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INFLUENCE OF TEMPERATURE ON THE EFFICIENCY OF PHOTODESTRUCTION OF EHRLICH ASCITES CARCINOMA CELLS SENSITIZED BY HEMATOPORPHYRIN DERIVATIVE

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***Aim*: To elucidate the mechanism of the potentiating influence of heating associated with photoirradiation on the antitumor efficiency of photodynamic therapy (PDT) with hematoporphyrin derivative (HPD). *Methods*: The study was carried out on Ehrlich ascites carcinoma (EAC) cells, which were loaded with HPD in a serum-free medium and then irradiated with red light (λ_{max} = 630 nm) at various temperatures. Cytotoxicity was estimated by the trypan blue exclusion assay. *Results*: Our data support the view that in PDT the hyperthermia (around 44 °C) produced by irradiation can enhance synergistically the HPD-photoinduced tumor eradication; it was found that raising the irradiation temperature from 30 to 44 °C caused a substantial (~ 1.5 fold) increase in the rate of HPD-photosensitized inactivation of EAC cells, while hyperthermia (44 °C) itself showed little toxic effects towards the cells. *Conclusion*: Studies indicated that the potentiating effect of heating on the antitumor efficiency of HPD-PDT could be largely explained by the stimulation of reactive oxygen species formation such as H_2O_2, superoxide and hydroxyl radicals. It was also found that photosensitization of EAC cells by HPD caused a strong fall in the activity of catalase (CAT) and glutathione (GSH) peroxidase, and that heating sensitized the H_2O_2-detoxifying enzymes to HPD-photoinduced inactivation. Under HPD-PDT, these events could result in loss of protection against accumulating H_2O_2; we revealed that cell-bound CAT and the GSH redox cycle play an important role in the protection of EAC cells against HPD-PDT. Moreover, our findings suggest that during PDT with HPD, an increase in the temperature of tumors could enhance the efficiency of this therapy *via* the stimulation of a chlorin-type photoproduct formation.**
***Key Words*: photodynamic therapy, tumor, heating, reactive oxygen species, antioxidant enzymes.**

Photodynamic therapy (PDT) is a relatively new approach for the treatment of malignancies with only minimal side effects for the patient. It is based on the administration of a tumor–localizing dye (photosensitizer, PS) that becomes toxic to neoplastic cells when is activated by light (usually from a laser) at a specific wavelength in the presence of O_2. Clinically the most frequently used PS(s) are hematoporphyrin derivative (HPD) and its more purified and active form, commercially known as Photofrin–II (PF–II). Photoexcitation of HPD leads to the formation of singlet oxygen ($^1O_2$) and it was suggested [1] that this highly active oxidant is the main damaging agent in PDT. However, there are some indications that besides $^1O_2$, other reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide ($O_2^{-\bullet}$) and hydroxyl ($OH^\bullet$) radicals, might be involved in the HPD–PDT induced tumor eradication [2–4]. Investigations on the mechanism of action of HPD–PDT showed that this treatment modality may include not only a direct tumoricidal effect (damage of organelles within malignant cells), but also destruction of blood vessels in the tumor locus resulting in reduced supply of oxygen and nutrients [5, 6]. Although to date a number of patients with tumors practically of all histological types and in many locations have been treated with encouraging results by PDT with HPD or PF–II [7], the fundamental mechanisms by which PDT kills tumor cells as well as its optimal physical parameters remain poorly studied yet.

Though photochemical reactions of HPD seem to play a major role in the tumor response to PDT, clinical trials [8] and the data obtained on animal models [9–11] showed that during a standard regimen of PDT (630 nm laser light at a power density of 100–200 mW/cm^2) a considerable (5–13 °C) increase in the temperature of tumor tissues can take place. Moreover, these researchers supposed that at least part of the result attributed to PDT with HPD could be mediated by a hyperthermic contribution, because the optical radiation utilized during the therapy can produce intratumoral temperatures that exceed the threshold of hyperthermic effects, *i.e.* greater than 41 °C; hyperthermia has proven to be selectively lethal to several kinds of malignant cells at temperatures in the range of 41–46 °C [12]. The precise mechanisms leading to cell death following heat exposure are not clearly understood. Nevertheless, it is in–

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Abbreviations used: BCNU — 1,3–bis(2–chloroethyl)–1–nitrosourea; BSO — D, L–buthionine–(S,R)–sulfoximine; CAT — catalase; SOD — superoxide dismutase; DDC — sodium diethyldithiocarbamate; D_2O — deuterium oxide; DEF — deferoxamine mesylate; DOR — 2–deoxyribose; EAC — Ehrlich ascites carcinoma; EDTA — ethylenediaminetetraacetic acid; GSH — reduced glutathione; GSSG — oxidized glutathione; GPX — glutathione peroxidase; GR — glutathione reductase; HP — hematoporphyrin; HPD — hematoporphyrin derivative; H_2O_2 — hydrogen peroxide; i.p. — intraperitoneal; NADPH — reduced nicotinamide adenine dinucleotide phosphate; $^1O_2$ — singlet oxygen; $O_2^{-\bullet}$ — superoxide radical; $OH^\bullet$ — hydroxyl radical; PhP–640 — photoproduct–640; PF–II — Photofrin–II; PS — photosensitizer; PBS — phosphate–buffered saline; PDT — photodynamic therapy; PrSH — protein–bound sulfhydryl groups; ROS — reactive oxygen species; SE — standard error; TBA — 2–thiobarbituric acid; TB — trypan blue; Trp — tryptophan.

creasingly recognized that oxidative stress could be implicated in heat–induced cell death [13, 14]. Subsequently, more detailed studies on various experimental tumor systems clearly indicated that the thermal effects associated with photoirradiation may play a substantial role in the total cytotoxic effect of HPD–PDT and may potentiate (in a synergistic manner) the porphyrin–catalyzed photodestruction of tumor cells [15–17]. However, until now the mechanism of the synergism remains unclear, although these observations resulted in development of more effective treatment regimens in which HPD–based PDT is combined with a localized laser– or microwave–induced hyperthermia immediately before or simultaneously with the phototherapy [17, 18]. Several assumptions had been advanced to explain the potentiating effect of heating on the antitumor efficiency of HPD–PDT. Namely, it was suggested that in PDT the hyperthermia produced by a laser irradiation could promote tumor destruction *via* an increase in the reactivity of $^1O_2$ [19]. Further, based on the model experiments with glyceraldehyde–3–phosphate dehydrogenase, Prinsze et al [20] have proposed a mechanism for the synergistic interaction between HPD–PDT and hyperthermia in cancer treatment. Namely, their data suggest that even a minor photodamage of cellular proteins may cause a pronounced potentiation of their sensitivity to thermal denaturation; it is important to note that cell proteins are, as believed [5], a principal target of PDT with HPD. As known, the sensitivity of tumor cells to the phototoxic action of HPD depends largely on the presence of O_2. However, it was established that PDT with HPD can photochemically deplete ambient tumor O_2, causing acute hypoxia and limiting treatment efficiency [21]. Therefore, it was suggested that mild hyperthermia (42–44 °C) during PDT could improve the oxygenation status of tumors (due to a growth in tumor blood flow) and increase thereby the tumor response to the therapy [22].

However together with aforementioned, other important processes could be responsible for a heat–induced increase in the sensitivity of tumor cells to the phototoxic action of HPD. We believe that besides its direct antitumor effect, the hyperthermia associated with photoirradiation could promote the formation of $^1O_2$ and/or other cytotoxic ROS by activated molecules of the PS. Indeed, components of HPD are known to aggregate readily in certain solvents; in an aqueous environment, the PS exists as a complex mixture of non–aggregated or self–aggregated monoporphyrinic and oligomeric species [23]. At the same time, in a prior study [24] we established that in aqueous buffer the dimers and larger aggregates of HPD can be disrupted by an increase in the temperature. Such a thermal disaggregation of HPD–moieties may presumably occur in tumor cells during PDT and results in higher yields of $^1O_2$ and oxygen radicals formation, since the nonaggregated molecules of the PS can easily come in contact with the surrounding molecules of O_2. This assumption is supported by the data of [23], which showed that HPD in the aggregation state is a poor source of $^1O_2$. Furthermore, a plausible explanation for the potentiating action of light–induced heating on the efficiency of PDT with HPD could be that the hyperthermic influence during PDT might make tumor cells more susceptible to oxidative damage due to an inhibition of the cellular repair enzymes activity and/or antioxidant systems of the cell. Numerous studies utilizing laser–induced fluorescence have demonstrated that HPD is destroyed (photobleached) during the PDT procedure. This photoprocess under certain circumstances, notably when the initial concentration of HPD in the tumor is low, may limit the efficiency and applicability of PDT. However, our previous studies [25] and the data of other researchers [26] showed that illumination of HPD in aqueous buffer as well as in tumor cells leads to the formation of a photoproduct with peak absorption around 640 nm and peak fluorescence emission around 644 nm. Comprehensive studies on HPD led to the suggestion that the photoproduct with an absorption band at 640 nm (photoproduct–640, PhP–640) is a chlorin or a covalently linked porphyrin–chlorin system [26]. Moreover, it was reported that PhP–640 may take part in the PDT–induced tumor eradication, since it absorbs the radiation of light sources commonly used in clinical treatment and may act as a PS [27]. So, if the heating of tumors during PDT will promote the formation of PhP–640, it might be expected that this phenomenon could be also responsible for the potentiating effect of photoirradiation–induced hyperthermia on the efficiency of PDT with HPD. However, these assumptions require an experimental checking.

Thus, the main aim of this work was to elucidate the mechanism of the potentiating influence of heating, associated with the absorption of optical radiation, on the antitumor efficiency of PDT with HPD. This information is needed for the further development of more effective PDT protocols.

MATERIALS AND METHODS

Chemicals. Deferoxamine mesylate (DEF), deuterium oxide (D_2O), H_2O_2 (30%, w/v), Cu/Zn superoxide dismutase (Cu/Zn–SOD; from bovine erythrocytes, 3300 units/mg protein), catalase (CAT; from bovine liver, thymol free, 16700 units/mg protein) and other chemicals (of analytical grade or better) were purchased from Sigma (St. Louis, USA) unless noted otherwise. HPD was prepared from hematoporphyrin (HP) dihydrochloride as described in [28]. The obtained product was diluted with 0.9% NaCl solution (pH 7.4) to a final porphyrin concentration of 5 mg/ml, and stored in the dark at –20 °C. Working solutions of HPD (0.5 mg/ml) were prepared immediately before use by further diluting of the stock solution with a medium (see below). In the work, all solutions were sterilized by filtration through 0.22 μm filter units.

