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Photosensitized Inactivation of Tumor Cells by Porphyrins and Chlorins

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Declaration: Hereby I declare that this doctoral thesis, my original investigation and achievement, submitted for the doctoral degree at Tallinn University of Technology has not been submitted for any degree or examination.

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CONTENTS

ABBREVIATIONS

ALA	5-aminolaevulinic acid
AlSPc	sulphonated aluminium phthalocyanine
ATP	adenosine triphosphate
BCNU	1,3-bis(2-chloroethyl)-1-nitrosourea
BPD-MA	benzoporphyrin derivative monoacid ring A
BSA	bovine serum albumin
BSO	D, L-buthionine-[S,R]-sulfoximine
CAT	catalase
DEF	deferoxamine mesylate
DOR	2-deoxy-d-ribose
EAC	Ehrlich ascites carcinoma
E6	chlorin-e_6 trimethyl ester
GSH	reduced glutathione
GPX	Se-dependent glutathione peroxidase
GR	glutathione reductase
HP	hematoporphyrin IX
HE	hydroethidine
His	histidine
HPD	hematoporphyrin derivative
LD_{50}	the light exposure time at which 50% of cells were stained by trypan blue
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
M-THPC	meso-tetrahydroxyphenylchlorin
NBT	nitro blue tetrazolium
NPe6	mono-L-aspartyl chlorin-e_6
$^1O_2$	singlet oxygen
$O_2^{-\bullet}$	superoxide anion radical
$OH^\bullet$	hydroxyl radical
PBS	phosphate buffered saline
PDT	photodynamic therapy
PP	protoporphyrin IX
PPO	protein peroxides
PS	photosensitizer
PII	Photofrin II
PUFA	polyunsaturated fatty acids
PhP-640	photoproduct-640
PrSH	protein-bound sulfhydryl groups
ROS	reactive oxygen species
SOD	superoxide dismutase
TB	trypan blue
Trp	tryptophan
Tyr	tyrosine
XOD	xanthine oxidase
XDH	xanthine dehydrogenase

LIST OF PUBLICATIONS INCLUDED IN THE THESIS

The present dissertation is based on the following papers, which will be referred to in the text by their Roman numerals I-IV:

I Shevchuk, I.N., Chekulayev, V.A., **Chekulayeva, L.V.** 2002. The role of lipid peroxidation in the photodestruction of Ehrlich ascites carcinoma cells sensitized by hematoporphyrin derivative. - Experimental Oncology, vol. 24, no. 3, p. 216-224.

II **Chekulayeva, L.V.**, Shevchuk, I.N., Chekulayev, V.A., Ilmarinen, K. 2006. Hydrogen peroxide, superoxide, and hydroxyl radicals are involved in the phototoxic action of hematoporphyrin derivative against tumor cells. - Journal of Environmental Pathology, Toxicology and Oncology, vol. 25, nos. 1-2, p. 51-77.

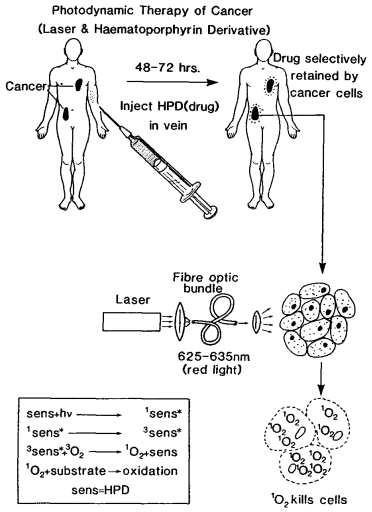
III **Chekulayeva, L.V.**, Shevchuk, I.N., Chekulayev, V.A. 2004. Influence of temperature on the efficiency of photodestruction of Ehrlich ascites carcinoma cells sensitized by hematoporphyrin derivative. - Experimental Oncology, vol. 26, no. 2, p. 125-139.

IV **Chekulayeva, L.**, Shevchuk, I., Chekulayev, V. 2003. Photodynamic therapy of tumours with chlorin-e_6 is pH dependent. - Proceedings of the Estonian Academy of Sciences – Biology/Ecology, vol. 52, no. 1, p. 40–54.

In the appendix to this thesis, copies of the articles I-IV have been included. These articles were reproduced with permission from corresponding publishers. Some unpublished data were also presented.

INTRODUCTION

Since cancer continues to plague humanity there is large need for development of new modalities for both diagnosis and therapy. Most of the currently available methods suffer from serious disadvantages. The treatments, e.g. ionising radiation, chemotherapy, surgery, may themselves induce malignancies or the patient may be physically impaired for a longer period of time. However, successes in the development of laser and fibre-optical techniques have provided new possibilities for treatment of the disease. Today, low-power lasers in combination with certain photosensitizing agents are used in treatment of malignant tumors by photodynamic therapy (PDT). It is based on the administration into an organism of a tumor localizing drug (usually, some porphyrins or chemically related compounds) followed by exposure of the tumor locus to visible light of an appropriate wavelength corresponding to the absorbance band of the used photosensitizer (PS).



Scheme 1. Principle of the photodynamic therapy of cancer

Hematoporphyrin derivative (HPD) and its improved version called Photofrin II (PII) are the most widely used PS(s) for PDT of malignancies (Dougherty, 2002). The principle steps involved in PDT are illustrated in Scheme 1. HPD is administered intravenously, and then a period of time (usually 48 to 72 hours) is allowed to pass. This enables the PS to accumulate to a maximum possible concentration in the tumor. Then the tumor is irradiated with red light (typically 630 nm laser light) with a dose of between 50 and 500 J/cm^2. Photoexcitation of HPD leads to the formation of singlet oxygen ($^1O_2$, a highly reactive oxidant) that as believed is responsible for induction of tumor necrosis. Studies on the mechanism of PDT showed that this treatment modality may involve a direct antitumor effect (damage of organelles within the malignant cells) as well as destruction of blood vessels in the tumor locus resulting in reduced supply of O_2 and nutrients (Peng et al., 1996). It was also discovered that local immune

reactions, e.g. macrophages, could be responsible for the tumor necrotizing action induced by PDT with HPD (Korbelik, 1996). Besides, this treatment modality strongly suppresses the metastatic potential of transformed cells. It is important to note that the intravenous administration of HPD or PII, which are strong fluorophores, can be used for visual detection of tumor localization (Lipson et al., 1961). The obtained clinical experience indicates that PDT has many advantages as compared with commonly used modalities for cancer treatment, such as: higher selectivity, low traumatic effects and systemic toxicity. It was also established that PDT does not induce genetic abnormalities and may successfully combine with traditional approaches for the disease treatment. Its main limitation is related to the small penetration depth of light into tissues. Tumor tissue further away from the light source (fiber tip etc.) than 1 cm can usually not be inactivated.

