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EFFECTS OF LIGHT EXPOSURE ON THE UPTAKE AND DESTRUCTION OF HEMATOPORPHYRIN DERIVATIVE IN EHRLICH CARCINOMA CELL SUSPENSION

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Abstract. A mixture of porphyrins derived from hematoporphyrin and termed hematoporphyrin derivative (HpD) has been used for localization and photodynamic therapy (PDT) of tumours. Our data showed that HpD photobleached more rapidly when incorporated into tumour cells than in simple aqueous solutions. At light doses that are usually used in PDT about 30–40% of the HpD added to the cell suspension can be destroyed. In addition to photochemical modification, which leaves the porphyrin ring intact, more "drastic" reactions destroying the ring took place. At least one photoproduct was detected. This photoproduct (presumably a reduced porphyrin) has an emission peak at 640–650 nm and is optimally excited at 400 nm. However, the identity of the reactive oxygen species mediating HpD photobleaching in the cells is not clear. In cells, HpD may produce hydrogen peroxide (as a result of photosensitized oxidation of some biomolecules) and very reactive hydroxyl ($OH^{\bullet}$) radicals. We established that HpD is very reactive towards $OH^{\bullet}$ radicals. Therefore, besides singlet oxygen, H_2O_2 and $OH^{\bullet}$ radicals may be involved in photobleaching. It was found that light exposure (λ_{max} = 630 nm) accelerates the cellular uptake of HpD. This process was induced, however, by lethal light doses. It could be supposed that light exposure in the presence of HpD results in a membrane damage that allows HpD to penetrate faster into the cells or facilitates the binding of the sensitizer to the membrane itself. However, a long-term dark incubation of tumour cells, previously subjected to limited injury by means of HpD–PDT, did not induce similar levels of the sensitizer accumulation. Our data suggest that the light-induced increase in the HpD uptake by the tumour cells may be the result of covalent binding of HpD with certain intracellular molecules, probably with protein.

Key words: photodynamic therapy, hematoporphyrin derivative, photodestruction, binding, tumour cells.

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Abbreviations: PDT = photodynamic therapy; HP = hematoporphyrin IX; HpD = hematoporphyrin derivative; PII = photofrin II; PP = protoporphyrin; PS = photosensitizer; PBS = phosphate buffered saline; $^1O_2$ = singlet oxygen; EAC = Ehrlich ascites carcinoma; TB = trypan blue; SDS = sodium dodecyl sulphate; BSA = bovine serum albumin; PhPP = photoprotoporphyrin; AO_2 = oxygenated amino acid; GSH = glutathione.

INTRODUCTION

Photodynamic therapy (PDT) of cancer is based on the administration of a tumour-localizing photosensitizer (PS) followed by exposure of the neoplastic tissue to red light. Hematoporphyrin derivative (HpD) and its improved version, called photofrin-II (PII), are the most widely used PS for PDT of neoplastic disease. HpD and PII are complex mixtures of hematoporphyrin (HP), monohydroxyethylvinyldeuteroporphyrin, protoporphyrin (PP), and their polymer forms [1]. Photoexcitation of the tumour-localizing components of HpD leads to the production of singlet oxygen ($^1O_2$), which is reported to be the main agent responsible for induction of necrosis and regression of malignancies [2]. It is clear that the amount of HpD in the tumour tissue is a main factor determining the efficacy of PDT. Therefore, many attempts have been made to enhance the absolute uptake of HpD or PII in tumours. Cowled & Forbes [3] demonstrated that the administration of verapamil, a calcium-channel-blocking agent, simultaneously with HpD, to mice bearing Lewis lung carcinoma increased the uptake of HpD in the tumour. Adriamycin and methotrexate, two chemotherapeutic drugs, were found to increase the accumulation of HpD in Lewis lung tumours in vivo [4]. Lonidamine, an antispermatogenic and antitumour agent acting via inhibition of the energy metabolism, resulted in a significant increase in the uptake of HpD in Ehrlich ascites carcinoma (EAC) cells in vitro [5]. However, there is little information as to the effect of irradiation on the tumour uptake of a PS. Several authors have reported that the uptake of HP in tumour cells in vitro [6, 7] and PII in vivo is enhanced by light exposure. So, Ma et al. [8] showed that the accumulation of PII in the tumour of mice with CaD2 mammary carcinoma could be significantly increased when small light doses (25–75 J/cm^2) were given to the tumour area 1.5 h after intraperitoneal (i.p.) injection of the PS. However, mechanisms of the light-induced increase in the uptake of HpD in the tumour are not well understood.

It must be remembered that most PS used for PDT of tumours, including HpD or PII, photobleach on illumination [9, 10]. Hence, tumour destruction will be incomplete if the PS bleaches too rapidly during illumination. It has been suggested that the photobleaching of some tetrapyrroles, such as HP, PP, HpD, etc., is mediated by self-photogenerated $^1O_2$ that attacks the sensitizer macrocycles at the methine bridges to give pyrrole derivatives that do not absorb strongly in the visible region of spectrum and which do not act as a PS [11, 12]. These porphyrins do not photobleach in the absence of oxygen [13]. However,

the photobleaching yields and the mechanisms of HpD photodestruction in tumour area may be different from those observed in simple aqueous solutions.

In the present work we studied the effects of light exposure on the uptake and degradation of HpD in cells in vitro.

MATERIALS AND METHODS

Chemicals

HpD was prepared from HP dihydrochloride (Aldrich) according to the original method of Lipson et al. [14] modified by Kessel et al. [15]. Other chemicals were purchased from Sigma Chemical Co., St. Louis, USA, unless noted otherwise.