Animals and cells. White, mongrel, three month old female mice obtained from the National Institute for Health Development (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 National Institute of Chemical Physics and Biophysics

(Tallinn, Estonia) were maintained by intraperitoneal (i.p.) transplantation of 0.2 ml ascites fluid (~ 2.5 x 10^7 cells) from mouse to mouse every 7 days.

Light source. 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 infrared radiation was removed by a 4 cm water filter). The flux of the light was focused as a spot (2.54 cm^2) and directed on the front face of a quartz cuvette containing EAC cell suspension or HPD solutions. In some experiments, EAC cells and HPD solutions were also irradiated with green light at 510 nm (the range between 480–570 nm). The intensity of the emitted light at 510 and 630 nm was always 260 mW with a power density of 102 mW/cm^2, as measured by an IMO–2N radiometer (Russian Federation).

Preparation of cell suspensions, the irradiation conditions, and cytotoxicity assay. Six to seven days old EAC cells were withdrawn from the sacrificed animals and suspended in phosphate–buffered saline (PBS) containing 154 mM NaCl, 6.2 mM KCl, 5.55 mM glucose and 10 mM sodium phosphate buffer (pH 7.3). The cells were centrifuged at 600 *g* for 5 min at 4 °C, washed twice, counted, and resuspended in the same buffer at a concentration of 4 x 10^7 cells/ml. The viability of the cells was about 95–98% as found by light microscopy in the presence of trypan blue (TB). Further, the cells were loaded with HPD. For this purpose, an aliquot of the cell suspension (4 x 10^7 cells/ml) was placed into a plastic flask (Nunclon, Denmark) and after the addition of HPD (up to a final concentration of 20 μg/ml) the cells were incubated for 25 min in the dark at 30 °C with shaking at 80 oscillations/min. After the incubation, the cell suspension was transferred quantitatively to a 10–ml centrifuge tube and the cells were sedimented by centrifugation at 2800 rpm for 5 min at 4 °C. The supernatant containing the unbound fraction of HPD was removed by aspiration, whereas the packed cells were resuspended in PBS at a concentration of 4 x 10^7 cells/ml and kept in an ice bath until use. For the simultaneous thermal and PDT treatment, the cells loaded with HPD were diluted with PBS that was pre–heated in a water bath to a desired temperature. An 8 ml sample of the cell suspension (5 x 10^6 cells/ml) was quickly transferred into a 2 x 2 cm quartz cuvette and a microstirring magnet was added. The cuvette with the cells was then placed in a thermostatted (by circulating water) holder and illuminated in air with stirring at different temperatures, which were maintained within the errors limits of ± 0.5 °C. The time interval between introduction of the cells into the preheated PBS and the beginning of light exposure was 5 min.

Cytotoxicity was determined by the TB exclusion assay. Immediately after photoirradiation, an aliquot of the cell suspension (5 x 10^6 cells/ml) was diluted 1 : 1 (v/v) with 0.2% TB and 2 min later the cells were counted using a Goryaev's chamber. Results were expressed as the percentage of dead cells calculated as the ratio of TB–stained versus the total number of cells.

Assessment of cell respiration. The respiration of EAC cells (1 x 10^7 cells/ml in air–saturated PBS) was estimated by measuring the rate of oxygen consumption by the cells using a Clark–type oxygen electrode. The measurements were performed in a water–thermostatted incubation chamber under continuous magnetic stirring at a required temperature.

Depletion of glutathione in EAC cells. This was achieved by treatment of the cells with D, L–buthionine–(S,R)–sulfoximine (BSO), a potent inhibitor of glutathione biosynthesis [29], using *in vivo* protocols. These experiments were performed on 8 female mice to which 0.2 ml EAC was injected i.p. On the sixth day after the tumor inoculation, some of the animals were injected i.p. with BSO (up to 4 mM/kg body weight) dissolved in 0.9% NaCl. Control animals with the tumor received an i.p. injection of 0.9% NaCl. Fourteen hours later, the carcinoma cells from control and BSO–treated mice were removed by aspiration, washed twice with PBS, and the levels of glutathione in these cells were measured as described below.

Determination of the intracellular levels of tryptophan, protein–bound sulfhydryl groups, reduced and oxidized glutathione. The tryptophan (Trp) content of cellular proteins was measured according to the method of Spies & Chambers with p–dimethylaminobenzaldehyde exactly as described in [5], whereas protein–bound sulfhydryl groups (PrSH) were assayed by the method of Ellman with 5,5'–dithiobis–2–nitrobenzoic acid as described in [30]. Reduced glutathione (GSH) and its oxidized form (GSSG) were assayed by the spectrofluorometric method [31]. Briefly, GSH in the acid–soluble supernatant fraction of EAC cells was 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). GSSG was determined by the same reagent, but at pH 12 and in the presence of N–ethylmaleimide.

Assay for $O_2^{-\bullet}$, H_2O_2, and $OH^\bullet$ generation. The intracellular levels of $O_2^{-\bullet}$ were measured using an adaptation of the method employed by [32]. We used the dye hydroethidine (HE; Fluka BioChemica, Germany) that is oxidized by $O_2^{-\bullet}$ within the cell to produce ethidium bromide, which emits red fluorescence after intercalation into DNA. Briefly, following photoirradiation EAC cells (at a density of 1.5 x 10^6 cells/ml in PBS) were incubated with 10 μM HE (made as a 10 mM stock solution in N,N–dimethylformamide) for 15 min in a shaking water bath at 37 °C. After the incubation, the fluorescence from ethidium bromide in the cells was measured at 610 nm (λ_{ex} = 488 nm) in 1 x 1 cm quartz cuvettes; slits were 5 and 15 nm for excitation and emission, respectively. The blank corresponding to HE alone in the buffer was subtracted from the value.

The production of H_2O_2 during photodynamic treatment of EAC cells with HPD was estimated by measurement of its concentration in cell free supernatants. Namely, immediately after light exposure aliquots (0.2 ml) of the cell suspension (5 x 10^6 cells/ml) were placed into

pre–cooled (on melting ice) plastic tubes. Further, the cells were precipitated on an Eppendorf table centrifuge at maximal speed (8500 *g*, for 4 min at room temperature) and the concentration of H_2O_2 in the supernatants was determined by the method [33] that is based on the oxidation of nonfluorescent leukodiacetyl–2,7–dichloro–fluorescin to a fluorescent compound by H_2O_2 in the presence of horseradish peroxidase.

The generation of $OH^{\bullet}$ radicals during photodynamic treatment of EAC cells with HPD was estimated by the 2–deoxyribose (DOR) method of Gutteridge [34]; the sugar is oxidized by $OH^{\bullet}$ with the formation of products, which upon heating with 2–thiobarbituric acid (TBA) at low pH yield a pink chromogen. In our studies, the TBA–reactive products of DOR were determined as described previously in [34, 35] with minor modifications. Briefly, during the whole irradiation procedure aliquots (0.3 ml, typically containing 3 μmoles of DOR) of the cell suspension (5×10^6 cells/ml in PBS) were collected into test tubes with 0.9 ml of 154 mM NaCl. After the addition of 0.2 ml of cold 15% trichloroacetic acid solution, the samples were vortexed and stored on an ice bath for 20 min. The tubes with the samples were then centrifuged and 0.8 ml of 1% TBA in 0.05 M NaOH was added to 0.9 ml of the supernatant. These samples were heated at 100 °C for 15 min, cooled rapidly to room temperature and their fluorescence was read at 553 nm (excitation at 532 nm). The obtained results were expressed as relative fluorescence units against a standard containing rhodamine B (1.0 μM, in 10 mM sodium phosphate buffer of pH 7.2). This standard was set to 100 units at excitation 480 nm, emission at 580 nm. In these experiments, the contribution of lipid peroxidation to TBA reactivity was negligible and the values were subtracted from those observed during HPD–PDT of EAC cells in the presence of DOR.

Studies of the photochemical transformations of HPD in EAC cells. In these experiments, EAC cells loaded with HPD were diluted with PBS to a concentration of 5×10^6 cells/ml. Further, an 8 ml sample of the cell suspension was transferred into a 2 x 2 cm thermostatted quartz cuvette and irradiated in air under magnetic stirring with red light illumination at 630 nm. During light exposure, the photodegradation of HPD in EAC cells as well as the formation of PhP–640 was monitored by fluorescence spectroscopy. Namely, following photoirradiation aliquots (0.2 ml) of cell suspension were withdrawn and placed into test tubes with 1.3 ml of 1.0 M NaOH. One hour later, fluorescence spectra (excitation at 399 nm) of the cell lysates were recorded using 1 x 1 cm quartz cuvettes. The fall of light–induced fluorescence of HPD in the 614 nm band was measured to determine the rate of its photodegradation in the cells, while the yields of PhP–640 formation were estimated by measuring the fluorescence of cell lysates at 644 nm. The concentration of HPD in cell lysates was determined from a calibration curve, which was obtained by measuring the fluorescence of known porphyrin concentration at 614 nm in 1.0 M NaOH.

Photodestruction of tryptophan in aqueous solution. In these experiments, an 8 ml sample of a 10 μg/ml HPD solution (containing 0.2 mM Trp) was placed into a 2 x 2 cm quartz cuvette and irradiated in air under magnetic stirring with light at 510 or 630 nm. Under photoexcitation of HPD, the absorbance (differential spectrum) of Trp at 280 nm was registered to determine the rate of its photooxidation.

Determination of enzyme activities. Following HPD–PDT or pretreatment with corresponding inhibitors, EAC cells were lysed with 0.2% Triton X–100. Any non–solubilized material was removed by centrifugation, and the supernatant solutions were analyzed for CAT, glutathione peroxidase (GPX), glutathione reductase (GR) or Cu/Zn–SOD activity.

CAT activity was determined at 20 °C by the method of Aebi [36]. CAT decomposes H_2O_2 to give H_2O and O_2. The decomposition of H_2O_2 was followed by a decrease in the absorbance at 240 nm. The reaction mixture contained 50 mM sodium phosphate buffer (pH 7.0), 10 mM H_2O_2 and ~ 150 μg protein of cell lysate, final volume was 1.5 ml. CAT activity was expressed as μmoles H_2O_2 decomposed/min per mg protein, using an excitation coefficient of 43.6 M^{-1} cm^{-1} for H_2O_2. The total cell proteins were determined by the well–known procedure with fluorescamine, using bovine serum albumin as the standard.

