycolysis. The integrity of mitochondria in EAC cells was estimated by measuring the rate of oxygen consumption by the cells ($1 \cdot 10^7$ cells/ml, in air–saturated PBS) with a Clark–type oxygen electrode. The measurements were performed in a water–thermostatted incubation chamber under continuous magnetic stirring at 37 °C.

The rate of glycolysis was estimated by glucose consumption. EAC cells at a concentration of $1 \cdot 10^7$ cells/ml were incubated for 1 h in a water bath (37 °C) in PBS. At the end of the incubation, 0.5 ml of 9% trichloroacetic acid (TCA) solution was added to 1 ml of cellular suspension. The samples were vortexed and stored for 1 h at 4 °C. The cells were then sedimented by centrifugation at 3000 *g* for 10 min at 4 °C. In the supernatant, the glucose concentration was measured colorimetrically by standard o–toluidine reagent using a "Sigma" Kit (procedure No. 635).

Measurement of the intracellular content of ATP. Immediately after HPD–PDT, adenosine triphosphate (ATP) was extracted from $1 \cdot 10^6$ cells by the addition of 0.1 ml of an ice–cold solution containing 5% TCA and 4 mM ethylenediaminetetraacetic acid. The cell extracts were then assayed with luciferin/luciferase using Luminometer 1251 (BioOrbit, Turku, Finland), essentially as described by Kahru et al. [19].

Assay for lipid peroxides. The peroxidation of PUFA in EAC cells was estimated immediately after HPD–PDT by two different methods: by measurement of accumulation of conjugated dienes (CD) and malondialdehyde (MDA). CD (i.e. primary products of the peroxidation of unsaturated fatty acids) were assayed according to [20]. In this assay, $16 \cdot 10^6$ cells were extracted in 5 ml of chloroform–methanol 2 : 1 (v/v). The chloroform–methanol extract was forced into a two–phase system by addition of water. The recovered chloroform phase was evaporated to dryness over nitrogen at 40 °C. The lipid residues were redissolved in 3 ml of methanol, vortexed for 30 s and the absorbance at 233 nm (ascribed to CD) was recorded against a methanol blank on a Specord M–40 spectrophotometer (Germany). The concentration of CD was calculated using a molar extinction coefficient of $2.52 \cdot 10^4$ M^{-1} cm^{-1}. MDA was determined colorimetrically using 2–thiobarbituric acid as described in [21]. These measurements were expressed in terms of MDA normalized to the cell protein content. The total cell proteins were determined by the method of Lowry et al. [22], with bovine serum albumin as the standard.

Determination of the intracellular content of tryptophan, histidine, protein–associated sulfhydryl groups, reduced glutathione and cholesterol. Tryptophan (Trp) was measured by the method [23]. For each sample, $2 \cdot 10^7$ cells were collected in 1 ml PBS and 9 ml of 22.3 mM solution of p–dimethylaminobenzaldehyde in 21.1 N H_2SO_4 was added (the Ehrlich reagent was prepared immediately before use). After 16 h at a room temperature, 0.1 ml of 0.045% $NaNO_2$ solution was added, and 30 min later the absorbance was measured at 590 nm.

The histidine (His) content of cellular proteins was estimated with 5–aminotetrazole according to the procedure [24], whereas protein–associated sulfhydryl groups (PrSH) were assayed by the method of Ellman with 5,5′–dithio–bis–2–nitrobenzoic acid as described in [25].

Reduced glutathione (GSH) was assayed by the spectrofluorometric method [26]. Briefly, GSH in the acid–soluble supernatant fraction of EAC cells reacted with o–phthaldialdehyde (Fluka BioChemica, Germany) at pH 8 to yield a highly fluorescent cyclic product; its fluorescence was measured by a Hitachi 650–60 fluorescence spectrophotometer (Japan).

The content of total cholesterol in EAC cells was assessed colorimetrically by the reaction of Liberman and Burchard as described in [27]. For each estimation, $12 \cdot 10^6$ cells were used.

RESULTS

It was found that photodynamic treatment of EAC cells with HPD as a sensitizer led to significant alterations in the shape and dimensions of the cells; two–