Animals and cells

White mongrel 4-month-old female mice obtained from the Institute of Experimental and Clinical Medicine (Tallinn, Estonia) were used in the experiments. EAC cells obtained from the Institute of Chemical Physics and Biophysics (Tallinn, Estonia) were weekly transplanted intraperitoneally into mice, 3×10^7 cells per animal.

Irradiation procedure

In all the experiments a 1 kW xenon arc-lamp, which has a continuous character of the light emission from 200 nm up to infrared domain of the solar spectrum, equipped with glass filters (KS-10 together with SZS-25), which transmit 50% of light at 630 nm (range 590–830 nm), and a focusing optical system served as the radiation source. The flux of light was focused as a spot (D = 0.8 or 1.8 cm) and directed on the front face of a quartz cuvette (with optical path length of 1 or 2 cm) containing EAC cell suspension or HpD solutions. The samples were air-equilibrated and irradiated under continuous magnetic stirring at 20°C. In this study the intensity of the emitted light at 630 nm was changed with respect to the types of the experimental procedures from 220 up to 335 mW. The HpD solutions were also irradiated with 330 nm light, which was isolated by means of a glass filter UFS-5 (bandpass range 250–400 nm), including a 4 cm water filter. The intensity of the emitted light at 330 nm was 170 mW with a power density 67 mW/cm^2, as measured with an IMO-2N radiometer (Russia).

Preparation of EAC cell suspensions and determination of the amount of injured cells

For in vitro experiments 6–8-day-old tumour cells were withdrawn with a syringe from the mice's abdominal cavity. A 4-fold amount of an isotonic solution (either Hanks'-balanced salt solution without phenol red and serum or

154 mM NaCl with 6.2 mM KCl and 5.55 mM glucose) was added to the cells. The cell suspension was stirred and centrifuged for 8 min at 4 °C. The packed cells were resuspended in one of the isotonic solutions described above and stored on ice bath until the beginning of the experiments. The viability of the cells was about 95–98%, as the trypan blue (TB) test indicated.

The amount of injured cells in the suspension was determined immediately after irradiation by staining the cells with 0.05% TB in phosphate buffered saline (PBS) (pH 7.2).

Study of HpD photodestruction

A 5 mg/ml solution of HpD in 0.9% NaCl (pH 7.4) was added into quartz cuvettes containing 2.5 or 8 ml of EAC cell suspension in an isotonic solution (supplemented with 10 mM of Tris-HCl or Hepes buffer, pH 7.4), to final concentration of HpD up to 1 μg/ml. Further the cell suspension (5×10^6 cells per ml) was irradiated with red light (λ_{max} = 630 nm) under magnetic stirring at 20 °C. Additional information on irradiation conditions (the intensity and light fluence rate) is given in figure legends.

The fall of the porphyrin absorption in the Soret peak (around 400 nm) and of the fluorescence intensity in the 617 nm band was measured to determine the rate of HpD photodegradation. Absorption spectra of HpD solutions were recorded by means of a Specord M-40 spectrophotometer (Germany). The fluorescence from HpD in simple aqueous solutions and in EAC cell suspensions (under magnetic stirring at 20 °C) was registered by means of a Hitachi 650-40 spectrofluorimeter (Japan). Quartz cuvettes with optical path lengths of 1 cm were used.

The kinetics experiments were repeated three times. The standard error (SE) was less than 5% for the kinetics measurements.

Determination of cellular uptake of HpD

In a typical experiment, 8 ml of EAC cell suspension (2×10^7 cells per ml) in Hanks' medium (supplemented with 10 mM of Tris-HCl buffer, pH 7.4) was placed to a quartz cuvette with an optical path length of 2 cm. Two minutes after the addition of HpD (up to 4 μg/ml) the cell suspension was irradiated with red light (λ_{max} = 630 nm; intensity 220 mW; fluence rate 86.6 mW/cm^2) under magnetic stirring at 20 °C. For the determination of HpD content in the EAC cells, the fractions of 0.2 ml were withdrawn in duplicate and were placed in 2.0 cm^3 plastic tubes (Sigma). The cells were then sedimented by centrifugation at 3000 g for 5 min at 20 °C. The supernatant was removed. The total amount of HpD in the cells was measured by dissolving the cells in 1.5 ml of 0.1 M NaOH and by measuring the fluorescence (λ_{ex} = 400 nm) of this solution at 620 or 644 nm. The porphyrin concentrations were determined from a calibration curve, which was obtained by measuring the fluorescence intensity of known porphyrin concentrations in 0.1 M NaOH. The calibration curve was linear up to 0.5 μg HpD

per ml. Therefore, the analysed samples were diluted below this value. Protein concentrations were determined according to the method of Lowry et al. [16].

Light-scattering measurements

The 90° scattering intensity in EAC cell suspension (5×10^6 cells per ml) was estimated with a Hitachi 650-40 spectrofluorimeter at wavelength of 400 nm. Quartz cuvettes with 1 cm optical path lengths containing 1.0 ml of the cell suspension (in Hanks' medium with 10 mM of Hepes buffer, pH 7.4) each were used. These measurements were performed under magnetic stirring at 20 °C.