The activities of GR and GPX were determined at 37 °C. GR was measured essentially as described in [37]. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.4), 1.0 mM ethylenediamine–tetraacetic acid (EDTA), 0.2 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH), 2.5 mM GSSG, and cell sample (~ 250 μg protein), in a total volume of 1.5 ml. GR activity was determined by tracking the decrease in absorbance of NADPH at 340 nm during the reduction of GSSG. The enzyme activity was expressed as nmoles NADPH oxidized/min per mg protein, using an excitation coefficient of 6.22 mM^{-1} cm^{-1} for NADPH. GPX activity was determined by the GR–coupled method reported by [38], using H_2O_2 as the substrate. GPX catalyses the oxidation of GSH to GSSG by H_2O_2. The rate of GSSG formation was then measured by following a decrease in the absorbance of the reaction mixture containing NADPH and GR at 340 nm as NADPH is converted to NADP. The assay mixture contained 50 mM potassium phosphate buffer (pH 7.4), 1.0 mM EDTA, 0.9 unit/ml yeast GR, 1.0 mM GSH, 0.15 mM NADPH, 1.0 mM NaN_3 (to inhibit CAT), 0.25 mM H_2O_2 and ~ 200 μg protein of cell lysate; final volume, 1.5 ml. GPX activity was calculated as nmoles NADPH oxidized/min per mg protein.

The activity of Cu/Zn–SOD was measured at 25 °C according to the procedure [39] that utilizes a xanthine–xanthine oxidase system to generate a superoxide flux and nitro blue tetrazolium as an indicator substance. The enzyme activity was expressed as units/mg protein.

Statistics. Results were analyzed statistically by the Student's *t*–test. Values of $P < 0.05$ were considered statistically significant. All data in the text, tables, and figures are expressed as mean ± standard error (SE) of at least 3 separate experiments.

RESULTS AND DISCUSSION

Studies on the role of H_2O_2 and oxygen radicals in inactivation of tumor cells by HPD–PDT. In the work, practically all *in vitro* experiments on the mechanism of HPD–PDT were carried out at 30 °C unless indicated otherwise. Fig. 1 shows that under photoexcitation of HPD, the kinetics of EAC cells inactivation was of S–type. In our studies, the light exposure time at which 50% of the cells were stained by TB (LD_{50}) was utilized as a criterion to evaluate the efficiency of HPD–PDT induced cytotoxicity *in vitro*. It is necessary to note that, photoirradiation of EAC cells in the absence of HPD, as well as their incubation with the PS in the dark at 30 °C did not cause any notable increase in the number of dead cells.

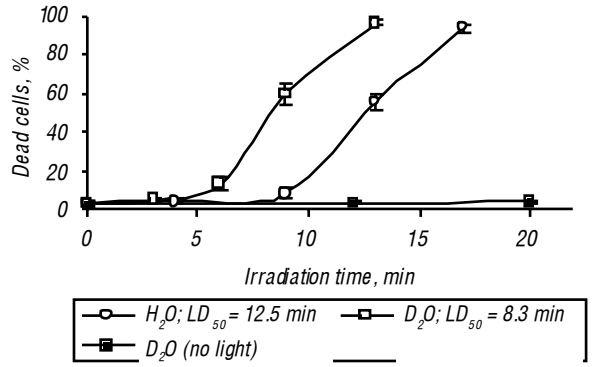

Fig. 1. The influence of D_2O on the rate of HPD–photosensitised inactivation of EAC cells (5 x 10^6 cells/ml). Before irradiation, the cells were loaded with HPD (at 20 μg/ml) in PBS made of H_2O. Further, the cells loaded with HPD were brought into suspension in PBS made of either H_2O or 99.8% D_2O and illuminated with red light at 630 nm under magnetic stirring at 30 °C. LD_{50}, the light exposure time at which 50% of the cells were stained by TB. Bars are SE as estimated from 3 independent experiments

There is much experimental evidence indicating that the cytotoxic action of HPD and light is mediated by the photosensitized production of $^1O_2$ *via* a Type II reaction [1]. On the other hand, some *in vitro* [3, 4] and *in vivo* studies [2] suggest the production of H_2O_2, $O_2^{-\bullet}$ and $OH^\bullet$ in cells and tissues exposed to visible light in the presence of HPD. However, in these published works it has not been proven unequivocally that these ROS are involved in the phototoxic action of HPD against tumor cells. For instance, Hariharan et al [4] using Chinese hamster V–79 cells have demonstrated the generation of $OH^\bullet$ when the cells were exposed to HPD and red light, but these researchers did not investigate the contribution of this oxidant in the cytotoxicity resulting from photoexcited HPD. Therefore, it seemed important to assess the possibility that in addition to $^1O_2$ other ROS (such as H_2O_2, $O_2^{-\bullet}$ and $OH^\bullet$) might be involved in the phototoxic action of the sensitizer against tumor cells. In this relation, we performed a comparative study of the influence of D_2O, CAT as well as selective traps of certain oxygen radicals on the intensity of HPD–sensitized photoinactivation of EAC cells *in vitro*.

One of the most reliable methods to prove the participation of $^1O_2$ in photochemical processes is using D_2O as a solvent. Replacing H_2O by D_2O must increase the efficiency of photochemical reactions proceeding *via* a Type II reaction, because the lifetime of $^1O_2$ in D_2O exceeds that in H_2O almost 15–fold. Studies showed that HPD–photosensitized inactivation of EAC cells is enhanced, in a synergistic manner, when the cells were suspended in D_2O–PBS (see Fig. 1). This indicates that $^1O_2$ is involved in the inactivation. However, the potentiating effect of D_2O was relatively small; in PBS made of D_2O the LD_{50} value of HPD–PDT was decreased by a factor of about 1.5, as compared with cells suspended in PBS made of H_2O (see Fig. 1). Similar effects of D_2O on the yield of photoinactivation of tumor cells were observed earlier by others [40], who used HP as a PS. HPD, as a lipophilic compound, is concentrated predominantly in plasma and subcellular membranes, making these structures especially sensitive to toxic photodamage. In this connection, we believe that the small effect of D_2O on the rate of HPD–photoinduced injury of EAC cells could be explained by dissolution of the photodrug in membrane lipids where the lifetime of $^1O_2$ (50–100 μs) is close to that observed in D_2O (~ 60 μs) [41]. Another explanation for the effect of D_2O would be that other ROS along with $^1O_2$ are involved in the cytotoxicity resulting from photoexcited HPD. Further studies confirmed this assumption. We revealed that $O_2^{-\bullet}$ might also participate in HPD–photoinduced killing of neoplastic cells. Indeed (Fig. 2), photoirradiation of EAC cells containing HPD led to a very rapid and significant increase in the intracellular level of $O_2^{-\bullet}$ that correlated with cell death. It was also found that upon photoirradiation of EAC cells loaded with HPD, the presence of 1.0 mM 4,5–dihydroxy–1,3–benzene–disulfonic acid (Tiron, a cell–permeable trap of $O_2^{-\bullet}$ [42]) suppressed (by 25%) the PDT–induced cytotoxicity that was associated with a substantial (~ 2–fold) inhibition in the rate of $O_2^{-\bullet}$ production (see Fig. 2, *a* and *b*, respectively). To provide more convincing evidence of the participation of $O_2^{-\bullet}$ in the phototoxic action of HPD–PDT, further experiments were performed on EAC cells pretreated with diethyldithiocarbamate (DDC), an inhibitor of Cu/Zn–SOD [43]. It is well known that cells can scavenge $O_2^{-\bullet}$ with the help of this constitutive antioxidant enzyme. Studies showed that pretreatment of HPD–loaded EAC cells with 0.1 mM DDC caused a considerable (~ 60%) decrease in the activity of Cu/Zn–SOD and, as a result, a notable (~ 30%) increase in the rate of their HPD–photosensitized inactivation (Table 1). It is important to emphasize that this pretreatment of EAC cells with DDC did not induce any appreciable decrease in the number of viable cells and had no effect on the activity of other antioxidant enzymes, such as CAT, GR and GPX. Our findings are consistent with the recent data of other researchers [44], who demonstrated that SOD(s) in tumor cells play an important role in their resistance to PDT with PF–II. Further, we found that HPD–photosensitized inactivation of EAC cells could be mediated by the production of H_2O_2 and very reactive $OH^\bullet$. As shown in Fig. 3, photoirradiation of the cells led to the formation of significant (μM) amounts of H_2O_2 and caused a substantial oxidation of DOR. It was also found that the levels of H_2O_2 and $OH^\bullet$, which were formed during photosensitization of EAC cells

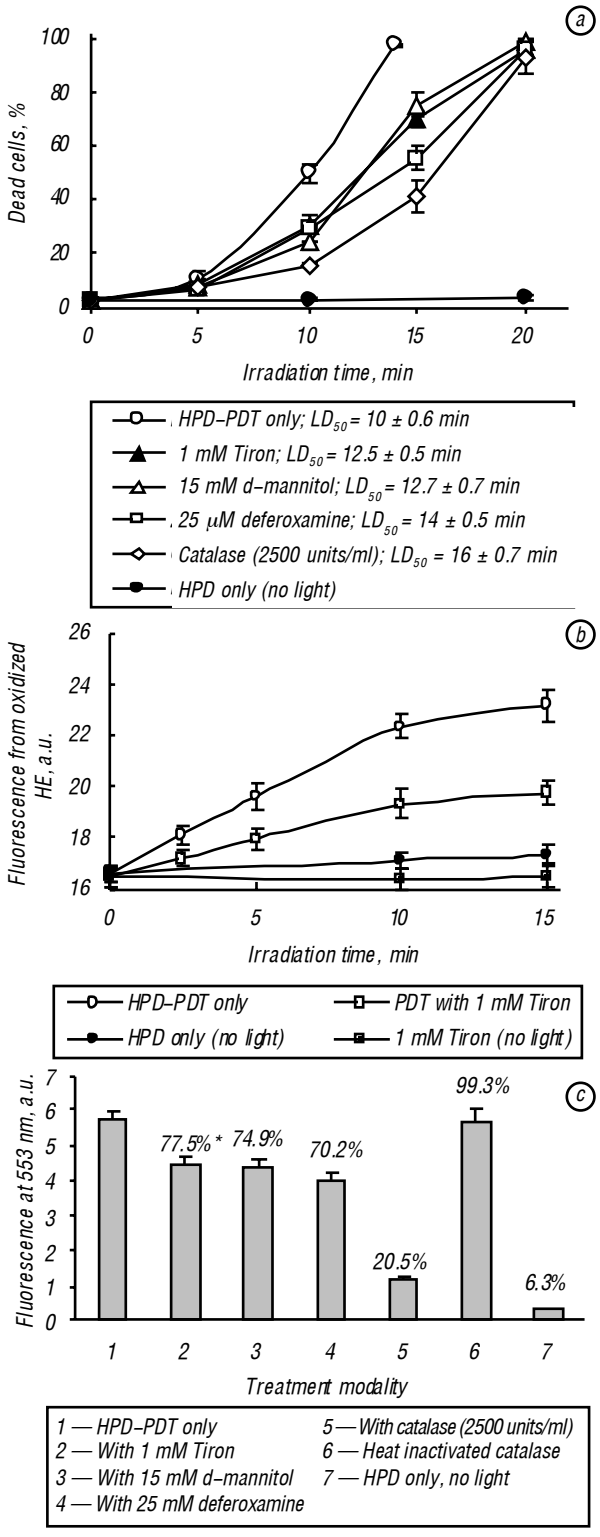

Fig. 2. Effects of traps of various ROS and exogenously added catalase on the intensity of HPD–photosensitized (λ_{max} = 630 nm; temperature, 30 °C) inactivation of EAC cells (5 x 10^6 cells/ml in PBS) (*a*), the production of $O_2^{-\bullet}$ (*b*) and the yields of $OH^\bullet$ formation (*c*), which were detected after 10 min PDT by measuring the photooxidation of 2–deoxyribose (initial concentration, 10 mM). The intracellular levels of $O_2^{-\bullet}$ were measured by the hydroethidine (HE) method. LD_{50}, the light exposure time at which 50% of the cells were stained by TB. * % from the value at HPD–PDT. Points, mean of at least 3 separate experiments. Bars, SE

by HPD, are sufficient to induce cell death. Namely (Fig. 2, *a*), the LD_{50} value of HPD–PDT was increased by 60% after addition of the H_2O_2–scavenging enzyme