HPD and PII, as sensitizers for PDT, have serious drawbacks. Namely, they have relatively weak light absorption in red region of the spectrum where the maximal light penetration of tissues is observed (maximum at 700-800 nm) that may limit the depth of tumor necrosis or result in unacceptably long light-exposure times. A drawback of the use of PII is also the prolonged cutaneous photosensitivity. The problems encountered with HPD have led to the development of new dyes. Some of them, so-called second-generation PS(s), are proposed as alternative agents for PDT: (1) metallo-phthalocyanines; (2) modified porphyrins, such as sulfonated tetraphenylporphines, tetra(m-hydroxyphenyl)porphines; (3) reduced porphyrins, such as some derivatives of chlorin-e_6 and phephorbide-a, meso-tetrahydroxyphenylchlorin (m-THPC) and its bacteriochlorin analogue, benzoporphyrin derivative monoacid ring A (BPD-MA), and Sn(IV)-etiopurpurin; and 4) texaphyrins. They are effective generators of $^1O_2$ and have a strong absorption peak in the range of 650-800 nm, wavelength at which light penetration in tissue is enhanced (Peng et al., 1996). Among the newly synthesized PS(s) several compounds, such as sulphonated aluminium phthalocyanine (AlSPc), m-THPC, BPD-MA, mono-L-aspartyl chlorin-e_6 (NPe6) and lutetium texaphyrin, attained the phase 2 of clinical trials (Peng et al., 1996; Dougherty et al., 1998; Tuulik et al., 2002).

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 PII (Dougherty, 2002), the fundamental mechanisms by which PDT kills tumor cells as well as its optimal physical parameters are still incompletely clarified. So, the photoactivated HPD is an efficient producer of $^1O_2$ in simple chemical systems (Tanielian et al., 2001) and it was suggested (Weishaupt et al., 1976) that this oxidant is the main damaging agent in PDT. At the same time, 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. However, to date the role of H_2O_2 and oxygen radicals in the phototoxic action of HPD against tumor cells remains still unclear. Similar situation occurs for chlorin-type PS(s); namely, for derivatives of chlorin-e_6.

Previous studies showed that proteins in tumor cells are a target for HPD-PDT (Peng et al., 1996). At the same time, recently it was found that these biomolecules can be oxidized by $^1O_2$ with the formation of semistable peroxides (Davies, 2003). As $^1O_2$ is involved in the phototoxic action of HPD against tumor cells, we supposed that formation of these peroxides could occur in tumor tissues subjected to PDT with the PS. The ability of photoexcited HPD to the generation of protein peroxides (PPO) in target cells could have important consequences for more precise understanding the mechanism of the tumor ablating effect of HPD-PDT; currently PPO are regarded as a new form of ROS, since decomposition of such peroxides has been demonstrated yield reactive species, including oxygen- and carbon-centered radicals (Headlam and Davies, 2003). These radicals can inactivate cellular enzymes, destroy antioxidants, damage lipids and DNA. In the presented thesis, we evaluated the possibility that in addition to $^1O_2$ other ROS could be involved the antitumor effect of PDT with HPD or certain derivatives of chlorin-e_6.

As known both of the two main constituents of cellular membranes, lipids and proteins, may be damaged by the photodynamic action of porphyrins. Studies from several laboratories strongly suggest that lipids in the membranes are the most critical targets upon photoexcitation of HPD (Thomas and Girotti, 1989b). However, in these works it has not been proven that photodamage of lipids is the main reason for cell inactivation, since the effects of PDT with HPD on the intactness of cellular proteins were not examined during these experiments. In this work we performed, therefore, a comparative study about the role of peroxidation of lipids and degradation of proteins in the phototoxic action of HPD against tumor cells.

Clinical trials showed that during a standard regimen of PDT a considerable (5-13 ºC) increase in the temperature of tumor tissues can take place (Berns et al., 1984). In this relation, studies on various experimental tumors 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-photoinduced destruction of tumor cells (Leunig et al., 1994). However, the mechanism of the synergism remains unclear yet, though these observations led to 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 this phototherapy (Mang, 1990). In this thesis, we made an attempt to clarify 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 to optimize the thermal regimen of PDT.

It is well known that the pH value in rapidly growing malignant tumors tends to be somewhat lower (due to an inadequate vascularization) than that of the surrounding normal tissue. The normal tissue pH in man varies from 7.0 to 8.06, whereas the pH values obtained in human tumors vary from 5.85 to 7.68 (Pottier and Kennedy, 1990). Tumor pH can be driven selectively even lower by the administration of glucose in high concentrations. Numerous attempts have been

made at exploiting this phenomenon to enhance the selective delivery and cytotoxicity of antineoplastic drugs. However, the possibility of using this approach to improve the efficiency of PDT with chlorin-e_6-type PS(s) has not been explored. In the presented work we, therefore, investigated the effect of pH and glucose administration on the antitumour efficiency of PDT with chlorin-e_6 trimethyl ester.

As the antitumor effect of PDT is mediated by the generation ROS, substantial interest has an elucidation of the role of antioxidant barriers of tumor cells in their resistance to the phototoxic influence of tetrapyrrolic compounds. However, current data on the aspect of PDT are still incomplete and fragmentary. This research trend is very important, since the obtained results could help in search of appropriate inhibitors to enhance the antitumor efficiency of PDT. In this work, we estimated the significance of superoxide dismutases (SOD(s)), catalase (CAT), and selected glutathione-dependent enzymes in the resistance of tumor cells to the cytotoxicity resulting from photoexcited HPD.