RESULTS AND DISCUSSION

Self-sensitized photooxidation of HpD in EAC cell suspension

As Fig. 1 shows, the rate of HpD photodestruction increased considerably in the presence of EAC cells, because a long-term dark incubation of HpD with the cells and the illumination of the porphyrin solution (at 630 nm) in the absence of EAC cells did not cause similar decay of HpD fluorescence at 617 nm. We established that the decay of HpD fluorescence (at 617 nm) is a result of the splitting of the tetrapyrrolic ring, since the porphyrin absorption (at the Soret peak) in EAC cell suspension also decreased during irradiation (data not shown). The kinetic curve of HpD photosensitized degradation had a very clearly expressed lag period (Fig. 1), after which the oxidation of the PS rapidly increased. Moan & Christensen [6] found that PDT-induced damage to the membranes of cells in culture leads to an increased uptake of sensitizer into the

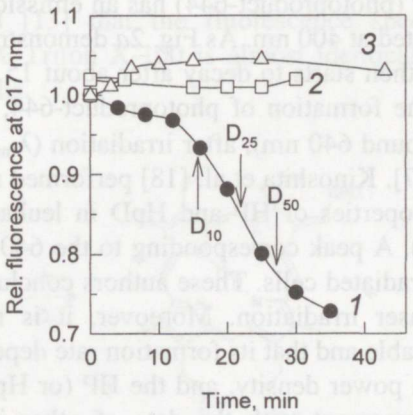

Fig. 1. Fluorescence intensities (λ_{ex} = 394 nm) from HpD (C = 1 μg/ml) in EAC cell suspension (the cells were resuspended in 2.5 ml of a medium containing 154 mM NaCl, 6.2 mM KCl, 5.55 mM glucose, and 10 mM Hepes buffer, pH 7.4): *1*, during light exposure (λ_{max} = 630 nm; 0.44 W/cm^2) and *2*, at dark incubation. *3*, irradiation of HpD, dissolved in the same medium, without EAC cells. D_{10}, D_{25}, D_{50} are the light doses needed for TB-staining of 10, 25, and 50% of the cells, respectively. A quartz cuvette with 1 cm optical path length was used in the experiment.

cells. One may speculate that the acceleration of HpD photobleaching was caused by the passage of the PS fraction unbound to EAC cells (about 80% of the initial HpD level) from the extra- to the intracellular area. Indeed (Fig. 1), a substantial enhancement of the permeability of the cell membrane (as measured by TB-staining) was observed during HpD photobleaching.

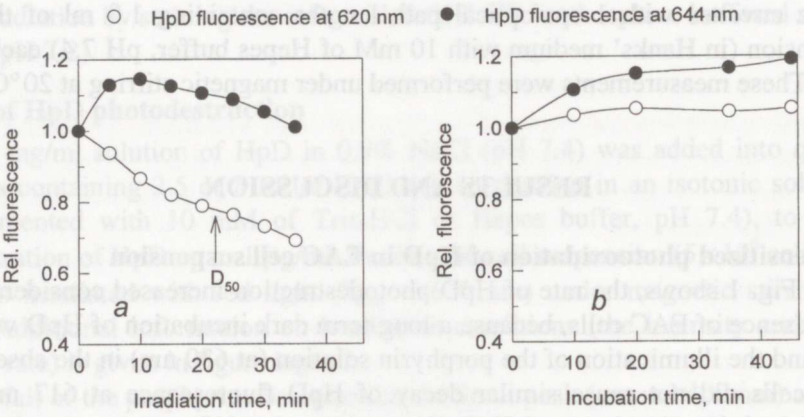

Fig. 2. Fluorescence intensities (λ_{ex} = 400 nm) from HpD (C = 1 μg/ml) in EAC cell suspension (the cells were resuspended in 8 ml of Hanks' medium with 10 mM of Tris-HCl buffer, pH 7.4): *a*, during light exposure (λ_{max} = 630 nm; 220 mW; 86.6 mW/cm^2) and *b*, at dark incubation. A quartz cuvette with 2 cm optical path length was used in the experiment.

The spectral changes in the fluorescence emission spectra of HpD in EAC cell suspension are shown in Fig. 2. At least one photoproduct was detected. We found that this product (photoproduct-644) has an emission peak at 640–650 nm and it is optimally excited at 400 nm. As Fig. 2*a* demonstrates, photoproduct-644 is rapidly formed and then starts to decay after about 15 min of light exposure. Earlier we observed the formation of photoproduct-644, absorbing light in the red spectral region (around 640 nm), after irradiation (λ_{max}= 405 nm) of HpD in an aqueous solution [17]. Kinoshita et al. [18] performed a detailed investigation of the fluorescence properties of HP and HpD in leukaemia cells under laser irradiation (at 515 nm). A peak corresponding to the 640 nm fluorescence band also appeared in the irradiated cells. These authors conclude that a photoproduct is produced during laser irradiation. Moreover, it is reported [18] that the photoproduct is very stable and that its formation rate depends on the presence of oxygen, the irradiation power density, and the HP (or HpD) concentration. Our results are in good agreement with the data of other investigators [10] who consider that photoproduct-644 is probably a chlorin or chlorin–porphyrin linked system formed primarily from aggregates of HP-like porphyrins by photo-reduction–photooxidation in a predominantly anaerobic environment (probably in consequence of type I processes). The photoreduction reaction and, consequently, the formation of photoproduct-644 may be stimulated by adding a

suitable proton donor. One of our earlier studies [17] showed that the rate of photoproduct-644 formation from HpD (in water medium) increased substantially in the presence of reducing agents (such as NADH and L-ascorbic acid). On the contrary, the yield of photoproduct-644 was inhibited when HpD photooxidation was carried out in the presence of bovine serum albumin (BSA), because the complexation of the sensitizer in BSA may lead to a disruption of the "sandwich"-type porphyrin aggregates in which, presumably, the formation of photoproduct-644 may occur [17]. Since the biologically active components of HpD contain vinyl groups [1], a chlorin-type photoproduct may also form from HpD with the participation of $^1O_2$ (type II process). Indeed, as Cox et al. [19] showed for PP (Fig. 3), the following reactions may lead to the formation of photoproduct-644: hydroxyaldehyde products may arise from the addition of $^1O_2$ to the vinyl groups of HpD and the adjacent double bonds may be reduced. Furthermore, chlorin derivative photoprotoporphyrin (PhPP) was detected in small amounts (<10%) during light exposure of WiDr cells containing PP [20]. However, the assumptions on the mechanisms of photoproduct-644 formation need experimental checking. Moan & Kessel [13] also observed the formation of a photoproduct (from PII in NHIK 3025 cells) with the emission peak at 640–650 nm, optimally excited at 395–400 nm. In addition, they showed the formation of another PII photoproduct that has an emission peak at 660–670 nm (optimally excited at 400–405 nm). It is known that the shape of the fluorescence emission spectrum of HpD is extremely sensitive to its molecular surroundings [21]. Therefore, Moan and Kessel [9, 13] believe that the increase of the fluorescence intensities from PII (at 640–650 and in the spectral region of 660–670 nm) in NHIK 3025 cells exposed to light (340–440 nm) may be largely due to a photoinduced displacement of the porphyrin in the cells. In particular, these authors showed [13] that the fluorescence spectrum of PII in PBS containing 0.005 vol.% Triton X-100 is almost identical with the spectrum of light-exposed PII in cells.