Table 1. The influence of sodium diethyldithiocarbamate (DDC) on the phototoxicity of HPD towards EAC cells

EAC cells[a]	Activity of Cu/Zn–SOD, units/mg protein	LD_{50}, min
Control	13.6 ± 0.5	14.5 ± 0.6
Pretreated with DDC	5.9 ± 0.3[b] (43.4%[c])	10.8 ± 0.5[b] (74.5%[c])

Notes: [a] The cells were withdrawn from tumor–bearing animals, washed and loaded with HPD (at 20 μg/ml). Further, the HPD–loaded cells were suspended in PBS (at a density of 5 x 10^6 cells/ml) and incubated for 20 min in a shaking water bath at 30 °C without or in the presence of 0.1 mM DDC. After the incubation, the control and DDC–treated cells were washed once with PBS, resuspended in the same buffer at a density of 5 x 10^6 cells/ml and illuminated with red light at 630 nm under magnetic stirring at 30 °C; LD_{50}, the light exposure time at which 50% of the cells were stained by TB; [c] % of control; each datum represents the mean ± SE of 3 separate experiments; [b] significant difference, $P < 0.01$.

CAT (2500 units/ml) and by ~ 30% in the presence of 15 mM d–mannitol, a specific trap of $OH^\bullet$. In addition, experiments with DOR showed that protective effects of these scavengers on the phototoxicity of HPD towards EAC cells were closely related to the suppression of $OH^\bullet$ generation (Fig. 2, *c*). The strong (~ 80%) inhibition of DOR breakdown by exogenously added CAT suggested that H_2O_2 is a crucial intermediate in generation of $OH^\bullet$ and that in EAC cells subjected to HPD–PDT the radical might be produced through a series of reactions called the Fenton–Haber–Weiss reaction, which consists of iron reduction by the $O_2^{-\bullet}$ ($Fe(III) + O_2^{-\bullet} \rightarrow Fe(II) + O_2$) and subsequent $OH^\bullet$ generation ($H_2O_2 + Fe(II) \rightarrow OH^\bullet + OH^- + Fe(III)$) [45]. Further studies supported this idea. In fact (see Fig. 2), we found that the HPD–photoinduced generation of $OH^\bullet$ in EAC cells and, as a consequence, the efficiency of PDT were substantially decreased during illumination of the cells in the presence of DEF, a well known chelator of iron, which can inhibit the decomposition of H_2O_2 in the Fenton–type reactions preventing thereby the formation of very cytotoxic $OH^\bullet$ [46]. Control experiments showed that EAC cells are capable of producing marked amounts of H_2O_2, $O_2^{-\bullet}$ and $OH^\bullet$. However, the levels of these ROS, which were produced by the cells during their incubation in the dark or upon light exposure in the absence of HPD, were found to be considerably lesser than those after PDT (see Fig. 3). The ability of mammalian cells, including transformed cells, to the generation of H_2O_2 and oxygen radicals is well documented [47, 48]. At present, mitochondria are considered as one of the main sources of intracellular free radicals; it has been estimated that up to 2% of the total oxygen consumed by the mitochondrial electron transport chain undergoes one electron reduction to generate $O_2^{-\bullet}$ and subsequently other ROS, such as H_2O_2 and $OH^\bullet$ [45].

In the present work, we established that photosensitization of EAC cells by HPD leads to the production of $O_2^{-\bullet}$, however its reactivity, like H_2O_2, is quite limited. Several ways can be envisioned in which $O_2^{-\bullet}$ might transform to a more damaging species. Firstly, it is reported [49] that in cells the spontaneous dismutation of $O_2^{-\bullet}$ can produce $^1O_2$, a highly reactive oxidant that is involved in the phototoxic action of HPD towards tumor cells, including EAC cells (see Fig. 1). Besides, the reaction of $O_2^{-\bullet}$ with H_2O_2 can also generate $^1O_2$ [50]. Sec–

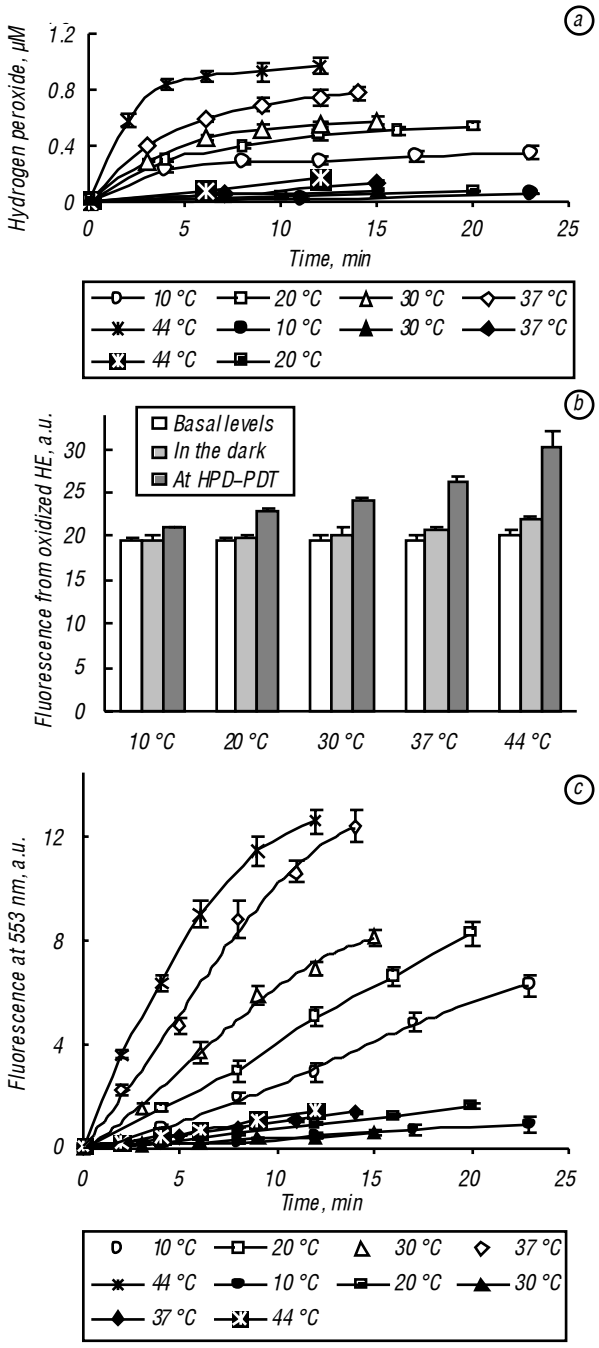

Fig. 3. Influence of temperature on the formation H_2O_2 (*a*), $O_2^{-\bullet}$ (*b*) and $OH^\bullet$ radicals (*c*) in EAC cells (5 x 10^6 cells/ml in PBS) during their photodynamic treatment (λ_{max} = 630 nm) with HPD (opened symbols) or incubation in the dark (filled symbols). The intracellular levels of $O_2^{-\bullet}$ were measured by the hydroethidine (HE) assay after 10 min light exposure, while the yields of $OH^\bullet$ formation were estimated by the 2–deoxyribose (DOR) method. For this purpose, the cells loaded with HPD were irradiated or incubated in the dark in the presence of 10 mM DOR and the oxidation products of the sugar by $OH^\bullet$ were assayed fluorometrically with 2–TBA. Points, mean of at least 3 separate experiments. Bars, SE

ondly, our studies indicated that under HPD–PDT a part of the formed $O_2^{-\bullet}$ could convert into $OH^\bullet$ that, as known, reacts at, or close to, a diffusion–controlled rate with almost all biological molecules. In fact, during photoirradiation of EAC cells loaded with HPD, the scavenging of $O_2^{-\bullet}$ by 1.0 mM Tiron led to a notable (~ 25%) decrease in the rate of DOR oxidation (Fig. 2, *c*). There are several processes by which the photochemically generated $O_2^{-\bullet}$ could be implicated in the formation of $OH^\bullet$ in tumor cells. It is known that $O_2^{-\bullet}$ can dismutate both enzymatically and nonenzymatically into H_2O_2, a precursor of $OH^\bullet$ in the Fenton reactions. Also, it was reported [51] that in cells $O_2^{-\bullet}$ can liberate iron from storage proteins; the released iron could then participate in the Fenton–reaction–mediated production of $OH^\bullet$.