1. REVIEW OF THE LITERATURE

1.1. Photodynamic Therapy - Background

1.1.1. Historical aspects

The introduction of the term “photodynamic” and several important discoveries within the field were occurred since the first decade of the XX century. In 1898, the medical student Oscar Raab revealed by chance that the toxicity of the dye acridine to paramecia was dependent on the ambient light. Von Tappeiner continued the work, and together with Jodlbauer, it was reported in 1904 that the presence of O_2 was required to achieve an effect. With this, he first minted the term PDT to describe the phenomenon of O_2 dependent photosensitization. Together with the dermatologist Jesoniek, he was the first to perform PDT on humans, reported in 1903, treating skin cancer, lupus of the skin and genital condylomas, with eosin as a PS. In 1908, the first studies of the biological properties of hematoporphyrin IX (HP) were performed by Hausmann in Vienna who sensitized mice. The first report on sensitization in humans was in 1913, when the German Meyer-Betz injected himself with 200 mg of HP and remained photosensitive to light for two months (Meyer-Betz.F., 1913). In 1924 Policard observed that endogenous porphyrins accumulated in tumors (Policard, 1924), but it was not before 1942 that exogenously administered porphyrins were shown to accumulate in tumors. Figge (1942) presented results indicating that the fluorescing properties of porphyrins could be used to detect tumors. At the same time Auler and Banzer (1942) demonstrated that light exposure of tumors in rats injected with HP induced tumor necrosis.

These early observations were not followed up for many years. The next and probably most important break-through came in 1961 when Lipson and co-workers tried to purity HP by sequential acetylation and reduction. Instead of obtaining a purified product they got a porphyrin mixture named HPD (Lipson et al., 1961). HPD contains monomeric and aggregated porphyrins and some of its compounds possess better tumor-localizing properties than that of HP.

Dougherty and co-workers initiated experiments with PDT of tumors in mice and rats using HPD as PS (Dougherty et al., 1975). Kelly and Snell (1976) reported the first clinical use of HPD. These initial studies and the continuous follow-up by Dougherty and his colleagues have led to a large international research activity in the field.

A new and interesting development in PDT is the use of 5-aminolaevulinic acid (ALA) in the treatment of skin lesions. In this technique the ALA is applied topically, as a cream, converts enzymatically to protoporphyrin IX (PP) and is irradiated with red light at 630 nm. The advantage of this technique being that, because the PS is localized almost totally in the tumor, skin photosensitivity is negligible and the effectiveness of tumor destruction improved. A wide clinical experience of ALA-based PDT has been acquired, e.g. excellent results, including cosmetic benefits, following clinical treatment of multiple basal-cell carcinoma by the phototherapy have been reported (Fink-Puches et al., 1998).

1.1.2. Commonly used photosensitizers

The field of research on various PS(s) is now quite extensive. Only some main groups of PS(s), which have been utilized in pre-clinical and clinical studies, will be mentioned here. The main feature of photosensitizing agents is light absorption in a wavelength range where the surrounding biological tissue is relatively transparent. Most PS(s) absorb light in several bands within the visible wavelength range. To reach a deeper penetration, the absorption peaks in the red region are used. HPD, the most common PS within the field of PDT, has an absorption peak in the red wavelength region at about 630 nm. The research within the field aims at finding compounds which absorb far out in the red wavelength region, towards the borders of the infrared, where the penetration depth of the treatment light is at its maximum. Larger tumor volumes can then be treated. Another important feature to consider is that the triplet states energies are lower for dyes absorbing in this region, and that there is an upper wavelength limit for the generation of $^1O_2$ via the Type II mechanism. This means that dyes absorbing at longer wavelengths than approximately 800 nm cannot be used. In addition, a useful PS should have a rather narrow absorption band, with little absorption at other wavelengths within the solar spectrum. This is to decrease the side effect of skin photosensitivity. Other basic properties that characterize an ideal photosensitizing drug, besides the absorption wavelength, are: a high quantum yield of $^1O_2$ generation, selective accumulation in malignant tumor tissue; a fast clearance; and a low toxicity. Photosensitizing compounds can be classified into three major groups:

1. Hydrophobic PS(s), which bear no charged peripheral substituents. They have negligible solubility in water or alcohol;
2. Hydrophilic PS(s), which have three or more charged substituents and are easily soluble in water at physiological pH; and
3. Amphiphilic PS(s), which have two or less charged substituents and are soluble in alcohol or water at physiological pH (Boyle and Dolphin, 1996).

Table 1.1. Overview of photosensitizers which have reached clinical trials phase II-III. The absorption maximum in the red wavelength region, utilized for treatment, is also shown

Agent short name	Agent full name	Agent trade name	Company	AW, nm
HPD	Hematoporphyrin derivative	Photofrin	QLT Photo-Therapeutics Inc., Vancouver, Canada	630
NPe6	mono-L-aspartyl chlorin-e_6			664
BPD-MA	Benzoporphyrin derivative monoacid ring A	Verteporfin	QLT Photo-Therapeutics, Inc., Vancouver, Canada	690
m-THPC	Meso-tetrahydroxy-phenylchlorin	Foscan	Scotia Pharmaceutical, Great Britain	652
AlSPc	Sulphonated aluminium phthalocyanine	Photosens	NIOPIC, Moscow, Russia	675
	Lutetium texaphyrin	LUTRIN	Pharmacyclics, Sunnyvale, CA, USA	732

Note: AW **-** absorption wavelength

HP(s) were the first PS(s) studied more in detail. The second generation of PS(s) includes compounds from other chemical groups. Some of them have attained clinical trials phase II-III, and all these PS(s) have absorption maxima located further out in the red wavelength region as compared to the absorption peak at 630 nm utilized for HPD (see Table 1.1.). A brief description of several of these groups, with some examples of individual agents, is given as follows.