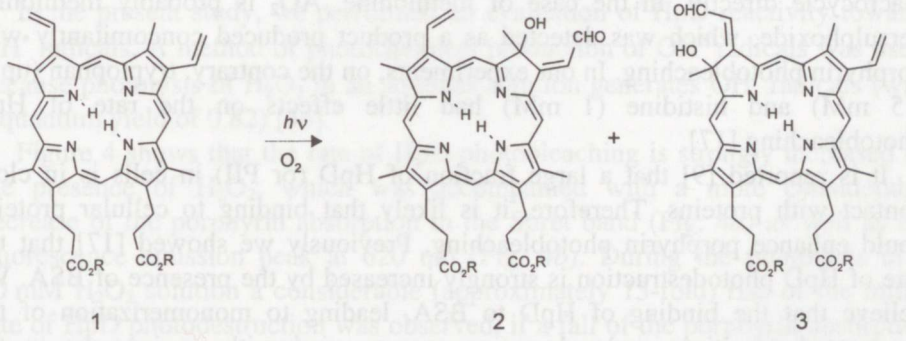

Fig. 3. Scheme (according to Cox et al. [19]) of the formation of chlorin-type photoproducts (2 and 3) during self-photosensitized oxidation of protoporphyrin IX (1). R = methyl.

We believe that the photoinduced displacement of porphyrins in tumour cells and photochemical modifications that leave the porphyrin ring intact (the formation of photoproducts that may have emission peaks in the red spectral region) are two additional processes (to the reactions that destroy the ring) that are responsible for the light-induced spectral changes in the fluorescence emission spectrum of HpD (or PII) in the cells. In our opinion, the yield of photoproduct-644 was relatively low and it was not the main product of HpD photodegradation. Nevertheless, the formation and retention in tumour loci of HpD photoproducts, absorbing light in the spectral region of higher tissue transmission, may play an important role in PDT, because these substances may possess photodynamic activity. In fact, on illumination, PhPP can generate $^1O_2$ with a quantum yield of 0.69 (in methylene chloride) [19]. Roberts et al. [22] performed a cell clonogenicity assay comparing photodegraded and fresh PII on CHO cells. The photodegraded PII exhibited significant phototoxicity although the fluorescence was bleached by more than 70%.

Until recently it was assumed that porphyrin is photochemically stable during treatment and could be activated indefinitely to produce the desired therapeutic effect. However, Moan et al. [23] first demonstrated the possible photooxidation of HpD in cells treated in culture. Similar photobleaching of PII had been observed in patients undergoing PDT [24]. We established (Fig. 1) that HpD incorporated into EAC cells photobleached more rapidly than in simple aqueous solutions. What are the mechanisms of the cells-mediated acceleration of HpD photodestruction? Krieg & Whitten [25, 26] showed that some porphyrins photobleach more rapidly when incorporated into erythrocyte ghosts (which contain photooxidizable lipids and proteins) or in microemulsions containing photooxidizable amino acids (such as methionine, tryptophan, and histidine) than in a pure solvent. These investigators suggested that the porphyrins sensitize $^1O_2$ efficiently but the $^1O_2$ is rapidly scavenged by substrates such as methionine and other amino acids. The oxygenated amino acids (AO_2) can subsequently act as agents to oxidize the porphyrins efficiently by attacking the porphyrin macrocycle directly. In the case of methionine, AO_2 is probably methionine persulphoxide, which was detected as a product produced concomitantly with porphyrin photobleaching. In our experiments, on the contrary, tryptophan (up to 0.5 mM) and histidine (1 mM) had little effects on the rate of HpD photobleaching [17].

It is reported [9] that a large fraction of HpD (or PII) in cells is in close contact with proteins. Therefore, it is likely that binding to cellular proteins could enhance porphyrin photobleaching. Previously we showed [17] that the rate of HpD photodestruction is strongly increased by the presence of BSA. We believe that the binding of HpD to BSA, leading to monomerization of the aggregated sensitizer molecules, was accompanied with an increase in the quantum yield of the HpD triplet state and, consequently, by the formation of $^1O_2$, which destroys porphyrins. However, it was found [18] that the

concentrations of HpD dimers and other aggregates are much higher in living cells than in the medium.