On the role of glutathione redox cycle and CAT in the resistance of tumor cells to HPD–PDT. Our experiments indicated that the phototoxic action of HPD against tumor cells could be mediated by the generation of H_2O_2. Therefore, it is of great interest to examine the importance of cellular H_2O_2–scavenging systems in the resistance of tumor cells to HPD–based PDT. A study showed that photoirradiation of EAC cells loaded with HPD induces the depletion of GSH (Fig. 4, *d*). Namely, at light doses causing an irreversible inactivation of about 95% of the cells, a strong (~ 60%) decrease in the intracellular content of the antioxidant was observed. The depletion of GSH could be attributed to the known ability of the thiol to direct chemical quenching of $^1O_2$ and other ROS, and/or its oxidation by Se–dependent GPX that detoxifies H_2O_2 and lipid hydroperoxides using GSH as hydrogen donor. Taken together, this suggests that the glutathione redox cycle could play a role in the protection of tumor cells against HPD–PDT. In the work, the importance of glutathione redox cycle in the sensitivity of tumor cells to the phototoxic action of HPD was examined by disrupting the cycle at several points; by lowering the cellular stores of glutathione with BSO as well as *via* inhibiting the activity of GR with 1,3–bis(2–chloroethyl)–1–nitrosourea (BCNU). First, EAC cells were pretreated with BSO (*in vivo*) to lower the level of glutathione and their sensitivity to cytotoxicity resulting from photoexcited HPD was then estimated *in vitro*. As shown in Table 2, the treatment of EAC cells with BSO resulted in a substantial (~ 70%) decrease in the content of both GSH and total glutathione, making the cells more sensitive to the phototoxic effect of HPD; the BSO–induced depletion of glutathione caused an almost 30% increase in the rate of EAC cells photoinactivation, as found by measuring the LD_{50} value of PDT. It should be pointed out that such a pretreatment of EAC cells by BSO had no effect on the cellular uptake of HPD and the number of viable cells. To provide more convincing evidence of the protective role of glutathione redox cycle, further ex–

Table 2. The effect of D, L–buthionine–(S,R)–sulfoximine (BSO) administrated *in vivo* on the content of glutathione in EAC cells and their sensitivity to HPD–induced PDT *in vitro*

EAC cells[a]	Reduced glutathione, μg per 1 x 10^7 cells	Total glutathione[b], μg per 1 x 10^7 cells	LD_{50}, min
From control mice, n = 4	3.74 ± 0.16	5.06 ± 0.21	12.1 ± 0.4
From BSO–treated, n = 4	1.19 ± 0.06[d] (31.8%[c])	1.84 ± 0.11[d] (36.4%[c])	9.5 ± 0.3[d] (78.5%[c])

Notes: [a] The cells from control and BSO–treated animals (details in "Material and methods") were removed by aspiration, washed twice with PBS, and loaded with HPD (at 20 μg/ml). Further, the cells were diluted with PBS (up to a density of 5 x 10^6 cells/ml) and illuminated with red light at 630 nm under magnetic stirring at 30 °C; LD_{50}, the light exposure time at which 50% of the cells were stained by TB; [b] reduced + oxidized glutathione; [c] % of control; n, number of animals; each datum represents the mean ± SE; [d] significant difference, $P < 0.02$.

periments were conducted on EAC cells pretreated with BCNU (Bristol–Myers Squibb SpA, Italy), an inhibitor of GR [52]; in the cycle, GR is responsible for regeneration of GSH. The data in Table 3 indicate that pretreatment of the cells with 0.1 mM BCNU (which inhibited GR activity by 65% without effect on the activities of GPX, CAT, or on the number of viable cells) markedly enhanced the phototoxic action of HPD; the LD_{50} exposure of PDT was decreased by 22% after treatment of EAC cells with the inhibitor. Thus, our experiments on EAC cells clearly demonstrated that the glutathione cycle plays an important role in the protection of tumor cells against HPD–PDT induced cytotoxicity. This finding is in agreement with the data of other investigators [53]. Namely, using murine L1210 leukaemia cells and human CaSki cervical carcinoma cells, they showed that GSH and GPX play a role in the protection of the cells against the phototoxic effect of HPD that was attributed to the detoxification of the formed lipid peroxides. However, our studies suggest that protective effects of these antioxidants could be mediated by reductive decomposition of H_2O_2 that, as indicated experiments with exogenously added CAT, is involved in the phototoxic action of the PS towards tumor cells (Fig. 2, *a*).

Table. 3. Effect of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) on the activity of glutathione reductase in EAC cells and their sensitivity to HPD-induced PDT *in vitro*

EAC cells[a]	Glutathione reductase activity, nmoles NADPH/min/mg protein	LD_{50}, min
Control	55.1 ± 1.5	11.0 ± 0.33
BCNU-treated	19.75 ± 0.65[b] (35.8%[c])	8.6 ± 0.32[b] (78.2%[c])

Notes: [a] The cells were withdrawn from tumor bearing animals, washed and loaded with HPD (at 20 μg/ml). Further, the HPD-loaded cells were suspended in PBS (at a density of 5 x 10^6 cells/ml) and incubated for 25 min in a water bath at 30 °C without or in the presence of 0.1 mM BCNU (was dissolved in dimethyl sulfoxide, DMSO). After the incubation, the BCNU-treated and control cells (instead BCNU the cells were treated with DMSO) were washed once with PBS, resuspended in the same buffer at a density of 5 x 10^6 cells/ml and illuminated with red light at 630 nm under magnetic stirring at 30 °C; LD_{50}, the light exposure time at which 50% of the cells were stained by TB; each datum represents the mean ± SE of 3 separate experiments performed in duplicate; [b] significant difference, $P < 0.01$; [c] % of control.

Under PDT, tumor cells could detoxify the formed H_2O_2 not only *via* the glutathione redox cycle, but also by endogenous CAT. However, in the literature we did not find any information about the importance of CAT in the resistance of tumor cells against the phototoxic influence of HPD. To elucidate the role of the H_2O_2–scavenging enzyme, experiments were performed on EAC cells pretreated with 3-amino-1,2,4-triazole, an irreversible inhibitor of CAT [54]. As shown in Table 4, pretreatment with aminotriazole (25 mM for 1 h) essentially (by 40%) inhibited activity of cellular CAT (without effect on the activities of GPX, GR, or on the intracellular content of GSH) and markedly (by 28%) increased the rate of HPD–photosensitized inactivation of the cells. It is important to note that incubation of EAC cells with 25 mM aminotriazole had a minor effect on the number of injured cells increasing it at the most by 5%. Thus, these experiments indicated that cell-bound CAT can protect tumor cells against HPD–PDT induced cytotoxicity *in vitro*.

Thus, our studies on the mechanism of action of PDT showed that along with $^1O_2$ other ROS (such as

Table. 4. Effect of aminotriazole (ATZ) on the activity of catalase in EAC cells and their sensitivity to HPD–photoinduced cytotoxicity *in vitro*

EAC cells[a]	Catalase activity, μmoles H_2O_2/min/mg protein	LD_{50}, min
Control	9.05 ± 0.41	19.3 ± 0.8
Pretreated with ATZ	5.60 ± 0.17[c] (61.9%[b])	15.1 ± 0.6[c] (78.2%[b])

Notes: [a] The cells were withdrawn from tumor bearing animals, washed and loaded with HPD (at 20 μg/ml). The HPD-loaded cells were then suspended in Hanks' balance salt solution (without phenol red, pH 7.3) at a density of 5 x 10^6 cells/ml and incubated for 1 h in a water bath at 37 °C without or in the presence of 25 mM ATZ. After the incubation, the control and ATZ-treated cells were washed once with PBS, resuspended in the same buffer at a density of 5 x 10^6 cells/ml and illuminated with red light at 630 nm under magnetic stirring at 30 °C; LD_{50}, the light exposure time at which 50% of the cells were stained by TB; [b] % of control; each datum represents the mean ± SE of 3 separate experiments; [c] significant difference, $P < 0.01$.

H_2O_2, $O_2^{-\bullet}$ and $OH^{\bullet}$) could be involved in the HPD–catalyzed photodestruction of tumor cells. Furthermore, they provided strong evidence that cell Cu/Zn–SOD, CAT and the glutathione redox cycle can protect the cells against the phototoxic action of the photodrug.

The influence of temperature on the response of tumor cells to HPD–PDT. In these studies, EAC cells loaded with HPD were irradiated with red light at 630 nm or incubated in the dark at five different temperatures of 10, 20, 30, 37, and 44 °C. The 30 °C group of cells was taken as the control, since there is increasing enthusiasm (due to excellent cosmetic results) for the use of porphyrin PS(s), including HPD, in PDT of skin cancers having the surface temperature close to 30 °C. In the work, we also assessed the sensitivity of tumor cells to the phototoxic influence of HPD at mildly hypothermic (10–20 °C) temperatures, because it was reported [55] that cooling of malignancies during interstitial laser photoirradiation can enhance the tumoricidal effect of PDT with HPD. Experiments showed that lowering the irradiation temperature from 30 to 10 °C markedly (by 44%) decreased, whereas growing the temperature from 30 to 44 °C, on the contrary, substantially (by about 1.5 fold) increased the rate of HPD–photosensitized inactivation of EAC cells, as found by measuring the LD_{50} value of PDT (Fig. 4, *a*). However, incubation of the cells in the dark, irrespective of temperature conditions, did not induce any noticeable increase in the number of injured cells. As cellular proteins are the target for HPD–PDT as well as for heat inactivation [12], we examined the influence of temperature on HPD–photosensitized damage of proteins in EAC cells; in one of our prior studies we already demonstrated that under HPD–PDT the cells die mainly *via* photooxidative injuries of their proteins [5]. As shown in Fig. 4, the photodynamic treatment of EAC 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 15 min illumination at 30 °C that caused an irreversible inactivation of ~ 90% of the cells, was determined as 15% for Trp, and 20% for PrSH. It was found that sub- (37 °C) and hyperthermic (44 °C) heating accelerated the HPD–catalyzed photoinjury of proteins in EAC cells. Namely, upon increasing the temperature from 30 to 44 °C a substantial (~ 55%) growth in the rates of Trp residues and PrSH photooxidation was registered that well cor-