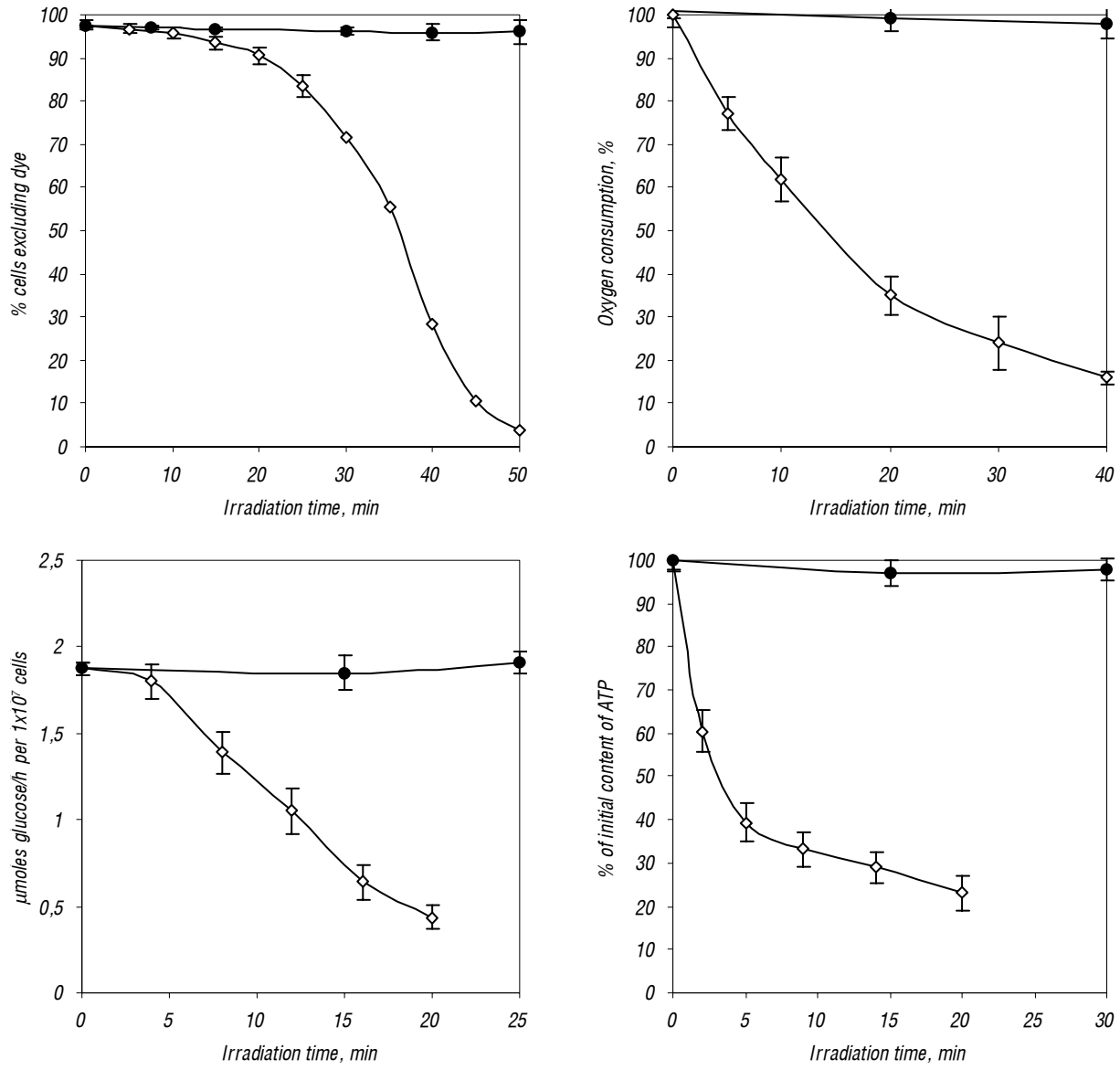

Fig. 1. The kinetics of trypan blue–staining (*a*), oxygen consumption (*b*), glycolytic activity (*c*) and the ATP content (*d*) of EAC cells during their photoirradiation in the presence of 5 μg/ml HPD (opened symbols) or at the dark incubation with the drug (filled symbols). The initial rate of oxygen consumption by the non–irradiated cells was measured as 1.42 ± 0.06 nmoles O_2/min per 1 · 10^6 cells. The initial level of ATP in the cells was 3.62 ± 0.24 nmoles per 1 · 10^6 cells. Points, mean of at least 3 separate experiments. Bars are standard errors (SE)

to three–fold increase in the cell volume, the appearance of numerous small protrusions on the cell surface (also known as "blebs"), the blending of microblebs into large protrusions of cytoplasm were observed. However, control experiments showed that illumination of EAC cells not exposed to HPD or incubation of the cells with the PS in PBS in the dark did not cause similar abnormalities in the cell morphology.

In addition to morphologic changes, the cells were tested for membrane permeability that can be detected by TB dye uptake (cells, whose membranes are intact, exclude TB; when the membrane is damaged, TB can enter the cell). As shown in Fig. 1, *a*, the uptake of TB by EAC cells pre–incubated with HPD increased slowly within 20 min of photoirradiation. Longer illumination of the cells induced a burst in the uptake of TB. At a later stage (after 45 min of irradiation), the appearance of ruptures on the outer membrane was also observed. By contrast, the TB uptake remained negligible in EAC cells incubated with HPD alone, as well as in cells irradiated in the absence of the PS.