Oxygen appears to be necessary for the photobleaching of HpD in tumour cells [13]. Nevertheless, the identity of the reactive oxygen species mediating HpD photodegradation is not clear, especially in the case of biological systems. It is known that the lifetime of $^1O_2$ is over 15-fold greater in D_2O than in H_2O [27]. Thus, if the $^1O_2$ lifetime is a rate-limiting factor in the photobleaching process, the efficiency of the reaction might be expected to increase in D_2O as solvent. However, as Moan & Kessel [13] showed, the rate of PII photodegradation in NHIK 3025 cells is enhanced only slightly when H_2O is replaced by D_2O. A plausible explanation seems to be that besides $^1O_2$, the hydroxyl ($OH^\bullet$) radical may be the reactive oxygen species that determines the photobleaching behaviour of PII in the cells. Indeed, the $OH^\bullet$ radical is the most reactive oxidizing free radical. The chemical rate constants occurring between porphyrins (such as HP or PP) and $OH^\bullet$ radical may exceed those for porphyrins and $^1O_2$ almost 10^5–10^6 fold [28, 29]. It is well known that HpD-sensitized photooxidation of some biomolecules may lead to the production of significant amounts of hydrogen peroxide (H_2O_2). In cells, the non-enzymatic decomposition of the formed H_2O_2 (for example, by means of the Fenton–Haber–Weiss reaction [30]) may cause the generation of very highly reactive $OH^\bullet$ radicals: $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^\bullet$. In fact, H_2O_2 was found as one of the products during photooxidation of NADPH in the presence of HP in D_2O [31]. Furthermore, the rat liver microsomal cytochrome P-450 undergoes rapid destruction in the presence of HpD and light. However, sodium benzoate, mannitol, and ethanol ($OH^\bullet$ radical scavengers) prevent deterioration of the microsomal haem-protein [32]. Previously we found [5] that HpD–PDT of EAC cells resulted in a considerable decrease of the reduced glutathione (GSH) content. The fall of the cellular GSH level may occur not only due to the enzymatic decomposition of the formed H_2O_2 and lipoperoxide compounds, but also as a result of a direct reaction of GSH with the $OH^\bullet$ radicals [33].

In the present study, we performed an evaluation of HpD reactivity towards $OH^\bullet$ radicals. A method of photochemical production of $OH^\bullet$ radicals was used, because photolysis of H_2O_2 in an aqueous solution generates $OH^\bullet$ radicals (with a quantum yield of 0.82) [34].

Figure 4 shows that the rate of HpD photobleaching is strongly increased by the presence of H_2O_2, which was accompanied with a more considerable decrease of the porphyrin absorption in the Soret band (Fig. 4*a*) as well as the fluorescence emission peak at 620 nm (Fig. 4*b*). During the photolysis of a 50 mM H_2O_2 solution a considerable (approximately 15-fold) rise of the initial rate of HpD photodestruction was observed, if a fall of the porphyrin absorption in the Soret peak was measured (Fig. 4*a*). The destruction of HpD, mediated by $OH^\bullet$ radicals, was associated with a sharp decrease of the sensitizer fluorescence at 644 nm (Fig. 4*c*) while at 460 nm the fluorescence of the HpD solution

increased (Fig. 4*d*). Hence, the interaction of $OH^{\bullet}$ radicals with HpD leads to a substantial inhibition of the photoproduct-644 production and, on the contrary, to an acceleration of the photoproduct-460 formation. We suggest that photoproduct-460 is a mixture of formylbiliverdins, which form as a result of a reaction of the splitting of the tetrapyrrolic ring [17]. So, it has been shown that certain metalloporphyrins are photooxygenated to yield formylbiliverdins as primary products [19]. A product with similar fluorescence properties in irradiated cells containing HpD (or PII) has also been detected [13, 35]. Thus, we can say that HpD is very reactive towards $OH^{\bullet}$ radicals and the formation of these radicals may explain the photobleaching behaviour of the sensitizer in tumour cells.

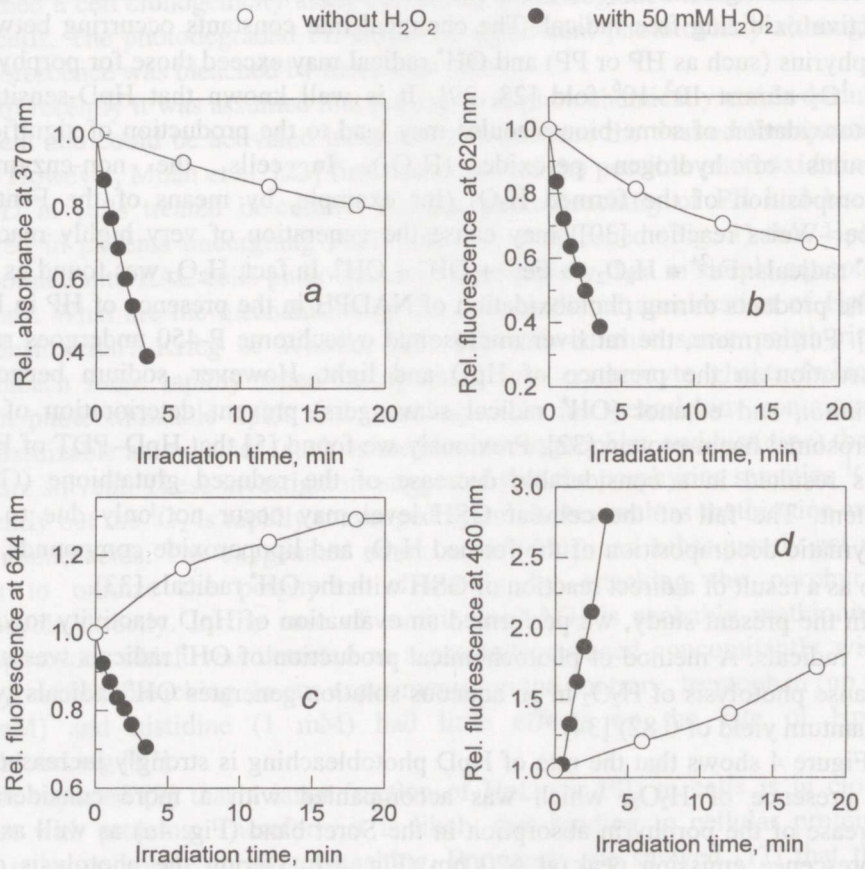

Fig. 4. Kinetics of HpD (C = 10 μg/ml) photodestruction (λ_{max} = 330 nm; 67 mW/cm^2; sample light path 2 cm; volume 8 ml) in 10 mM Tris-HCl buffer (pH 7.4) without or in the presence of 50 mM H_2O_2: HpD absorption in the Soret peak (*a*); and the fluorescence intensities (λ_{ex} = 400 nm) of HpD solution at 620 (*b*), 644 (*c*), and 460 nm (*d*).