1.1.2.1. Porphyrins

Porphyrins, which come from the Greek for "purple", constitute a group of pigments with a cyclic tetrapyrrolic macrocycle. Porphyrins are formally derived from porphin (Figure 1.1.) by substitution of some or all of the peripheral positions with various side chains. The structure of the porphyrin main core is a relatively flat, tablet-like molecular structure with a width of about 7 Å (Smith, 1975). A cavity in the middle of this structure can include a metal ion. Partly due to this structure, the agents easily aggregate by piling on top of each other. One main challenge when synthesizing PS(s) has been to produce compounds which stay mainly in the monomeric form in solution.

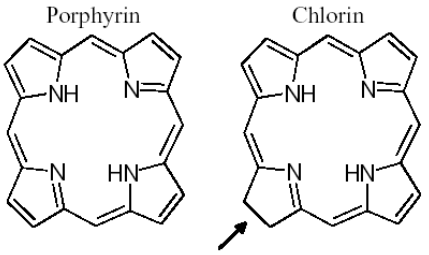


Figure 1.1. The main core of the porphyrin- and chlorin-type photosensitizers. As can be seen, the difference between the compounds is the lack of at least one double bond in one of the pyrrole rings in the chlorin (arrow)

Porphyrins are highly colored, their main absorption bands have very high extinction coefficients, and the intense "Soret" band, found around 400 nm is characteristic of the macrocyclic conjugation; rupture of the macrocycle (for example, to give biliverdin-type bile pigments) results in disappearance of this band (Smith, 1975). The chemical structures of some naturally occurring porphyrins and related compounds are shown in Figure 1.2.

PP is the immediate heme precursor in the biosynthesis of heme. Disordered metabolism of endogenous tetrapyrroles can lead to and/or is associated with diverse diseases, such as the porphyrias and some forms of cancer (Batlle, 1993). PP is, presently, one the most utilized PS(s) in PDT. Following the administration of its precursor ALA, PP is accumulated in malignant cells and upon light exposure can induce their irreversible injury (Shevchuk et al., 1996). HP is formally a hydration product of PP.

HPD was first described in 1960 by Lipson and Baldes and is obtained by the action of a mixture of sulphuric and acetic acids on HP (Lipson et al., 1961). The product of this treatment is mainly hematoporphyrin diacetate and this is treated with NaOH to obtain HPD. The neutralization treatment leads to hydrolysis to give HP, PP, hydroxy-ethyl-vinyl-deuteroporphyrin (HVD) and a complex mixture of their condensation products. Typically HPD consists of approximately 20% HP, 20-30% HVD, 3-5% PP. The other half, which is responsible for the antitumor effect of PDT in vivo, is a mixture of dimers, trimers and some higher molecular weight oligomers, up to eight porphyrin units (Dougherty, 1987). The porphyrin units are linked through ester and ether bonds, but a C-C linkage has also been suggested. PII, in comparison to HPD, is a porphyrin mixture enriched (by a gel filtration chromatography) in this oligomeric fraction to the extent of about 80-90%. The chemical structure of a major active component of HPD is given in Figure 1.2.

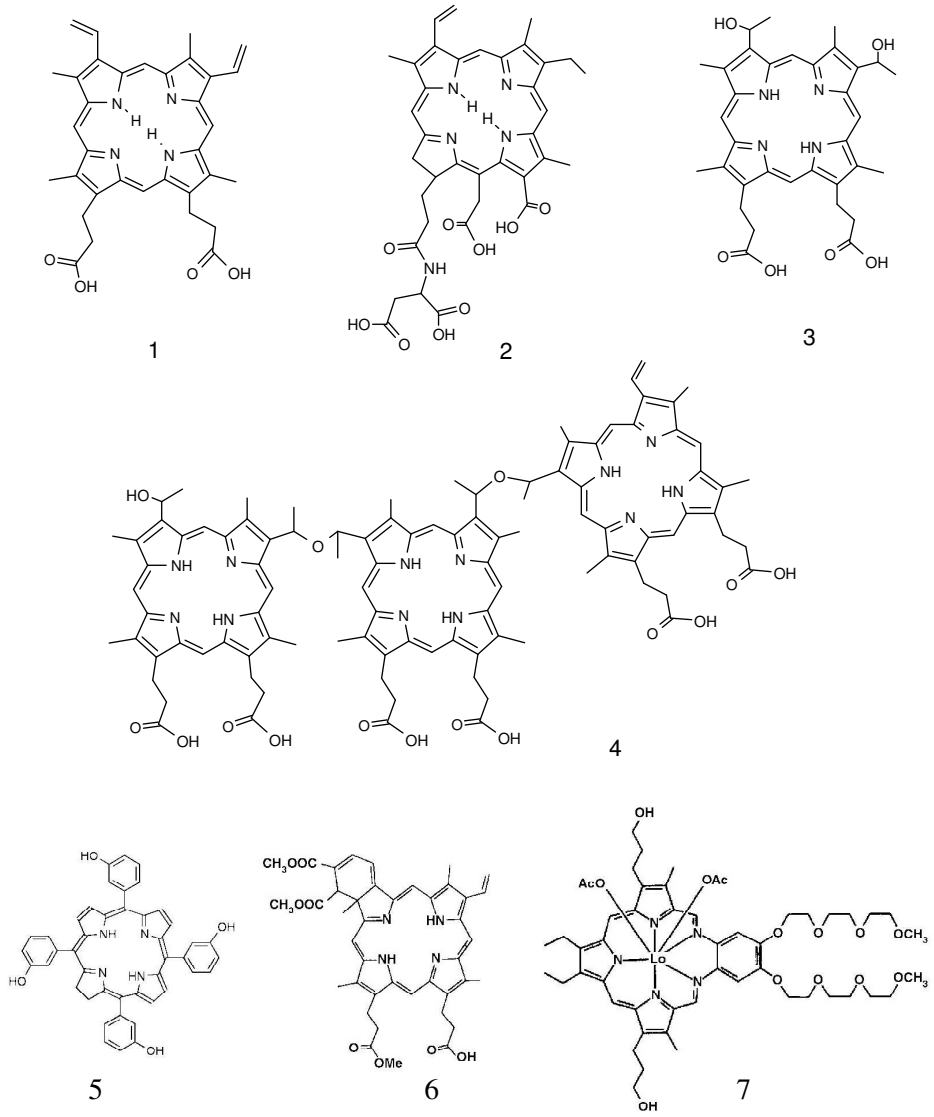


Figure 1.2. Chemical formulas of selected naturally occurring porphyrins and sensitizers used in photodynamic therapy of tumors: (1) protoporphyrin IX; (2) mono-L-aspartyl chlorin-e_6; (3) hematoporphyrin IX; (4) a porphyrin trimer is known to be a major active component of HPD; (5) meso-tetrahydroxyphenylchlorin; (6) benzoporphyrin derivative monoacid ring A; and (7) Lutetium texaphyrin