According to our results, the photoirradiation of EAC cells pre–incubated with HPD resulted in a serious damage of their mitochondria. In fact, after the 40 min of irradiation a considerable (about 90%) decrease in the rate of oxygen consumption by the cells was observed (Fig. 1, *b*). Furthermore, the HPD–based PDT led to a substantial inhibition of the glycolytic activity of EAC cells (Fig. 1, *c*). These events were associated with a dramatic (approximately 80%) fall in the intracellular content of ATP (Fig. 1, *d*). Thus, the HPD–induced photosensitization caused a strong damage not only to the cytoplasmic membrane but also to the energy homeostasis of EAC cells. It was also established that all these disturbances to the energy metabolism preceded the disintegration of the outer membrane in the cells.

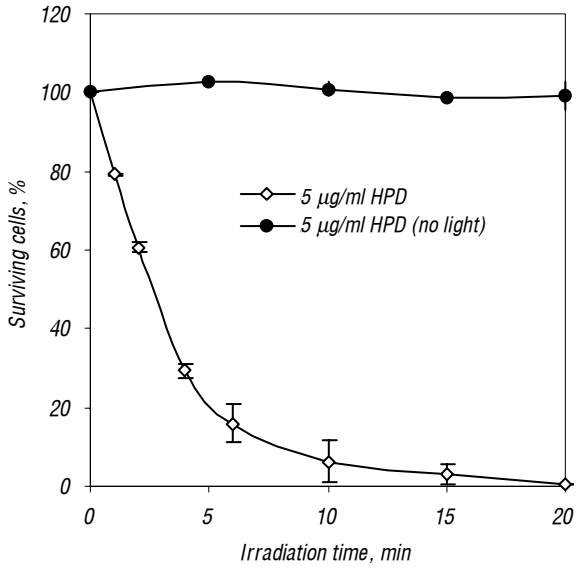

Fig. 2. The viability of EAC cells, as estimated by the MTT assay, after photodynamic treatment with HPD. All the data are from 3 independent experiments performed in duplicate. Bars, SE

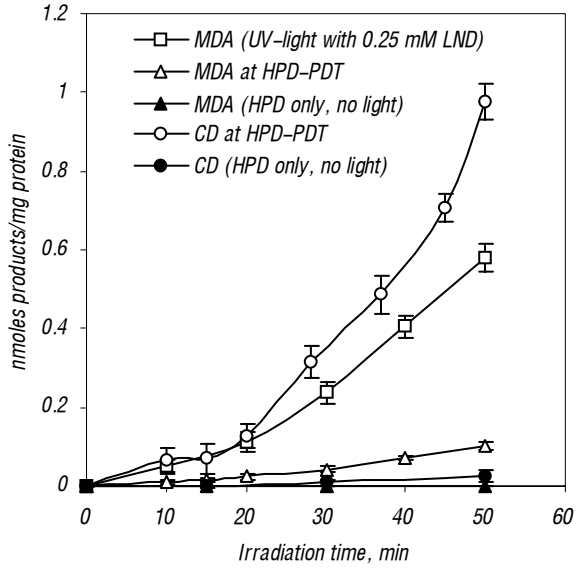

Fig. 3. Lipid peroxidation in EAC cells exposed to HPD– or LND–induced photosensitization as a function of time. MDA, malondialdehyde; CD, conjugated dienes; D_{10}, D_{50}, D_{90} are the light doses at which 10, 50 and 90% of the cells were stained by trypan blue. The content of CD in control cells was measured as 0.81 ± 0.04 nmoles/mg protein and the value was subtracted from those found in photodynamically treated cells. LND was added to EAC cells 10 min prior to the UV irradiation. All data are from 3 independent experiments. Bars, SE

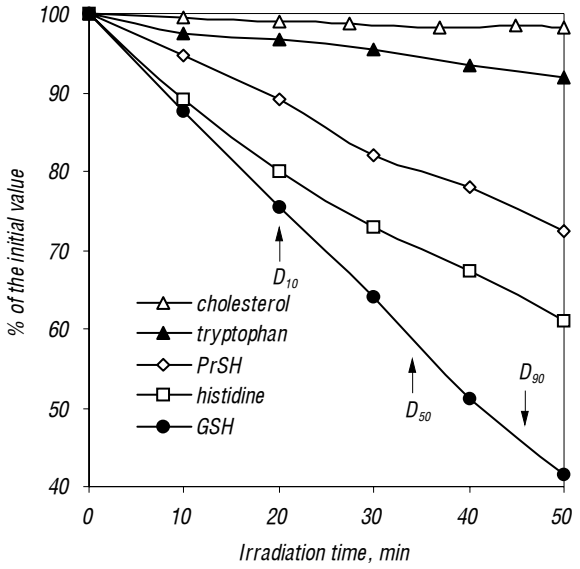

Fig. 4. Effects of HPD–induced photosensitization on the cholesterol, tryptophan, PrSH histidine and reduced glutathione (GSH) content of EAC cells. D_{10}, D_{50}, D_{90} are the light doses at which 10, 50 and 90% of the cells were stained by trypan blue. The points with error bars refer to 3 independent experiments. The initial level of GSH in the cells was measured as 1.41 ± 0.07 nmoles per $1 \cdot 10^6$ cells