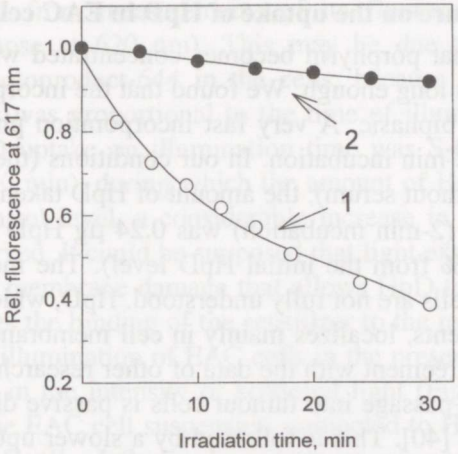

Fig. 5. Kinetics of HpD (C = 1 μg/ml) photodegradation (λ_{max} = 630 nm; 0.66 W/cm^2): *1*, in EAC cell suspension (the cells were resuspended in 2.5 ml of a medium containing 154 mM NaCl, 6.2 mM KCl, and 10 mM Hepes buffer, pH 7.4) and *2*, in the cells lysate (in 1% SDS). A quartz cuvette with 1 cm optical path length was used in the experiment.

Figure 5 indicates that the photobleaching yield of HpD in EAC cells was suppressed when the cells were lysed in 1% sodium dodecyl sulphate (SDS). We [36] and other researchers [37] showed that the decreased yields in detergents may result from partial incorporation of a PS into regions of the micelles having a low dielectric constant. On the other hand, the inhibition of HpD photodestruction may indicate that some biochemical processes in living cells may be involved. It is well known that the respiration chain of mitochondria is the main source of oxygen superoxide anion radicals ($O_2^{-\bullet}$) [38], which are able to catalyze the decomposition of H_2O_2 ($Fe^{3+} + O_2^{-\bullet} \rightarrow Fe^{2+} + O_2$) and, consequently, the formation of $OH^\bullet$ radicals that destroy porphyrins.

It was found [39] that metronidazole, an electrophilic "radiosensitizer", which efficiently quenches HP triplets with the formation of the metronidazole radical anion, increases the quantum yield of HP photobleaching. Because living cells may contain electron acceptors (Coenzyme Q, for instance), photobleaching of HpD in vivo may be affected by reactions of this type.

Thus, we found that HpD photobleached more rapidly when incorporated into EAC cells. In addition to photochemical modifications that leave the porphyrin ring intact, more "drastic" reactions that destroy the ring took place. However, the identity of the reactive oxygen species mediating HpD photobleaching in the cells is not clear. In cells, HpD may produce hydrogen peroxide (the result of photosensitized oxidation of some biomolecules) and very reactive $OH^\bullet$ radicals. Therefore, besides $^1O_2$, these reactive oxygen species may be involved in photobleaching.

Effect of light exposure on the uptake of HpD in EAC cells

Figure 6 shows that porphyrin becomes concentrated within the cells when the incubation time is long enough. We found that the incorporation of HpD into EAC cells is clearly biphasic. A very fast incorporation phase is evidenced by results obtained for 2-min incubation. In our conditions (the incubation of EAC cells in a medium without serum), the amount of HpD taken up during the phase of fast incorporation (2-min incubation) was 0.24 μg HpD per 1 mg of protein (that is about 15–20% from the initial HpD level). The mechanisms by which HpD is taken up by cells are not fully understood. HpD, which consists primarily of lipophilic components, localizes mainly in cell membrane structures [2]. Our results are in good agreement with the data of other researchers who believe that the first step of HpD passage into tumour cells is passive dissolution of the dye in the cell membrane [40]. This is followed by a slower uptake of HpD into the cytoplasm. So, Böhmer & Morstyn [41] showed that the loosely bound portion of HpD could be washed from tumour cells by a medium containing either fetal calf serum or serum albumin.

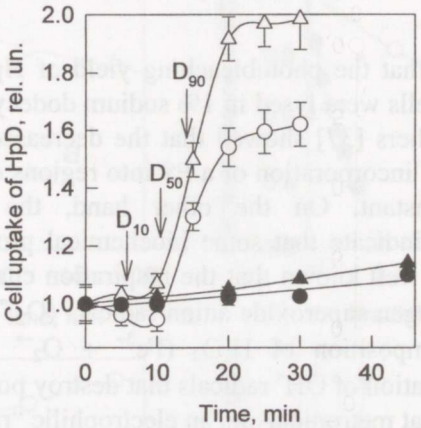

Fig. 6. Uptake of HpD (C = 4 μg/ml) to EAC cells (2×10^7 cells per ml; in Hanks' medium with 10 mM Tris-HCl buffer, pH 7.4) during light exposure (λ_{max} = 630 nm; 86.6 mW/cm^2) (open symbols) and at dark incubation (filled symbols). Rings designate the determination of HpD concentrations by means of the measurement of its fluorescence at 620 nm and triangles the same at 644 nm. The initial level of HpD (before irradiation) in the EAC cells was estimated as 0.24±0.01 μg HpD per mg of protein (according to the fluorescence intensity at 644 nm). D_{10}, D_{50}, D_{90} are light doses needed for TB-staining of 10, 50, and 90% of the cells, respectively. Bars = SE.