1.1.2.2. Chlorins and bacteriochlorins

Chlorins constitute a group of molecules very similar to porphyrins, as shown in Figure 1.1., illustrating the main core of a porphyrin and a chlorin. Chlorophylls belong to this group. In comparison to the porphyrin structure, the chlorin has at least one double bond missing in the pyrrole rings. In contrast to porphyrins,

chlorins have the strongest absorption peaks in the red part of the spectrum, which give the compounds a green colour. Therefore, some derivatives of chlorophyll-a, such as NPe6 and di-aspartyl chlorin-e_6, are proposed as alternative to HPD PS(s) for PDT of tumors. Clinical trials showed that NPe6-based PDT is very effective for the treatment of certain skin tumors and lung cancer (Dougherty et al., 1998; Taber et al., 1998). The chemical structure of NPe6 can be seen on Figure 1.2. BPD-MA is, despite the name, a chlorin, synthesized from PP. Besides the beneficial absorption wavelength at 690 nm, it has a rapid accumulation in the tumor, allowing light irradiation the same day as injection (Lui et al., 2004). It also has a relatively short half-life, with skin photosensitization lasting for less than a week. Chlorin-e_6 is a compound that has a strong absorption peak at 664 nm. Like BPD-MA, it possesses by a rapid fading of skin photosensitivity (Chan et al., 2005). M-THPC (see Figure 1.2.) is the most potent PS available for clinical use at present. It has a strong absorption peak in the red, at 652 nm. Drug and light doses utilized in clinical trials, are in the order of 10 times lower than for HPD (Ell et al., 1998). It was concluded that PDT with m-THPC is a safe and effective technique for the treatment of early carcinomas of the upper aerodigestive tract (Grosjean et al., 1996) and early cancers of the stomach (Ell et al., 1998).

Reduction of one of the pyrrole units on the porphyrin ring leads to a class of porphyrin derivatives called chlorins. Further reduction of chlorins gives another type of porphyrin derivative called bacteriochlorins, in which the reduced pyrrole units are diagonally opposite to each other. Now, some bacteriochlorins, (for example, 5,10,15,20-tetrakis(m-hydroxyphenyl)bacteriochlorin and bacteriochlorin-a) are proposed as the 2nd generation PS(s) for PDT of tumors and are on the stage 2 of clinical trials (Schuitmaker et al., 1990; van Duijnhoven et al., 2005). These PS(s) have an absorption band at a wavelength at which tissue penetration is optimal (around 760 nm). But, it is important to emphasise that on the whole bacteriochlorins are characterized, in comparison with porphyrins (e.g., PP and HP) by a decreased photostability. This drawback may potentially limit the antitumor efficiency of PDT with bacteriochlorins (Rovers et al., 2000).

1.1.2.3. Phthalocyanines and naphthalocyanines

Phthalocyanines and naphthalocyanines strongly absorb red light with maxima around 670 nm and 770 nm, respectively. When chelated with metal ions, such as, Zn and Al, they exhibit high triplet yields and longer triplet lifetimes and, as a consequence, high quantum yields of $^1O_2$ formation. The dyes can be sulphonated to various degrees to enhance the water solubility. The central macrocycle consists of a tetrapyrrole unit, but in contrast to porphyrin, the pyrrole sub-units are linked by nitrogen atoms rather than by methine bridges. Now, sulphonated Al phthalocyanine as the disulphonate and Zn phthalocyanines (in liposomes) are evaluated for use in PDT of tumors in some countries, including Estonia (Vakoulovskaia et al., 1999; Stranadko et al., 2001; Tuulik et al., 2002).

1.1.2.4. Texaphyrins

Texaphyrins (Figure 1.2.) are expanded, porphyrin-like macrocycles which complex large metal cations. The dyes have a strong absorbance peak in the near infrared, 730-770 nm. Due to the absorption profile, treatment light at these longer wavelengths can be used to obtain an increased penetration depth. These PS(s) can be used for thicker lesions and potentially also for pigmented tumors, such as melanomas, due to the decreased absorption of melanin in the near infrared. One compound that has reached clinical trials, is lutetium texaphyrin (Yuen A.R. et al., 1997).

Other PS(s) that should be mentioned are certain derivatives of pheophorbide-a and pyropheophorbide-a. At present, these green pigments are actively tested on various experimental models as possible PS(s) for PDT for tumors (Matroule et al., 1999; Hajri et al., 2002). They have a strong absorption of light ($\varepsilon \sim 50000$ M^{-1} cm^{-1} at 665 nm) and high quantum yields of $^1O_2$ production.

At the same time, clinical trials showed that all above mentioned 2nd generation PS(s) are still far from ideal. For example, BPD-MA is hardly soluble in water that makes difficulties for its clinical applications. Furthermore, like HPD, BPD-MA, NPe6 and AlSPc did not accumulate actively in tumor tissue; significant amounts of these compounds are taken up and retained in liver, spleen, kidney and skin. It has therefore been suggested that many of the problems associated with the use of HPD and other PS(s) might be circumvented by combination of a PS with carrier molecules that recognise the target tissue (Konan et al., 2002).