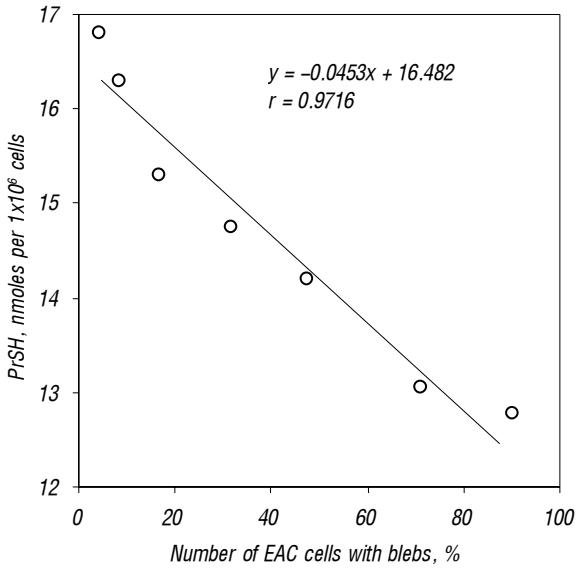

Fig. 5. The interrelationship between the number of EAC cells with blebs and their PrSH content. The cells ($4 \cdot 10^7$ cells/ml in PBS) were exposed to 5 μg/ml HPD–PDT. The total light exposure time was 45 min

Using the MTT–assay the *in vitro* phototoxicity of PDT towards EAC cells was estimated, too. It was found that irradiation of HPD–treated cells with 20 min light exposure led to a significant (about 98%) decrease in the number of surviving cells (Fig. 2). However, neither HPD nor light alone in the doses used had any effect on the viability of the cells.

To elucidate the role of damage to membrane lipids in PDT–induced cytotoxicity, the effects of light exposure with HPD on the oxidation of PUFA and cholesterol in EAC cells were examined. Fig. 3 shows that photosensitization of the cells by the porphyrin resulted in an enhancement of lipid peroxidation that was expressed as a gradual increase in the concentration of CD and MDA. Nevertheless, only very small amounts of these products of lipid peroxidation were found even after a prolonged (50 min) illumination of the tumor cells. Those results were unexpected, since the photodynamic treatment of EAC with HPD caused a strong decrease in the intracellular content of GSH (Fig. 4) that together with some GSH–dependent enzymes plays an important role in the detoxification of the formed lipoperoxides. Possibly, this phenomenon may be explaned by the fact that membrane phospholipids of EAC cells possess high content of saturated fatty acids. However, further ex–

periments showed that the cells containe a sufficient amount of the photooxidizable PUFA; the treatment of EAC cells with 0.25 mM LND, an antitumor agent having the photosensitizing activity [21], for 10 min followed by 50 min exposure to the UV light (λ_{max} = 330 nm) induced a 6–fold increase in the formation of MDA (see Fig. 3). It was also found that photodynamic treatment of EAC cells by HPD had a minor effect on the cholesterol content of the cells (see Fig. 4).

One of the mechanisms by which tumor cells might be damaged during the HPD–based PDT include the may direct oxidation of proteins by photodynamically generated $^1O_2$. Therefore, we evaluated the intactness of proteins in EAC cells after PDT. As it is shown in Fig. 4, the photodynamic treatment of the cells by HPD resulted in a substantial modification of the amino acid composition of their proteins; namely, the part of photooxidized amino acid residues after 50 min illumination was determined as 8% for Trp, 39% for His and 28% for PrSH. By contrast, a prolonged incubation of EAC cells with the PS in PBS in the dark or irradiation of the cells not exposed to HPD did not induce similar changes (data not shown).

DISCUSSION

Our experiments have demonstrated that HPD–PDT caused the strong inhibition of glycolytic and respiratory activity, and substantially lowered the ATP content in EAC cells (see Fig. 1). In addition, earlier [28] we showed that under the same experimental conditions the photoirradiation of HPD–sensitized EAC cells led to rapid and drastic decrease in the activity of succinate dehydrogenase. Other effects of HPD–PDT, such as an increased permeability of the plasma membrane to TB and the alterations in cell morphology (swelling, appearance of large protrusions on the cell surface), were observed at much larger light doses. The obtained results indicated a close relationship between HPD–PDT induced disturbances of the energy metabolism of irradiated cells and their ability to survive. For example, 20 min of light exposure caused the 80% decrease in the ATP level and inactivation of the approximately 98% of the cells (see Fig. 1, *d*, and 2). These findings suggested that after HPD–PDT carcinoma cells died mainly *via* the injury of their mitochondria as well as some glycolytic enzymes. Our results are in a good agreement with the data of other researchers [4, 6, 13] who consider the inhibition of the mitochondrial function as a crucial event in the cytotoxicity resulting from HPD–induced photosensitization *in vitro*.