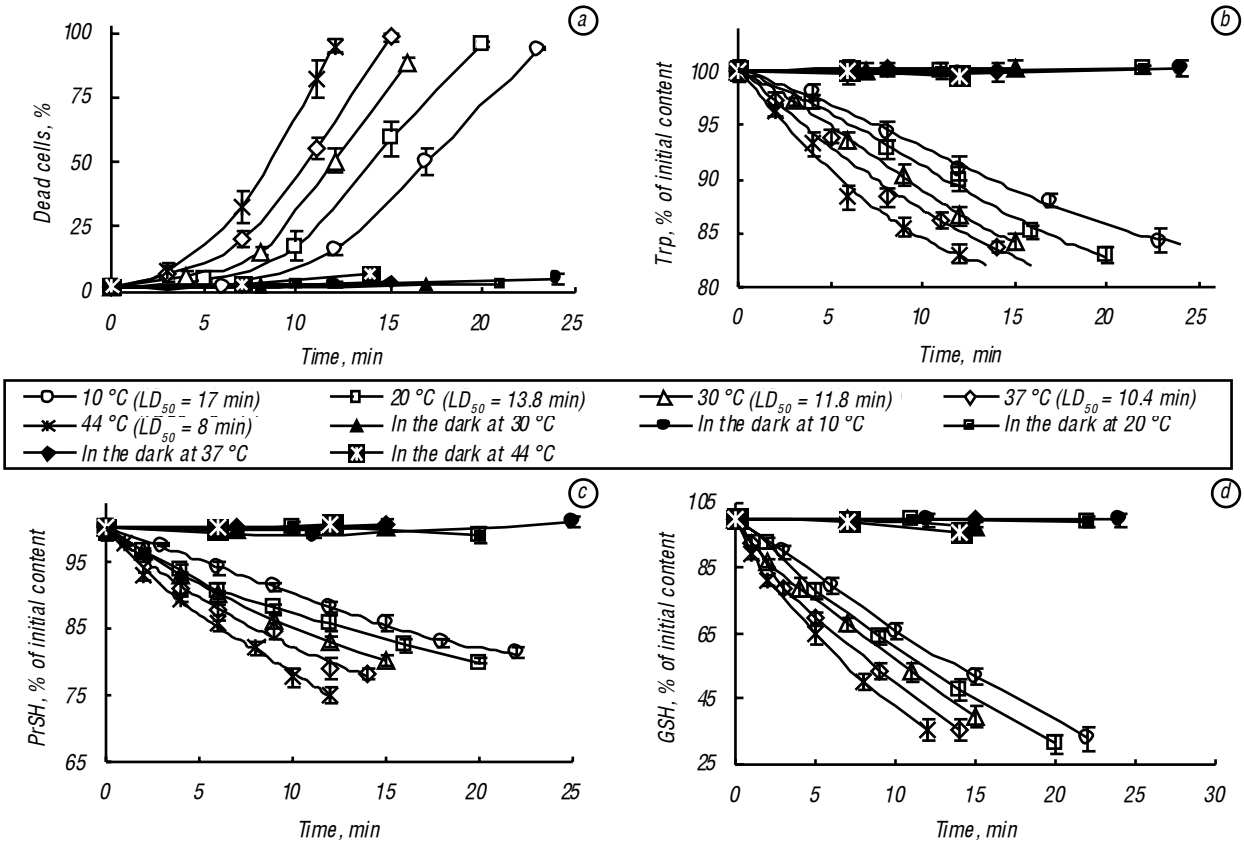

Fig. 4. Kinetics of inactivation of HPD–loaded EAC cells (5 x 10^6 cells/ml in PBS) (*a*), the intracellular content of tryptophan (Trp) (*b*), protein–bound SH groups (PrSH) (*c*) and reduced glutathione (GSH) (*d*) after irradiation with red light at 630 nm (opened symbols) or during incubation in the dark at different temperatures (filled symbols). The initial levels of Trp, PrSH and GSH in the non–irradiated cells were determined as (per 1 x 10^6 cells): 20.4 ± 0.2 nmoles for Trp, 17.05 ± 0.68 nmoles for PrSH, and 1.15 ± 0.05 nmoles for GSH. LD_{50}, the light exposure time at which 50% of the cells were stained by TB. All data are from 3 independent experiments. Bars, SE

related with an increase in the rate of HPD–PDT induced inactivation of the cells. On the contrary, decreasing the irradiation temperature from 30 to 10 °C made cell proteins less sensitive to the photooxidative injuries, resulting in protection of the cells against HPD–PDT induced cytotoxicity. However, both heat shock and cold stress itself did not induce similar changes in the amino acid composition of cell proteins.

Further, we evaluated the influence of heat shock on the oxidative potency of PDT with HPD, using GSH as a biomarker of oxidative stress. Studies showed that heating enhances the HPD–PDT induced oxidative stress in tumor cells. Indeed, we found that EAC cells subjected to HPD–PDT at 37 and 44 °C had lesser levels of GSH, as compared with the control cells treated by the PDT at 30 °C (Fig. 4, *d*). It is important to note that incubation of the cells in the dark even at a hyperthermic (44 °C) temperature caused only a minor (~ 5%) decrease in the intracellular content of GSH. On the contrary, a shift in the temperature from 30 to 10 °C led to a substantial (~ 40%) decrease in the rate of HPD–photoinduced oxidation of GSH in the cells. This indicates that cooling suppressed the PDT induced oxidative stress in EAC cells. Thus, our *in vitro* experiments clearly indicated that heating intensifies the HPD–PDT induced oxidative stress in tumor cells, promotes the photooxidative damage of proteins in the cells and increases in a synergistic manner their susceptibility to the cytotoxicity resulting from photoexcited HPD, while hypothermia (cooling up to 10 °C) inhibits the phototoxic action of the PS. A remarkable observation of the study is also that the potentiating effect of heating on HPD–photosensitized killing of tumor cells may take place at 37 °C, *i.e.* at temperatures below hyperthermic. This observation is in agreement with the results of other researchers [56].

On the generation of ROS in tumor cells during HPD–PDT at elevated temperatures. In these studies, we concentrated our efforts on elucidation the mechanism of pro–oxidative effects of heat stress in PDT with HPD, because that permits to explain an increased sensitivity of tumor cells to the phototoxic influence of the drug at elevated temperatures. First, we tested the possibility that heating could induce the disaggregation of HPD components in tumor cells, since the event must, in turn, lead to an enhanced production of $^1O_2$ [23, 24]. The relative proportion of monomeric and aggregated moieties of HPD in solution can be evaluated by measuring its fluorescence intensity that, as known, decreases in the aggregated state. Fig. 5 depicts the changes in the fluorescence spectrum of HPD in non–illuminated EAC cells depending on the temperature. An analysis of the spectral changes showed that the PS becomes more aggregated when the cells were heated in a water bath, since the heating of EAC cells from 10 to 44 °C resulted in a 25% decrease in the fluorescence intensity of HPD

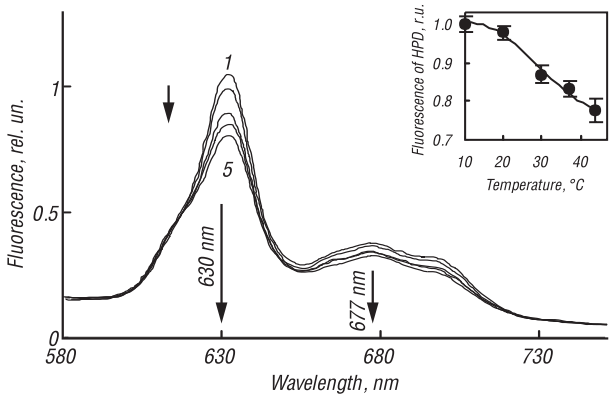

Fig. 5. Fluorescence emission spectra of HPD (λ_{ex} = 505 nm) in EAC cells (5 x 10^6 cells/ml in PBS) at different temperatures (sample light path, 1 cm). Spectra from 1 to 5 represent temperatures of 10, 20, 30, 37 and 44 °C, respectively. Inset: fluorescence intensities of HPD in the cells at 630 nm as a function of temperature. Before the spectroscopic measurements, EAC cells loaded with HPD were placed in a water bath and incubated for 5 min in the dark at a desired temperature. Bars, SE

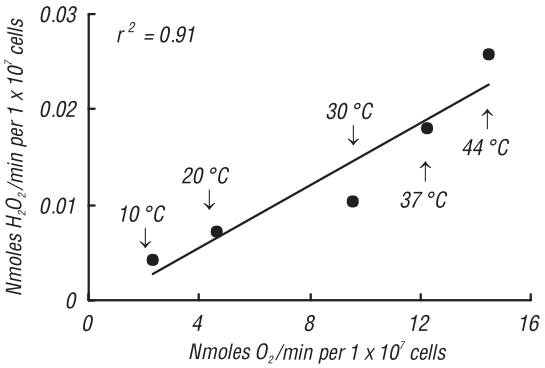

Fig. 6. The interrelationship between the rate of H_2O_2 production by non–illuminated EAC cells and their respiratory activity at various temperatures

at 630 nm (without any shifts in the position of its main fluorescence peaks at 630 and 677 nm). Studies indicated that the event (a rise in the content of aggregated moieties of HPD) could be attributed to a heat–induced shortening in the cell volume; namely, upon raising the temperature from 10 to 44 °C a substantial (1.5–2–fold) decrease in the volume of EAC cells was registered microscopically. Thus, we cannot explain the potentiating effect of heating on the antitumor efficiency of HPD–PDT *via* an enhancement in the formation of $^1O_2$ due to disruption of self–aggregated moieties of the PS.

Heat stress during PDT could promote the HPD–induced photooxidative damage of cellular constituents and, as a consequence, tumor cell eradication *via* an increase in the reactivity of $^1O_2$ [19] as well as through the stimulation of other ROS formation. In fact, we found that upon HPD–PDT a rise in the temperature from 30 to 44 °C strongly enhanced the production of H_2O_2, $O_2^{-\bullet}$ and $OH^\bullet$ in EAC cells that directly correlated with an increase in the rate of HPD–photoinduced inactivation of the cells (Fig. 3 and 4, respectively). On the contrary, lowering the irradiation temperature from 30 to 10 °C led to an inhibition of these ROS formation in EAC cells and, as a result, to a decrease in the efficiency of PDT with HPD. Besides, our investigations showed that heat stress itself promotes the generation of ROS in tumor cells. In fact, raising the temperature from 30 to 44 °C caused a marked increase in the rates of H_2O_2, $O_2^{-\bullet}$ and $OH^\bullet$ production by non–irradiated EAC cells (Fig. 3). The finding is in a good agreement with the observations of other researchers, e.g. [47]. Until now, the precise location and mechanisms of increased formation oxygen radicals during heat stress remain unclear. Nevertheless, there are some indications that the mitochondrial electron transport chain could be responsible for an increased production of $O_2^{-\bullet}$ and H_2O_2 (precursors of $OH^\bullet$) in cells subjected to a thermal stress [57]. Our data are consistent with these findings; it was found that within the temperature range of 10–44 °C, the ability of EAC to the production of H_2O_2 is directly related to the respiratory activity of the cells (Fig. 6). It was suggested that the cellular damage associated with hyperthermia could be mediated in part by an increased flux of oxygen radicals [13]. On this basis, we believe that heat shock during HPD–PDT can enhance the generation of ROS by tumor cells, which along with photochemically generated oxidants could take part in the therapy–induced eradication of diseased tissues. Thus, our studies on the mechanism of the potentiating effect of light–induced heating on the antitumor efficiency of PDT with HPD suggest that the phenomenon could be explained *via* heat–induced increases in the formation of cytotoxic ROS such as H_2O_2, $O_2^{-\bullet}$ and $OH^\bullet$. Moreover, the heating during PDT could promote the HPD–mediated photodestruction of tumor cells not only by increasing generation, but also reactivity of oxygen radicals [58]. It was also reported that hyperthermia (42 °C) can enhance the cytotoxicity of H_2O_2 [14].