1.1.3. Uptake of photosensitizers by tumors

The retention of certain porphyrins by solid tumors, as compared to normal surrounding tissues, has been recognized for many years. Nevertheless, the mechanisms involved in the preferential accumulation of porphyrin-type PS(s) in tumors after their administration into an organism are not fully understood yet. Six hypotheses have been proposed to account for the selective concentration and/or retention of porphyrins in malignant tissue (Korbelik, 1992):

1) Cancer cells, in common with other rapidly proliferating cells, may have an increased requirement for cholesterol for membrane biosynthesis. They may therefore upregulate the expression of the low-density lipoprotein receptor, which recognises the apoB/E lipoproteins. It is known that lipoproteins are major carriers of lipophilic porphyrins (including HPD and PII) in the bloodstream and may therefore be a means of entry of these compounds into cells (Candide et al., 1986).

2) A decreased intratumoral pH value may affect the ionization of porphyrin species with weakly acidic p*K*a, thus retaining them within tumors. In other words, it was demonstrated that porphyrins (like HP, HPD and PII) in an environment with lowered pH can be protonated, leading to an increased lipophilicity and cellular uptake (Pottier and Kennedy, 1990).

3) Tumors often contain an increased amount of lipid bodies and particularly neutral lipid droplets. In addition, membranes of tumor cells may be more

hydrophobic than those of normal cells. Both phenomena might explain the preferable accumulation of hydrophobic PS(s).
4) The abnormal structure of tumor stroma characterized by a large interstitial space, a leaky vasculature, reduced lymphatic drainage, and a high amount of newly synthesized collagen (that binds porphyrins) also may favor a preferential uptake of PS(s).
5) Tumor cells may have increased capabilities for phagocytosis or pinocytosis of porphyrin aggregates.
6) Tumor-associated macrophages (TAM) could be largely responsible for the concentration of a PS (in particular HPD and PII) in tumors. It was established that TAM may contain up to 9 times the porphyrin levels present in tumor cells. Besides, many experimental tumors can comprise up to 80% TAM and even in human cancers TAM can make up to 20-50% of the cellular content.

1.1.4. Mechanisms in photodynamic therapy

There are three essential constituents in the PDT reaction: the PS molecule, light with appropriate wavelength and the presence of O_2. The interaction among these 3 constituents is the main part of the treatment effect. In the following, the mechanisms involved in the tumor ablating effect of PDT will be divided into direct cell damage and secondary effects, mostly for didactic purposes, since the mechanisms are dependent on each other and presumably interact in a complex way. The various effects may also be different for the various classes of PS(s). In the section, the mechanisms of PDT will be reviewed with a special impact on HPD. Most of the discussion will, however, also be applicable to other PS(s).

1.1.4.1. Primary molecular photo-processes involved in PDT

The most important messengers in the processes causing cell damage during PDT are oxygen species in various toxic states. Indeed, in vitro and in vivo studies showed that during de-oxygenation of the surroundings, photoinactivation of tumor cells by HPD is totally halted (Henderson and Fingar, 1987). The reactions involved in PDT are divided into two groups depending on the nature of the reaction. Upon absorption of a photon, the molecule of porphyrins or related PS(s) undergoes one or more energy transitions and usually emerges in its excited triplet state. The triplet can participate in a one-electron oxidation-reduction reaction with a neighbouring molecule, producing free radical intermediates that can react with O_2 to produce peroxy radicals and various ROS (Type I reactions). A Type II reaction is a process where, the triplet-state PS transfers energy to ground state oxygen, generating $^1O_2$, a highly reactive form of oxygen that reacts with many biological molecules, including unsaturated lipids, proteins, and nucleic acids (Thomas and Girotti, 1988; Ochsner, 1997; Wright et al., 2002). Most sensitizers for PDT are efficient producers of $^1O_2$ in simple chemical systems, and it is assumed that Type II photochemistry is the dominant mechanism for PDT in most circumstances in cells and tissues. Alternatively, there are some suggestions that H_2O_2, $O_2^{-\bullet}$ and $OH^\bullet$ could be involved in the tumor necrotizing effect of PDT with porphyrin-type PS(s) and phthalocyanines, e.g. (Das et al., 1985; Gilaberte et al., 1997;

Hadjur et al., 1997). It should be noted that in cells $^1O_2$ could convert to $O_2^{-\bullet}$ and H_2O_2 in the presence of reducing agents such as thiols (Buettner and Hall, 1987).

1.1.4.2. Targets and mechanisms of PDT-induced cell killing

1.1.4.2.1. Sub-cellular targets of PDT

The diffusion distance of $^1O_2$ in biological tissues has been estimated to be of the order of 0.01-0.02 μm. Because of the limited migration of $^1O_2$ from the site of its formation, sites of initial cell and tissue damage of PDT are closely related to the localization of the PS (Peng et al., 1996). Hence, PDT with a PS located in the nucleus will mainly damage DNA and nuclear proteins, while a PS located in the plasma membrane will destroy the cell target.