In the present study we found that photoirradiation of EAC cells pre–incubated with HPD resulted in an enhancement of lipid peroxidation and caused a serious damage to cellular proteins. These molecular changes were associated with disturbances in the cell morphology, an increased permeability of the outer membrane to TB, an inhibition of the mitochondrial function, and cell photokilling. What is the significance of HPD–sensitized photooxidation of cellular lipids in the phototoxic effects of the porphyrin? It is well documented that the oxidative degradation of PUFA and cholesterol is detrimental to membrane structures and functions. Indeed, when the lipids are peroxidized, membranes, which consist of lipids and proteins, undergo the physical change and are finally destroyed. Moreover, it was also established that in the presence of certain transition metal ions the breakdown of lipid hydroperoxides *via* a radical chain reaction produces reactive oxygen species, various aldehydes and other toxic products that can modify proteins, DNA and lead to cell death [29, 30]. It was suggested that lipid peroxidation might be causally related to cytolethality in HPD–photosensitized cells. Thomas & Girotti [11], using murine leukaemia cells, showed that photodynamic treatment of the cells by HPD resulted in the formation of large amounts of lipid hydroperoxides, and concluded that lipid peroxidation plays an important role in tumor cell eradication. Buettner et al. [8], using leukaemia cells of the same line (L1210), showed that PDT with Photofrin® produces membrane–derived lipid free radicals and that increasing the polyunsaturation of cellular lipids enhances radical production as well as the resulting phototoxicity. Furthermore, this research group reported [9] that under PDT with Photofrin® the presence of iron and ascorbic acid accelerates this free radical formation and that the process correlates with an increase in TB–detectable membrane leakage and cell disintegration. By contrast, our experiments on EAC cells indicated that there is a very low probability that the HPD–PDT induced impairment of mitochondria and, as a consequence, cell death was mediated by the peroxidation of membrane lipids. In fact, after 20 min of light exposure, which caused a powerful (more than 2–fold) decrease in the respiratory activity of the cells as well as a 98% decrease in cell survival, only traces of oxidation products of unsaturated fatty acids were detected (see Fig. 1, *b*, 2 and 3 respectively). In addition, the photodynamic treatment of EAC with HPD (for 20 min) had no effect on the intracellular content of cholesterol (see Fig. 4). Nevertheless, we cannot exclude entirely the involvement of lipid peroxidation in the PDT–induced deterioration of the plasma membrane integrity in EAC cells.

At the same time, we found that in EAC cells proteins are more sensitive to the damaging influence of HPD–PDT than their lipid constituents. Namely, in the cells the initial rates of HPD–photosensitized oxidation of PrSH, Trp and His residues were more than 10 times higher than those for unsaturated lipids (Table). What is the mechanism of the phenomenon? In cell mem–

Table. The oxidation rates of unsaturated lipids, proteins and some amino acids in solution as well as in EAC cells subjected to HPD–induced photosensitization *in vitro*

Components	Initial levels in non–irradiated cells, nmoles per $1 \cdot 10^6$ cells (± SE)	Initial rates of photo–oxidation, nmoles h^{-1} per $1 \cdot 10^6$ cells (± SE)	k_r, M^{-1} s^{-1}
Unsaturated fatty acids	–	0.010 ± 0.001[(a)] 0.051 ± 0.008[(b)]	$0.74–2.4 \cdot 10^5$ [37]
Cholesterol	16.54 ± 0.84	0.34 ± 0.12	$0.67 \cdot 10^5$ [37]
Tryptophan	22.34 ± 1.03	1.97 ± 0.21	$3.6 \cdot 10^7$ [32]
Histidine	53.7 ± 3.8	35.2 ± 1.9	$5 \cdot 10^7$ [38]
Cysteine	17.05 ± 0.68	6.14 ± 0.64	$0.89 \cdot 10^7$ [39]
Albumin	–	–	$2–5 \cdot 10^8$ [40]

Notes: [(a)] — this value was calculated by measuring the formation of MDA, and [(b)] — by measuring the production of CD; k_r — rate constants of $^1O_2$ reactions with different substrates in solution.

branes, lipids and proteins provide a competing environment for the formed $^1O_2$. However, proteins, as known, are more reactive toward $^1O_2$ than unsaturated lipids. As shown in Table, the typical rate constants reported for unsaturated lipids (PUFA and cholesterol) reacting with $^1O_2$ in organic solvents correspond to the order of 10^5 M^{-1} s^{-1}. In contrast, rate constants for $^1O_2$ reacting with photooxidizable amino acids and proteins are considerably greater, 10^7–10^8 M^{-1} s^{-1}. Moreover, Kanofsky [31] has shown that only approximately 7% of the $^1O_2$ produced in membranes are quenched by cellular lipids. This is in agreement with our data, which suggest that lipids may not be the primary target for HPD–PDT–induced cytotoxicity *in vitro*.