Effects of hyperthermia alone or jointly with HPD–PDT on H_2O_2–detoxifying systems of tumor cells. Heat shock during HPD–PDT could promote an increase in oxidative stress and thereby enhance the tumor response to the therapy by inactivating cellular antioxidant defenses. In this connection, we investigated the intactness of CAT and the glutathione redox cycle in EAC cells during their incubation in the dark as well as upon HPD–PDT at elevated temperatures; in this work, we already demonstrated that the H_2O_2–detoxifying systems play an important role in the protection of the cells against the phototoxic action of HPD (Tables 2, 3, and 4). This research trend presents substantial interest, since in the literature there is only limited information as to the influence of hyperthermia and HPD–photosensitized reactions on the activity of CAT or enzymes of the glutathione cycle in transformed cells. We found that hyperthermia (44 °C) itself had a minor effect on the levels of GSH in EAC cells and did not induce any noticeable decrease in the activity of cellular GPX. However, further studies revealed that the H_2O_2–destroing enzyme is inactivated during photosensitization of EAC cells with HPD and that heating promotes the photoinduced degradation of GPX in the cells. In fact, the photoirradiation of HPD–loaded EAC cells at control (30 °C) temperature caused a 30% decrease in the GPX activity, and upon elevating the temperature to

44 °C, a strong (~ 2–fold) increase in the rate of the enzyme inactivation was observed (Fig. 7, *a*). In contrast, lowering the temperature from 30 to 10 °C protected GPX against HPD–photoinduced inactivation. Similar results were obtained during a study of the effects of hyperthermia alone or jointly with HPD–PDT on the activity of CAT in EAC cells. It was found that like GPX, the H_2O_2–detoxifying is inactivated during photoirradiation of HPD–loaded EAC cells and that heating sensitizes CAT to HPD–photoinduced inactivation. Namely (Fig. 7, *b*), raising the temperature from 30 to 44 °C resulted in a considerable (~ 2–fold) increase in the rate of HPD–PDT mediated inactivation of CAT in the cells. Moreover, experiments showed that in EAC cells CAT, in comparison with GPX, is much more sensitive to the inactivating influence of HPD–PDT or heat treatment. Indeed (Fig. 7), at light doses which caused an irreversible inactivation of about 90% of the cells only a small (~ 30%) fall in the activity of GPX took place, while the activity of cellular CAT was inhibited by almost 95%. It was also established that hyperthermia, a 12 min incubation of EAC cells in the dark at 44 °C, caused a marked (~ 10%) decrease in the activity of cellular CAT, whereas after the same incubation the activity of GPX in the cells remained practically unchanged. Thus, our studies suggest that under PDT the HPD–mediated photoinactivation of cell–bound GPX and CAT could result in loss of protection against accumulating H_2O_2, providing an additional pathway of phototoxicity. In addition, they suggest that during PDT with HPD the heating associated with the absorption of optical radiation may promote the phototherapy–induced oxidative stress and, as a consequence, the tumor eradication *via* increasing in the rates of HPD–photosensitized inactivation of cellular CAT and GPX.

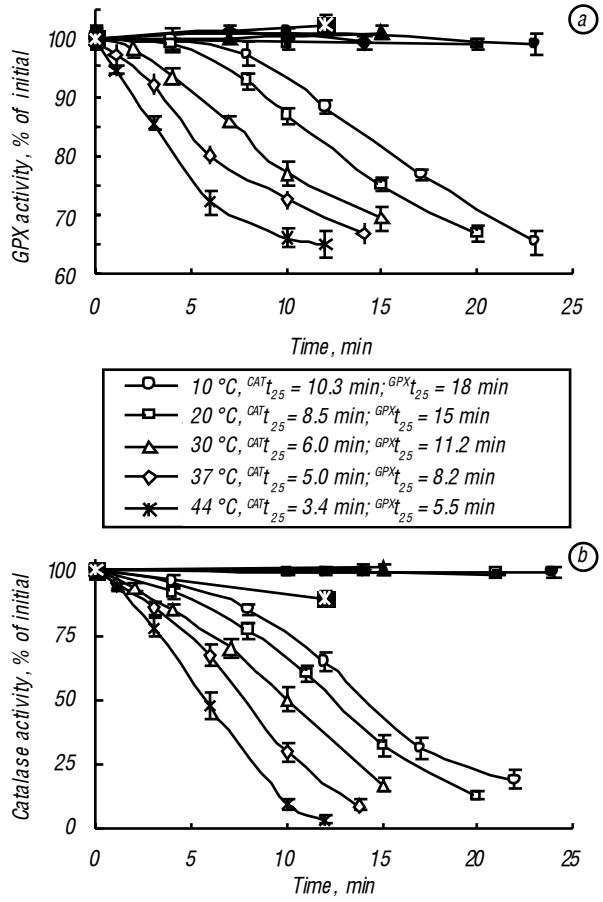

Fig. 7. The activity of glutathione peroxidase (GPX) (*a*) and catalase (*b*) in HPD–loaded EAC cells (5 x 10^6 cells/ml in PBS) after their irradiation with red light at 630 nm (opened symbols) or incubation in the dark at various temperatures (filled symbols). The initial activities of catalase and GPX in the cells were determined as 9.46 ± 0.41 μmoles H_2O_2 decomposed/min/mg protein and 49.3 ± 2.1 nmoles NADPH oxidized/min/mg protein, respectively. $^{CAT}t_{25}$ and $^{GPX}t_{25}$ are the light doses at which 25% inhibition in the activity of catalase and GPX was observed; in this work, the parameters were used to estimate the efficiency of HPD–mediated photoinactivation of the H_2O_2–detoxifiyng enzymes. The points with error bars refer to 3 independent experiments

The effect of temperature on photochemical transformations of HPD in tumor cells. In this series of our studies, we examined the effect of heating on the photochemical transformations of HPD (its degradation as well as PhP–640 formation) in tumor cells, since the aspects of PDT remain undetermined. At the same time, this information could clarify some discordance in the literature data concerning an influence of temperature on the antitumor efficiency of PDT with the PS. For the most part, studies on the mechanism of HPD–PDT suggest that heating associated with photoirradiation enhances the tumoricidal action of the phototherapy, e.g. [16]. However, it was reported [55] that cooling of tumors during interstitial HPD–PDT also improves the tumor response to the therapy indicating that a PDT associated heating of tumors may reduce the treatment efficiency. One possible explanation for the phenomenon might be that lowering the temperature of tumor tissues during light exposure inhibits the photodecay of HPD in these tissues and, as a consequence, results in a increased yield of inactivation of tumor cells per incident photon. In this connection, we examined the influence of temperature on photostability of HPD in tumor cells. Experiments on EAC cells loaded with HPD showed that the PS is progressively destroyed during illumination and that the rate of HPD photobleaching in the cells depends largely on the temperature of a medium. Namely (Fig. 8 *a*), we revealed that elevating the temperature from 30 to 44 °C resulted in a considerable (~ 3–fold) increase in the rate of HPD photodecay in EAC cells, whereas cooling of the cells from 30 to 10 °C caused, on the contrary, a substantial (> 4–fold) inhibition in the rate of the sensitizer photodegradation (as found by measuring a slope on the kinetic curves of HPD photobleaching). It is important to note that independently of temperature conditions, a prolonged (20–25 min) incubation of EAC cells in the dark did not induce any marked lowering in the initial content of HPD in the cells. At the same time, experiments showed that HPD is a relatively photostable drug. Indeed (Fig. 8, *a*), at a normal (30 °C) temperature and at the light dose causing an irreversible inactivation of about 95% of the cells, only a small (~ 11%) decrease in the intracellular level of HPD took place. Furthermore, at the light doses causing a 95% decrease in the number of viable cells, the yield of HPD photobleaching in EAC cells was changed only slightly (at the most by 2%) upon heating (to 44 °C) or

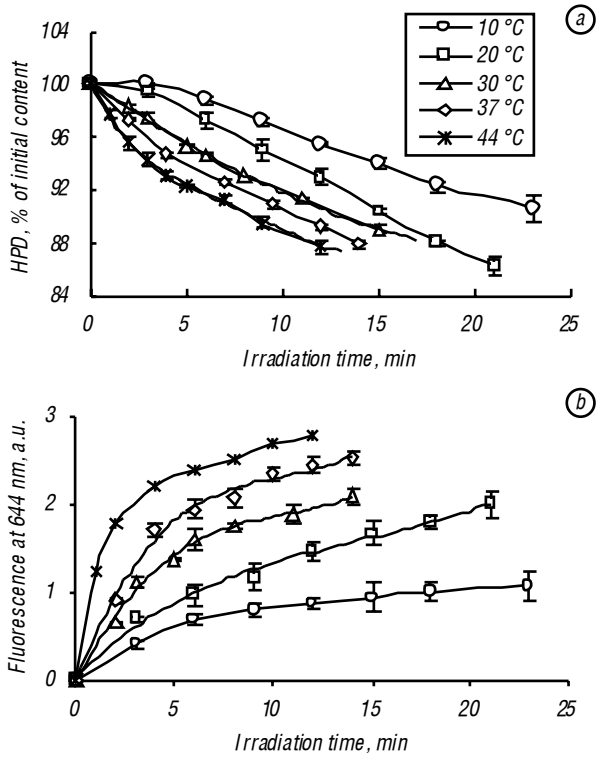

Fig. 8. Kinetics of HPD photodegradation (*a*) and photoproduct–640 formation (*b*) in EAC cells (5 x 10^6 cells/ml in PBS) during their irradiation (λ_{max} = 630 nm) at different temperatures. The initial content of HPD in the cells was determined as 77.7 ± 5.3 ng per 1 x 10^6 cells. Points, mean of at least 3 separate experiments. Bars, SE

cooling of the cells to 10 °C. Thus, although the rate of HPD photobleaching in tumor cells is found to be largely dependent on the temperature, our data suggest the very low probability (due to high photostability of HPD) that at a sufficient concentration of the photodrug in tumor tissues the efficiency of PDT could be appreciably affected by a stimulatory influence of sub– or hyperthermic temperatures on the photobleaching of HPD in malignancies. Hence, most likely, another causes underlie the potentiating effect of cooling on the antitumor efficiency of interstitial PDT with HPD. In the further experiments, we tested the hypothesis that the potentiating influence of heating on HPD–PDT induced tumor eradication could be mediated by a heat–catalyzed increase in the formation of PhP–640, since it was suggested that accumulation of the chlorin–type photoproduct can cause an increase in the absorption in the red spectral region at around 620–650 nm during illumination of tumors *in vivo* and that it possesses photosensitizing activity [27]. Using fluorescence spectroscopy, we found that photoirradiation of EAC cells loaded with HPD resulted in a very rapid and substantial growth in the intracellular level of the red–absorbing photoproduct and that its formation in the cells exhibited a strong tendency to increase with increasing temperature (Fig. 8, *b*). Namely, upon elevating the temperature from 30 to 44 °C a considerable (> 3 times) increase in the rate of PhP–640 formation was observed (the energy activation of the photoproduct formation was calculated as 42.3 ± 2.9 kJ/mole). It must be noted that a prolonged (25 min) incubation of HPD–loaded EAC in the dark even at a hyperthermic (44 °C) temperature did not generate any detectable levels of the chlorin–type compound. Thus, these findings speak in favor of our hypothesis that the potentiating effect of photoirradiation–induced heating on the efficiency of HPD–PDT could be mediated, in part, by an increased generation of PhP–640.