The uptake kinetics and localization of a PS depends on chemical and physical parameters such as lipophilicity, type and number of electrical charges, charge-to-mass ratio, type and number of ring and core substituents, and whether entry into the cell occurs by diffusion or endocytosis (Oleinick and Evans, 1998). Lipophilic anionic sensitizers (like HPD and PII) are concentrated in the mitochondria, endoplasmic reticulum, cytoplasmic and nuclear membranes, and perinuclear region of the cytoplasm of cells in vitro, much lower concentrations are seen in lysosomes and nuclei (Peng et al., 1996). Following light exposure, the sites of photodamage are observed mainly in the biomembranous system of cells. HPD-PDT induced damage of the plasma membrane can be observed within minutes after light exposure. This type of damage is manifested as swelling, bleb formation and shedding of vesicles (Volden et al., 1981), reduction of active transport (Penning et al., 1994), depolarization of the plasma membrane, increased permeability to chromate, inhibition of the activities of plasma membrane enzymes such as Na^+/K^+-adenosine triphosphatase (ATPase) and Mg^{2+}-ATPase (Gibson et al., 1988; Penning et al., 1994), a rise in Ca^{2+} (Penning et al., 1992), up- and down-regulation of surface antigens (Dougherty et al., 1998), lipid peroxidation (Thomas and Girotti, 1989b) and protein cross linking (Moan and Vistnes, 1986). In several in vitro studies a mechanism of the HPD-PDT mediated killing of cells was causally linked with inhibition of amino-acid transport and Na^+/K^+-ATPase, adenosine triphosphate (ATP) depletion and blocking of DNA excision repair (Penning et al., 1994). Since HPD and especially PII are preferably concentrated in mitochondria, it was suggested that killing of neoplastic cells (by necrosis) both in vitro and in vivo could be largely mediated by photodamage of these organelles leading to the shortage in ATP (Hilf et al., 1986). It has been shown that in cells the activities of several important mitochondrial enzymes are inactivated at a very early stage after HPD-PDT such as succinate dehydrogenase (Hilf et al., 1984), F_oF_1 ATPase (Perlin et al., 1985) and cytochrome c oxidase (Gibson and Hilf, 1983). In particular, by the ^{31}P-NMR technique has been demonstrated that the fall of ATP levels in HPD-PDT-treated mammary carcinomas is a direct and initial response of tumor tissue to PDT and is most likely caused by inhibition of mitochondrial function (Hilf et al., 1987).

Aggregated as well as hydrophilic PS(s) localize preferentially inside lysosomes and disruption of these organelles leads to the release of hydrolytic enzymes and necrotic cell death (Peng et al., 1996).
Dyes that are present in the cytosol can sensitize tubulin to photodamage. This leads to accumulation of cells in mitosis, in some cases followed by cell death (Berg et al., 1992).
The probability of cell inactivation per quantum of absorbed light is widely different among PDT sensitizers (Berg et al., 1992). Generally, this probability is lower for hydrophilic than for lipophilic sensitizers, indicating that membrane structures are notably vulnerable. It was also established that PS(s) localizing in mitochondria are more efficient in photokilling of cells.

1.1.4.2.2. PDT can induce apoptosis

PDT can kill tumor cells not only by necrosis (as stated above), but also via apoptosis, both in vitro and in vivo (Oleinick et al., 2002). The mechanism of normal removal of cells from tissue during growth, morphogenesis and differentiation was termed apoptosis or programmed cell death in 1972 by Kerr et al. (1972). Apoptosis is a biochemical process for elimination of individual cells, while programmed cell death refers to the genetically determined elimination of cells at prescribed times and in response to prescribed signals.
Apoptotic cells are morphologically shrunken, retracted from the neighbouring cells, have a characteristic condensation of nuclear chromatin, and breaks into membrane delineated fragments called apoptotic bodies. In tissue apoptotic bodies are phagocytosed by macrophages. Apoptosis ensures that intracellular components are not released to induce an inflammatory response. Necrosis, on the other hand, is characterized by cell swelling, blebbing of the plasma membrane, loss of plasma membrane integrity and induction of inflammation in tissue. The mode of cell death (via necrosis or apoptosis) induced by PDT depends on cell line, site of initial photodamage, treatment dose and cell density (He et al., 1994; Dahle et al., 2000; Oleinick et al., 2002).
PDT induced apoptosis was first demonstrated by Agarwal et al. (1991) in mouse lymphoma cells loaded with Al phthalocyanine. PDT triggers the activation of several signal transduction pathways in cells, which can both be involved in protection against cell death and in induction of cell death.
The molecular mechanisms of PDT-induced apoptosis are not fully understood. Briefly, the most common pathway to PDT induced apoptosis goes via damage to mitochondria. This results in a so-called permeability transition and disruption of the outer mitochondrial membrane (Oleinick et al., 2002). Cytochrome c and Apoptosis Inducing Factor enter the cytosol from the intermembrane space of the mitochondria and eventually lead to activation of caspase 3 (Granville et al., 1998). However, apoptosis can also be initiated by damage to the plasma membrane by activating phospholipase C and phospholipase A_2, which generate inositol-3-phosphate and arachidonic acid, respectively that can induce apoptosis (Agarwal et al., 1993). Cleavage of caspase 3 starts the caspase cascade culminating in activation of DNA fragmentation factor, which activates endonucleases that are responsible for DNA fragmentation, activation of Acinus

that is involved in chromatin condensation and cleavage of poly-ADP-ribose-polymerase (Oleinick et al., 2002).
The BcL-2 family of proteins exercises the most proximal and important controls over apoptosis. Some, like BcL-2 and BcL-xL, are anti-apoptotic, while others, like Bax and Bad, promote apoptosis. A role for BcL-2 in suppressing PDT-induced apoptosis was first shown by He et al. (1996). BcL-2 may act in several ways to inhibit apoptosis, including binding of the apoptosis inducing factors Bax and Bap31 (Granville et al., 1998), inhibition of lipid peroxidation, and stabilization of the permeability transition pore. Protein kinases are involved in the phosphorylation and inactivation of both Bax and BcL-2. For instance, PDT can activate phosphatidylionsitide-3'OH kinase that phosphorylates the tyrosine kinase Etk/Bmx. Etk/Bmx protects against apoptosis, probably by phosphorylation of Bad (Xue et al., 1999).
PDT-induced DNA damage leads to p53 mediated transcription of p21 and arrest of the cell cycle followed by apoptosis of the arrested cells (Oleinick et al., 2002). Similarly, PDT dephosphorylates pRB that also causes cell cycle arrest followed by apoptosis (Ahmad et al., 1999).
Nitric oxide (NO) can induce apoptosis by triggering mitochondrial permeability transition. In this relation, PDT has been shown to upregulate the constitutive NO synthase followed by increased NO production and apoptosis (Gupta et al., 1998).
Furthermore, mitogen activated protein kinases (MAPK-proteins) can be activated by PDT (Oleinick et al., 2002). MAPK-proteins are important intermediate molecules in signalling pathways that turn extracellular stimuli into intracellular responses by activation of other protein kinases and transcription factors. The MAPK-proteins p38/HOG and JNK1/SAPK have, in different cell lines, been shown both to be involved in promotion and protection against apoptosis (Oleinick et al., 2002).