In the work we have demonstrated that photoirradiation of EAC cells in the presence of HPD resulted in a serious damage of their proteins. It was also established that in cellular proteins the Cys and, especially, His amino acid residues degrade more easily than the Trp (see Fig. 4). Trp can be easily oxidized by HPD + light in model experiments [32]. In our experiments with EAC cells, however, it was destroyed only slightly (by 8%), although in aqueous solution the rate constant for $^1O_2$ reacting with Trp exceeds considerably that for Cys (see Table). Hence, it seems likely that not all of the Trp molecules can be reached by excited HPD. Taken together, our results suggest that at HPD–PDT the direct photodamage of cellular proteins, but not the peroxidation of membrane lipids, is responsible for cell death. In fact, at a light dose that kills about 98% of EAC cells (see Fig. 2) only traces of PUFA and cholesterol were oxidized, but SH groups and His residues of proteins appeared to be very sensitive targets; of the His and PrSH content, 20 and 12% was respectively destroyed during the PDT with HPD (see Fig. 3, 4). Moreover, by the experimental protocol utilized in the work, the mechanism of some cytotoxic effects of HPD–PDT might be uncovered to some degree.

It was found that photodynamic treatment of EAC cells with HPD resulted in the appearance of numerous plasma membrane protrusions (blebs). Similar changes in cell morphology after HPD–induced photosensitization were also registered in other laboratories, e.g. [3]. However, the mechanism of bleb formation and its significance in cell death remain unclear yet, especially, in the case of PDT–induced cytotoxicity. In our experiments on EAC cells the formation of blebs was usually associated with intensive staining of the cells by TB. We assume that at PDT with HPD the appearance of large protrusions on the surface of tumor cells might be associated with cytoskeletal abnormalities, in particular, with detachment of cortical (actin–containing) microfilaments from plasma membrane. Fingar & Wieman [33] have shown that photoirradiation of HPD–loaded endothelial cells results in disruption of their microfilament organization. Our findings, in turn, suggest that the decrease in the intracellular content of ATP (see Fig. 1, *d*) and the oxidation PrSH (see Fig. 4) might be responsible for the cytoskeletal disturbances produced by HPD–PDT. In fact, the formation of blebs was observed in the energy depleted EL–4 thymoma ascites tumor cells after their treatment with rotenone (an inhibitor of respiration) [34]. On the other hand, the same morphological changes were found in cells exposed to toxic levels of menadione (2–methyl–1,4–naphthoquinone) [35]. These researchers reported that the oxidation of SH groups in cytoskeletal proteins (particularly, actin) is mainly responsible for menadione–induced cell surface abnormalities. At the same time, we found that during HPD–PDT the amount EAC cells with blebs was inversely related with their PrSH content (Fig. 5).

The ability of photoexcited porphyrins to damage proteins is well documented. In tumor cells, such photodamage to proteins, in addition to the peroxidation of unsaturated lipids, can lead to a severe injury and, as a consequence, to cell death. So, in our experiments the HPD–PDT–induced disturbance of the plasma membrane integrity (as evaluated by TB–test) was directly related to the decrease in the Trp, His and PrSH content (see Fig. 4). This observation is in agreement with the suggestion of Dubbelman et al. [36] that the main photodynamic effect of porphyrins on red cell membranes is caused by the photooxidation of amino acid residues of proteins, mainly His. In the study, we also found that photodynamic treatment of EAC cells with HPD caused a strong reduction in the intracellular level of ATP, which preceded the disintegration of the outer membrane in the cells (see Fig. 1). Because in mammalian cells practically all vital functions are dependent on the presence of ATP, we believe that upon HPD–PDT the shortage of energy could contribute to the TB–detectable membrane leakage. Our data suggest that after PDT with HPD the EAC cells died mainly *via* the inactivation of their energy producing systems; a powerful inhibition of the mitochondrial function as well as the glycolytic activity of the cells was registered (see Fig. 1). The destructive impact of HPD–PDT was associated with oxidation of the PrSH (see Fig. 4) and could be consequently ascribed to the inactivation of some enzymes containing in their active sites SH groups. For example, it was reported [13] that oxidation of SH groups in the ADP/ATP translocator is the main cause of oxidative phosphorylation impairment when isolated mitochondria were exposed to HPD and light.