The amounts of HpD taken up by the cells as a function of the time of illumination were also determined. Figure 6 shows that the light exposure (λ_{max} = 630 nm) accelerates the cellular uptake of HpD. The HpD uptake was measured by recording the fluorescence from the cells lysed in 0.1 M NaOH at 620 or 644 nm. The data in Fig. 6 indicate that at illumination the amounts of HpD taken up by the cells were much higher if the intracellular concentration of

HpD was estimated from measurements of its fluorescence at 644 nm (in comparison with those at 620 nm). This may be due to the formation and accumulation of photoproduct-644 in the cells, because the difference in the HpD concentrations was proportional to the time of illumination (Fig. 6). The dependence of HpD uptake on illumination time was S-type. Indeed (Fig. 6), after a lag period (10 min), during which the amount of HpD bound to the cells remained at the control level, a considerable increase in the rate of uptake of porphyrin was observed. It could be supposed that light exposure in the presence of HpD results in a membrane damage that allows HpD to penetrate faster into the cells or facilitates the binding of the sensitizer to the membrane itself. It was established that the illumination of EAC cells in the presence of HpD leads to a substantial increase in the intensity of scattered light (Fig. 7). The increase of light scattering in the EAC cell suspension, subjected to HpD–PDT, may be the result of swelling of cells. Indeed, phase-contrast microscopy showed that the HpD–PDT of EAC cells led to significant alteration in the shape and dimensions of the cells. The following events took place: 2–3-fold increase in the cell volume, the appearance of multiple small bumps on the surface (also called "blebs"), and the blending of microblebs into large bumps of cytoplasm. During further illumination an intensive staining of cells with TB was observed. This indicates an essential injury of plasma membranes of carcinoma cells. The results obtained demonstrate that the light-induced enhancement of cellular HpD uptake coincided in time with a considerable increase of the cell size and with an enhancement of the permeability of their plasma membranes (Fig. 6). Further-

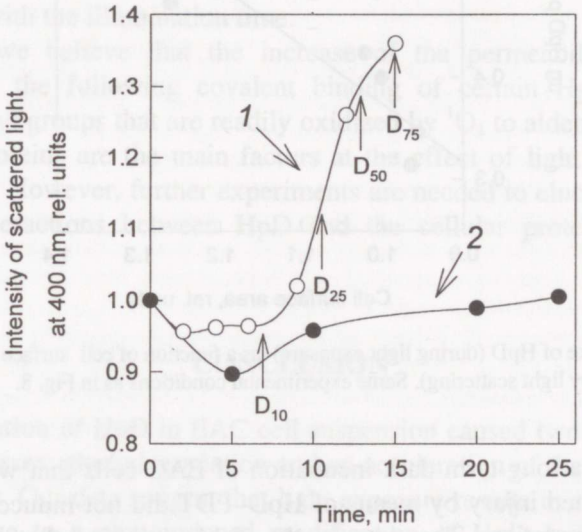

Fig. 7. Intensities of scattered light in EAC cell suspension (5×10^6 cells per ml; in Hanks' medium with 10 mM Hepes buffer, pH 7.4; volume 8 ml): *1*, during HpD–PDT (HpD 2 μg/ml; λ_{max} = 630 nm; 86.6 mW/cm^2) and 2, at dark incubation in the same medium. A quartz cuvette with 2 cm optical path length was used in the experiment. SE<10%.

more, during the photodynamic treatment HpD uptake was proportional to the number of TB-stained cells (Fig. 8) and to the cell surface area (Fig. 9).

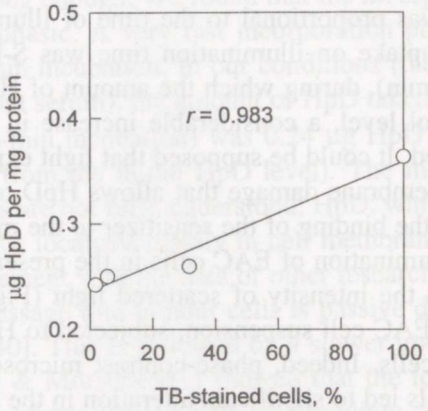

Fig. 8. Relationship between the binding of HpD (during light exposure) and HpD–PDT induced injury of EAC cells (as measured by TB-staining). Same experimental conditions as in Fig. 6.

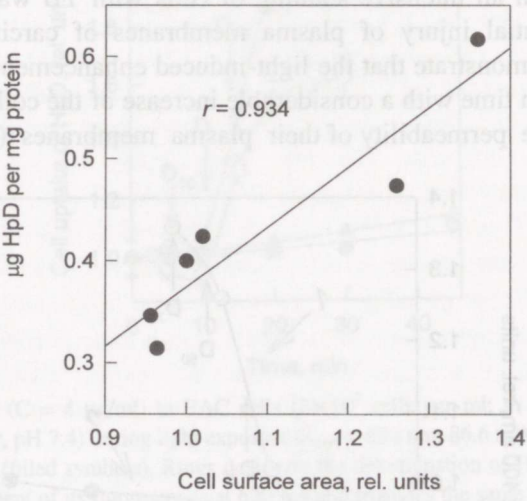

Fig. 9. Cellular uptake of HpD (during light exposure) as a function of cell surface area (as measured by light scattering). Same experimental conditions as in Fig. 8.

Surprisingly, a long-term dark incubation of EAC cells that were previously subjected to limited injury by means of HpD–PDT did not induce similar levels of the PS accumulation. The data in the Table indicate that the membrane damage (as measured by TB-staining) is not the only determinant of the effect of light on the cellular uptake of HpD. Other factors besides membrane permeability may be involved.