In this work, an attempt was also made to estimate the significance of PhP–640 in the phototoxic action of HPD against tumor cells at elevated temperatures. For the purpose, a heat–induced increase in the phototoxic action of HPD to EAC cells after their irradiation with red light at 630 nm (which can excite both HPD and PhP–640) was compared with that at 510 nm (which cannot excite PhP–640, since the photoproduct did not absorb appreciably in this spectral region [26]). It was found that the potentiating influence of a thermal stress on HPD–photosensitized inactivation of EAC cells was less effective when the cells were irradiated with green light at 510 nm, as compared with that at 630 nm. In fact (Table 5), under the PDT with red light at 630 nm a shift in the irradiation temperature from 30 to 44 °C caused a 55% increase in the rate of HPD–photoinduced eradication of EAC cells, but substantially lesser (by 26%) growth in the rate was observed when the cells were exposed to green light at 510 nm. It was reported [59] that visible light itself, *i.e.* in the absence of exogenously added photosensitizing agents, can cause serious changes in cellular homeostasis parameters (a shift in the redox state of mitochondria, cellular pH, the level of adenosine triphosphate, Ca^{2+}, and etc.) and that these light–induced alterations in cellular homeostasis are largely depended on the wavelength of radiation. Hence, it could be assumed that under HPD–PDT the irradiation of EAC cells with red light at 630 nm caused, as opposed to green light at 510 nm, such a change in cell homeostasis that made the cells more vulnerable to a thermal stress. To discard this possibility and to obtain more convincing evidence of the possible contribution of PhP–640 to a heat–mediated increase in the tumoricidal effect of HPD–PDT *in vivo*, we examined the influence of heating on the formation of PhP–640 as well as the temperature dependence of photosensitizing activity of HPD on the wavelength of radiation using a simplest photochemical system — Trp and HPD dissolved in PBS (it is important to note that in these model experiments the concentration of HPD was brought into correspondence with that found in EAC cells, 10 μg/ml). It was found that when the system was exposed to red light at 630 nm, raising the temperature

Table 5. Influence of temperature on the efficiency of HPD–sensitized photooxidation of tryptophan (Trp) in aqueous solution as well as photoinactivation of EAC *in vitro* depending on the wavelength of incident light

Irradiation conditions	Rates of Trp oxidation[a], μM/min ± SE (n = 3)	LD_{50}, min ± SE (n = 3)
30 °C, λ_{max} = 510 nm	6.74 ± 0.22	7.7 ± 0.4
44 °C, λ_{max} = 510 nm	9.95 ± 0.31 (147.6%[b])	6.1 ± 0.3[c] (79.2%[b])
30 °C, λ_{max} = 630 nm	1.81 ± 0.05	13.8 ± 0.5
44 °C, λ_{max} = 630 nm	3.02 ± 0.09 (166.9%[b])	8.9 ± 0.3[c] (64.5%[b])

Notes: [a] The reaction mixtures consisted of 10 μg/ml HPD and 0.2 mM Trp in PBS; [b] % from the value obtained at 30 °C; [c] significant as compared with the LD_{50} value of PDT obtained at 30 °C, n — number of experiments; LD_{50}, the light exposure time at which 50% of the cells were stained by TB; $P < 0.01$.

from 30 to 44 °C led to a considerable (67%) increase in the rate of HPD–sensitized photooxidation of Trp that well correlated with a heat–induced increase in the rate of PhP–640 formation (Table 5 and Fig. 9, respectively). However (Table 5), the same heating of a reaction mixture produced markedly less (by 47.6%) increase in the rate of HPD–photoinduced oxidation of Trp if the mixture was irradiated with green light at 510 nm that, as mentioned above, cannot excite the PhP–640. Thus, our findings strongly suggest that during clinical PDT, employing a 630 nm laser light to activate HPD, an increase in the temperature of tumor tissues, associated with the absorption of optical radiation, could enhance the tumoricidal effect of this therapy *via* the stimulation of PhP–640 formation.

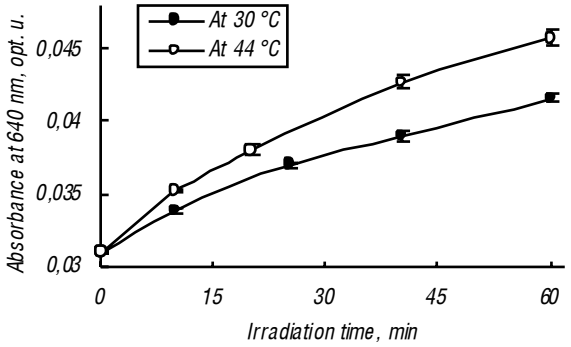

Fig. 9. The effect of heating on the formation of photoproduct–640 during irradiation of HPD aqueous solutions with red light at 630 nm. The samples contained 10 μg/ml HPD and 0.2 mM tryptophan in PBS. These measurements were performed in 2 x 2 cm quartz cuvettes. Bars, SE

In conclusion, although PDT utilizing HPD or PF–II as photosensitizing agents has already proved its usefulness in the treatment of cancer, the fundamental mechanisms of its antitumor effects are still incompletely understood. First, it concerns the nature of the ROS, which could be responsible for the HPD–PDT induced tumor cell eradication. Previous studies concerning the aspect in the mechanism of action of HPD–PDT have been inconclusive; some indicate $^1O_2$ as the main damaging agent, whereas others suggest the participation of oxygen radicals. In this work, we clearly demonstrated that H_2O_2, $O_2^{-\bullet}$ and $OH^\bullet$ could mediate, along with $^1O_2$, the tumoricidal action of PDT with HPD. However, further work is needed to clarify the mechanism of H_2O_2 and oxygen radicals formation in tumor cells treated with HPD–PDT. At the same time, our studies suggest that in tumor cells subjected to HPD–PDT the Fenton–like reactions could play an important role in the generation of $OH^\bullet$. Furthermore, our experiments provided strong evidence that cell–bound Cu/Zn–SOD, CAT and the glutathione redox cycle can protect tumor cells against the phototoxic influence of HPD. These findings suggest that the efficiency of PDT with HPD in a clinical setting could be improved by its combination with chemotherapeutic agents, which can inhibit SOD(s) as well as H_2O_2–detoxifying systems of transformed cells.

Earlier investigations on the mechanism of HPD–PDT showed that during PDT may happen a significant increase in the temperature of tumor tissues and that the heating potentiates the porphyrin–mediated photodestruction of neoplasms. However, until now the mechanism of the phenomenon remained unclear. Our studies support the view that in laser PDT the mild hyperthermia (42–44 °C) produced by irradiation can enhance synergistically the HPD–photoinduced tumor eradication. A remarkable observation of the present study is also that the potentiating effect of heating on HPD–photosensitized killing of tumor cells may take place at temperatures below hyperthermic. Our data indicate that the potentiating effect of heating, associated with photoirradiation, on the efficiency of PDT with HPD could be largely explained by the stimulation of cytotoxic ROS formation such as H_2O_2, $O_2^{-\bullet}$ and $OH^\bullet$. In addition, our experiments showed that photosensitization of tumor cells by HPD causes an inactivation of cell–bound CAT and GPX, and that heat stress sensitized the H_2O_2–detoxifying enzymes to HPD–photoinduced inactivation; upon HPD–PDT, these events could result in loss of protection against accumulating H_2O_2. Our findings suggest that during clinical PDT, employing a 630 nm laser light to activate HPD, an increase in the temperature of tumors could enhance the tumoricidal effect of this therapy *via* the stimulation of a chlorin–type photoproduct formation that may act as a PS.

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ВЛИЯНИЕ ТЕМПЕРАТУРЫ НА ЭФФЕКТИВНОСТЬ ФОТОДЕСТРУКЦИИ КЛЕТОК АСЦИТНОЙ КАРЦИНОМЫ ЭРЛИХА, СЕНСИБИЛИЗИРОВАННЫХ ПРОИЗВОДНЫМ ГЕМАТОПОРФИРИНА

Цель: **выяснить механизм потенциирующего действия нагревания, ассоциированного с фотооблучением, на противоопухолевую эффективность фотодинамической терапии (ФДТ) с производным гематопорфирина (ПГП).** ***Методы:*** **исследование осуществлено на клетках асцитной карциномы Эрлиха (АКЭ), которые нагружали ПГП в бессывороточной среде, а затем облучали красным светом ($\lambda_{макс}$ = 630 нм) при различных температурах. Цитотоксичность оценивали посредством теста с трипановым голубым.** ***Результаты:*** **полученные нами данные подтверждают мнение, что в ФДТ гипертермия (около 44 °C), вызванная облучением, способна усиливать синергетически ПГП-фотоиндуцированное разрушение опухоли; обнаружено, что повышение температуры от 30 до 44 °C вызывало заметное (~ 1.5 кр.) повышение скорости ПГП-фотосенсибилизированной инактивации клеток АКЭ, тогда как гипертермия (44 °C) сама по себе оказывала лишь незначительное токсическое влияние на эти клетки.** ***Выводы:*** **потенциирующий эффект нагревания на противоопухолевую эффективность ФДТ с ПГП мог быть в значительной мере опосредованным стимуляцией генерации активных форм кислорода, таких, как: H_2O_2, супероксидного и гидроксильного радикалов. Обнаружено также, что фотосенсибилизация клеток АКЭ вызывала резкое снижение активности каталазы (КАТ) и глутатион(GSH)-пероксидазы и что нагревание повышало чувствительность этих H_2O_2-детоксифицирующих энзимов к ПГП-фотоиндуцированной инактивации. При ФДТ с ПГП эти события могут приводить к утрате защиты против аккумулирующейся H_2O_2; мы обнаружили, что КАТ и GSH редокс цикл играют важную роль в защите клеток АКЭ от фототоксического воздействия ПГП. Более того, полученные данные свидетельствуют о том, что при ФДТ с ПГП повышение температуры в опухоли способно потенциально усиливать эффективность этого лечения посредством стимуляции образования фотопродукта хлоринового типа.**

Ключевые слова**: фотодинамическая терапия, опухоль, нагревание, активные формы кислорода, антиокислительные энзимы.**