At present time, the prevalent concept is that under HPD–PDT the peroxidation of membrane lipids plays a crucial role in the initiation of cell death, although the ability of photoexcited HPD to damage a number of cellular proteins is also a well–documented event. The present work is apparently the first attempt to determine the significance of photodamage of lipids compared to that of proteins in the photodynamic killing of tumor cells with HPD. Experiments performed on EAC cells showed that cellular proteins, but not membrane lipids, are a principal target of PDT with the PS. Nevertheless, a comparison of our results with those from other laboratories [8, 9, 11] suggests that upon HPD–PDT the significance of lipid peroxidation in the initiation of cell death may be dependent on the cell type. However, this hypothesis will be the subject of future

studies. Our findings indicated that after PDT with HPD the EAC cells died mainly *via* the inactivation of their energy producing systems and that the PDT caused serious disturbances in cell morphology. However, further work is required to clarify the mechanism of cell surface blebbing.

REFERENCES

1. **Weishaupt KR, Gomer CJ, Dougherty TJ.** Identification of singlet oxygen as the cytotoxic agent in photo-inactivation of a murine tumor. Cancer Res 1976; **36**: 2326–9.

2. **Okunaka T, Kato H, Conaka C, Yamamoto H, Bonaminio A, Eckhauser ML.** Photodynamic therapy of esophageal carcinoma. Surg Endosc 1990; **4**: 150–3.

3. **Volden G, Christensen T, Moan J.** Photodynamic membrane damage of hematoporphyrin derivative-treated NHIK 3025 cells *in vitro*. Photobiochem Photobiophys 1981; **3**: 105–11.

4. **Penning LC, Tijssen K, Boegheim JPJ, van Steveninck J, Dubbelman TMAR.** Relationship between photodynamically induced damage to various cellular parameters and loss of clonogenicity in different cell types with hematoporphyrin as sensitizer. Biochim Biophys Acta 1994; **1221**: 250–8.

5. **Tatsuta M, Yamamoto R, Yamamura H, Iishi H, Noguchi S, Ichii M, Okuda, S.** Photodynamic effects of exposure to hematoporphyrin derivatives and dye-laser radiation on human gastric adenocarcinoma cells. JNCI 1984; **73**: 59–67.

6. **Hilf R, Murant RS, Narayanan U, Gibson SL.** Relationship of mitochondrial function and cellular adenosine triphosphate levels to hematoporphyrin derivative-induced photosensitization in R3230AC mammary tumors. Cancer Res 1986; **46**: 211–7.

7. **Oleinick NL, Morris RL, Belichenko I.** The role of apoptosis in response to photodynamic therapy: what, where, why, and how. Photochem Photobiol Sci 2002; **1**: 1–21.

8. **Buettner GR, Kelley EE, Burns CP.** Membrane lipid free radicals produced from L1210 murine leukemia cells by photofrin photosensitization: an electron paramagnetic resonance spin trapping study. Cancer Res 1993; **53**: 3670–3.

9. **Kelley EE, Buettner GR, Burns CP.** Production of lipid-derived free radicals in L1210 murine leukemia cells is an early oxidative event in the photodynamic action of Photofrin®. Photochem Photobiol 1997; **65**: 576–80.

10. **Thomas JP, Girotti AW.** Photooxidation of cell membranes in the presence of hematoporphyrin derivative: reactivity of phospholipid and cholesterol hydroperoxides with glutathione peroxidase. Biochim Biophys Acta 1988; **962**: 297–307.

11. **Thomas JP, Girotti AW.** Role of lipid peroxidation in hematoporphyrin derivative-sensitized photokilling of tumor cells: protective effects of glutathione peroxidase. Cancer Res 1989; **49**: 1682–6.

12. **Moan J, Vistnes A.** Porphyrin photosensitization of proteins in cell membranes as studied by spin-labeling and by quantification of DTNB-reactive SH-groups. Photochem Photobiol 1986; **44**: 15–9.

13. **Atlante A, Passarella S, Quagliariello E, Moreno G, Salet C.** Haematoporphyrin derivative (Photofrin II) photosensitization of isolated mitochondria: inhibition of ADP/ATP translocator. J Photochem Photobiol B: Biol 1989; **4**: 35–46.

14. **Gibson SL, Murant RS, Chazen MD, Kelly ME, Hilf R.** *In vitro* photosensitization of tumour cell enzymes by Photofrin II administered *in vivo*. Br J Cancer 1989; **59**: 47–53.

15. **Lipson R, Baldes E, Olsen A.** The use of a derivative of hematoporphyrin in tumor detection. JNCI 1961; **26**: 1–8.

16. **Kessel D, Thompson P, Musselman B, Chang CK.** Chemistry of hematoporphyrin-derived photosensitizers. Photochem Photobiol 1987; **46**: 563–8.

17. **Chekulayev V, Shevchuk I, Chekulayeva L.** Effects of light exposure on the uptake and destruction of hematoporphyrin derivative in Ehrlich carcinoma cell suspension. Proc Estonian Acad Sci Chem 1998; **47**: 73–91.