Concentrations of HpD in EAC cells at different times after photodynamic treatment*

Incubation time, min	TB-stained cells, % (SE < 10%)	μg HpD per mg protein (SE < 5%)
0	14.0	0.199
10	39.3	0.202
20	85.2	0.203
30	100.0	0.205
40	–	0.207
50	–	0.227

* 2 min after the addition of HpD (up to 2 μg/ml), the suspension of EAC cells (5×10^6 cells per ml; in Hanks' medium with 10 mM Tris-HCl buffer, pH 7.4) was irradiated (λ_{max} = 630 nm; fluence rate 86.6 mW/cm^2) up to a light dose of 41.6 J/cm^2. Illumination and the following incubation (in darkness) of the EAC cell suspension (volume 8 ml) were performed in a quartz cuvette (with 2 cm optical path length) under magnetic stirring at 20°C.

Our findings suggest that the light-induced increase in the HpD uptake by the tumour cells may be a result of covalent binding of HpD with some intracellular molecules, probably with protein. Indeed, one of our earlier studies [17] showed that HpD-sensitized photodegradation of BSA does not lead to a release of HpD components from their complex with the protein. In contrast, under illumination the affinity of BSA to HpD enhanced. It was expressed as a considerable decrease of the distribution coefficients of HpD between octanol-1 and 0.2% BSA solution with the illumination time.

Therefore, we believe that the increase of the permeability of the cell membrane and the following covalent binding of certain HpD components (containing vinyl groups that are readily oxidized by $^1O_2$ to aldehydes [19]) with intracellular proteins are the main factors at the effect of light on the cellular uptake of HpD. However, further experiments are needed to elucidate the nature of possible interactions between HpD and the cellular protein during light exposure.

CONCLUSION

The illumination of HpD in EAC cell suspension caused two simultaneously occurring processes: photodegradation and an acceleration of the cellular uptake of the sensitizer. Our data suggest that light exposure results in spectral changes that may be due to a photoinduced modification of HpD molecules without breakage of the porphyrin macrocycle (the formation of photoproducts which have a fluorescence peak around 640 nm). This may play a very important role in HpD–PDT of tumours, because these substances may possess photodynamic activity. Photochemical reactions involving breakage of the porphyrin

macrocycle also occur. Our findings show that HpD photobleached more rapidly when incorporated into cells. Hence, at PDT the quantum yield of HpD photobleaching may be considerable in tumour loci. We postulate that in living cells, H_2O_2, $OH^{\bullet}$ radicals, electron accepting compounds, and certain biochemical processes may be involved, besides $^1O_2$, in the photobleaching of HpD.

We found that lethal light doses increased the uptake of HpD in the tumour cells significantly. The results obtained are in good agreement with the data of other researchers [8] who consider that a way to improve the tumour-to-normal-tissue concentration ration of HpD is to expose the tumour loci to a small dose of light at a time when the concentration of the sensitizer in the blood is high. Hence, the PDT efficiency might be improved by fractionated light exposure.

ACKNOWLEDGEMENT

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VALGUSE MÕJU HEMATOPORFÜRIINI DERIVAADI ABSORPTSIOONILE JA DESTRUKTSIOONILE EHRLICHI KARTSINOOMI RAKKUDE SUSPENSIOONIS

Vladimir TŠEKULAJEV, Igor ŠEVTŠUK ja Ljudmila TŠEKULAJEVA

Hematoporfüriinist saadud ja hematoporfüriini derivaadiks (HpD) nimetatud porfüriinide segu on kasutatav kasvajate diagnostikas ja fotodünaamilises teraapias (FDT). Uuringutest nähtub, et rakku inkorporeeritud HpD laguneb valgustamisel kiiremini võrreldes vesilahusega. Kasvajate FDT-s tavaliselt kasutatavate valgusdooside puhul laguneb 30–40% rakkude suspensiooni lisatud HpD-d. Seejuures toimuvad fotokeemilised reaktsioonid, mille käigus võib porfüriini tsükkel jääda terveks või lõhustuda. Autoritel õnnestus tuvastada vähemalt üks fotoprodukt (arvatavasti porfüriini redutseerimise produkt), mille

fluorestsentsi lainepikkus on 640–650 nm ja ergastusmaksimum 400 nm. Pole teada, millised reaktiivsed hapnikuühendid põhjustavad HpD lagunemise rakus. HpD valgustamisel rakus võivad mõningate biomolekulide fotooksüdatsiooni tulemusel tekkida vesinikülihapend ning väga reaktsioonivõimelised hüdroksüülradikaalid ($OH^{\bullet}$). Uuringust selgub HpD kõrge reaktsioonivõime $OH^{\bullet}$-radikaalide suhtes. Seega on võimalik, et lisaks singletsele hapnikule põhjustavad HpD lagunemist ka H_2O_2 ja $OH^{\bullet}$-radikaalid. Valguse letaalsete dooside puhul ilmnes, et valgustamine lainepikkusel 630 nm suurendab HpD akumulatsiooni rakkudes. Tõenäoliselt tekitab kasvajarakkude kiiritamine valgusega HpD manulusel vigastusi rakumembraanis, mis omakorda suurendab sensibilisaatori membraanläbitavust või HpD seonduvust membraani endaga. Samas ei kaasnenud eelnevalt HpD FDT-ga osaliselt vigastatud kasvajarakkude pikaajalise inkubatsiooniga pimedas samaväärset sensibilisaatori akumulatsiooni taseme tõusu. Valgusega indutseeritud HpD akumulatsiooni suurenemine kasvajarakkudes võib olla põhjustatud HpD ja rakusiseste molekulide, arvatavasti valkude, kovalentsest interaktsioonist.