18. **Mossman T.** Rapid colorimetric assay for cellular growth and survival: application to proliferation and survival assays. J Immunol Methods 1983; **65**: 55–63.

19. **Kahru A, Liiders M, Vanatalu K, Vilu R.** Adenylate energy charge during batch culture of Thermoactinomyces vulgaris. Arch Microbiol 1982; **133**: 142–4.

20. **Recknagel RO, Ghoshal AK.** Lipoperoxidation of rat liver microsomal lipids induced by carbon tetrachloride. Nature 1966; **210**: 1162–3.

21. **Chekulayev V, Shevchuk I, Chekulayeva L, Kahru A.** Study of the photochemical and phototoxic properties of Lonidamine [1-(2,4-dichlorobenzyl)-1H-indazol-3-carboxylic acid. J Photochem Photobiol B: Biol 1997; **41**: 11–21.

22. **Lowry OH, Rosebrough NJ, Farr AL, Randall RJ.** Protein measurement with the Folin phenol reagent. J Biol Chem 1951; **193**: 265–75.

23. **Spies JR, Chambers DC.** Chemical determination of tryptophan. Anal Chem 1948; **20**: 30–9.

24. **Sokolovsky M, Vallee B.** The reaction of diazonium-1H-tetrazole with proteins. Determination of tyrosine and histidine content. Biochemistry 1966; **5**: 11: 3574–81.

25. **Edwards PG**. Evidence that glutathione may determine the differential cell-cycle phase toxicity of a platinum (IV) antitumor agent. JNCI 1988; **80**: 734–8.

26. **Hissin PJ, Hilf R.** A fluorometric method for determination of oxidized and reduced glutathione in tissues. Anal Biochem 1976; **74**: 214–26.

27. **Prohorova MI.** Methods of biochemical studies. Saint-Petersbourg: Saint-Petersbourg Univ. Press, 1982 (in Russian).

28. **Chekulayev V, Shevchuk I, Chekulayeva L, Jaalaid R.** Influence of extracellular magnesium on the antitumour efficiency of photodynamic therapy. Proc Estonian Acad Sci Chem 2000; **49**: 216–33.

29. **Girotti AW.** Photosensitized oxidation of membrane lipids: reaction pathways, cytotoxic effects, and cytoprotective mechanisms. J Photochem Photobiol B: Biol 2001; **63**: 103–13.

30. **Paillous N, Forgues-Ferry S.** Interest of photochemical methods for induction of lipid peroxidation. Biochïmie 1994; **76**: 355–68.

31. **Kanofsky JR.** Quenching of singlet oxygen by human red cell ghosts. Photochem Photobiol 1991; **53**: 93–9.

32. **Reddi E, Rodgers MAJ, Spikes JD, Jori G.** The effect of medium polarity on the hematoporhyrin-sensitized photooxidation of L-tryptopan. Photochem Photobiol 1984; **40**: 415–21.

33. **Fingar VH, Wieman JF.** Mechanisms of vessel damage in photodynamic therapy. Proc SPIE 1992; **1645**: 98–103.

34. **Gabai VL, Kabakov AE, Mosin AF.** Association of blebbing with assembly of cytoskeletal proteins in ATP-depleted EL-4 ascites tumour cells. Tissue Cell 1992; **24**: 171–7.

35. **Mirabelli F, Salis A, Perotti M, Taddei F, Bellomo G, Orrenius S.** Alterations of surface morphology caused by the metabolism of menadione in mammalian cells are associated with the oxidation of critical sulfhydryl groups in cytoskeletal proteins. Biochem Pharmacol 1988; **37**: 3423–7.

36. **Dubbelman TMAR, De Goeij AFPM, van Steveninck J.** Photodynamic effects of protoporphyrin on

human erythrocytes. Nature of the cross-linking of membrane proteins. Biochim Biophys Acta 1978; **511**: 141–51.

37. **Doleiden FH, Fahrenholtz SR, Lamola AA, Trozzolo AM.** Reactivity of cholesterol and some fatty acids toward singlet oxygen. Photochem Photobiol 1974; **20**: 519–21.

38. **Kraljic I, Sharpatyi VA.** Determination of singlet oxygen rate constants in aqueous solutions. Photochem Photobiol 1978; **28**: 583–6.

39. **Rougee M, Bensasson RV, Land EJ, Pariente R.** Deactivation of singlet molecular oxygen by thiols and related compounds, possible protectors against skin photosensitivity. Photochem Photobiol 1988; **47**: 485–9.

40. **Wilkinson F, Helman WP, Ross AB.** Rate constants for the decay and reactions of the lowest electronically excited singlet state of molecular oxygen in solution. An expanded and revised compilation. J Phys Chem Ref Data 1995; **24**: 663–1021.