1.1.4.2.3. The bystander effect in PDT

Until recently, it is generally believed that PDT-induced damage is limited to the cell in which the primary photooxidative injury takes place. However, in vitro experiments performed by Moan and co-workers (Dahle et al., 2000) indicated that photosensitized damage caused by PII or ALA-derived PP may be transmitted to cells not directly affected by the treatment; i.e. an inactivated cell can kill adjacent cells in colony. These observations led to suggestion that the antitumor effect of PDT could include a bystander killing of cells. It was reported that the degree of bystander effect is higher for cells dying by necrosis than for cell dying by apoptosis (Dahle et al., 2001). The intercellular signals and cytotoxic substances that could mediate the bystander effect in PDT have not been identified. Nevertheless, it was suggested that the most likely mechanism for the bystander effect is transfer of toxic substances via gap junction channels (Dahle et al., 2000). These authors supposed that mediators of the bystander effect might be cytotoxic aldehydes produced by PDT-induced lipid peroxidation or NO that readily passes through cell membranes.

1.1.4.3. Mechanisms of indirect killing of tumor cells by PDT

1.1.4.3.1. Killing of tumor cells due to photodamage of microvasculature

When originally developed, PDT of cancer was thought to be effective due to the selective localization of HPD in tumor cells and direct killing of these cells by $^1O_2$ produced from light-activated HPD. However, to date it was established that in most cases the PDT-mediated tumor damage is due primarily to vascular damage inducing hypoxia/anoxia of tumor cells and tumor necrosis (Peng et al., 1996). The mechanisms underlying the vascular effects of PDT differ greatly with different PS(s) (Peng et al., 1996; Dougherty et al., 1998). HPD/PII-based PDT leads shortly after irradiation to vessel constriction, macro-molecular vessel leakage, leukocyte adhesion and thrombus formation, all apparently linked to platelet activation and release of thromboxane. PDT with certain phthalocyanine derivates causes primarily vascular leakage and PDT with NPe6 results in blood flow stasis primarily because of platelet aggregation. All of these effects may include components related to damage of the vascular endothelium. PDT may also lead to vessel constriction via inhibition of the production or release of NO by the endothelium (Gilissen et al., 1993). Studies showed that the microvascular PDT responses can be partially or completely inhibited by the administration of agents that affect eicosanoid generation, such as indomethacin, various other thromboxane inhibitors, and aspirin, and this inhibition can markedly diminish the tumor response (Dougherty et al., 1998).

1.1.4.3.2. Immunological effects of PDT

The curative properties of PDT arise from the death of cancer cells spared from the direct cytotoxic effect by a combination of oxidative stress-initiated secondary tumoricidal activities. Contrary to the contemporary prevailing conception, these secondary effects are by no means limited to the ischemic death caused by the occlusion of tumor vasculature. Other events that are increasingly coming into focus are as follows: 1) antitumor activity of inflammatory cells and 2) tumor-sensitized immune reaction. They all can be elicited by phototoxic damage that is not necessarily lethal and bears an inflammatory impact (Korbelik, 1996).

Studies showed that photooxidative lesions of membranous lipids underlie the PDT-elicited inflammatory cellular damage, induced by the extensive release of lipid degradation fragments and metabolites of arachidonic acid (Korbelik, 1996). These products, together with mediators such as histamine and serotonin released from PDT damaged vasculature, are potent initiators of inflammation, which is a dominating event in the antitumor effect of PDT. The major hallmark of the PDT-induced local acute inflammatory reaction is a massive invasion of myeloid inflammatory cells into the treated tumor. This is a regulated process, characterized by a sequential arrival of neutrophils, mast cells and monocytes/macrophages. These cells were found to be highly activated and engaged in tumoricidal activity (Gollnick et al., 1997). So, increasing the numbers of circulating neutrophils in animals (by a treatment with granulocyte colony-stimulating factor) was found to improve the response of tumors to PDT

(de Vree et al., 1996). Also, selective enhancement of macrophage activity in tumor-bearing mice by treatment with the macrophage-activating factor DBPMAF was shown to markedly enhance the curative effect of PDT (Korbelik et al., 1997).

There have been substantial advances in the understanding of the PDT-induced tumor-specific immune reaction. This effect may not be relevant to the initial tumor ablation, but may be decisive in attaining long-term tumor control. Tumor sensitized lymphocytes can, under reduced tumor burden, eliminate small foci of viable cancer cells that have escaped other PDT mediated antitumor effects. Cancer immunity elicited by PDT has the attributes of an inflammation primed immune development process (Korbelik, 1996). The initial critical step of tumor-specific immune development is likely mediated by tumor-associated macrophages and/or dendritic cells serving as antigen presenting cells (Korbelik, 1996). These cells are prompted to phagocytize large numbers of cancer cells killed or damaged by the antitumor effects of PDT. Directed by powerful inflammation-associated signaling, the antigen presenting cells will process tumor-specific peptides and present them on their membranes in the context of major histocompatibility class II molecules. Presentation of tumor peptides, accompanied by intense accessory signals, creates conditions for the recognition of tumor antigens by helper T lymphocytes. These lymphocytes become activated and in turn sensitize cytotoxic T cells to tumor specific epitopes. The generation of CD4 and CD8 T cell clones that recognize tumor cells as their targets is followed by their rapid expansion and activation leading to fully developed tumor immunity. There are indications that B lymphocytes and natural killer cells also become activated and may contribute to PDT-elicited immune responses, but the role of these cells remains to be fully elucidated. The activity of tumor sensitized lymphocytes is not limited to the original PDT-treated site but can include disseminated and metastatic lesions of the same cancer (Korbelik, 1996). Thus, although the PDT treatment is localized to the tumor site, its effect can have systemic attributes due to the induction of an immune reaction.

The demonstration that lymphoid populations are essential for preventing the recurrence of PDT-treated tumors was provided by using a mouse sarcoma model growing in either immunocompetent or immunodeficient syngeneic hosts (Korbelik et al., 1996). PII-based PDT, which was fully curative for EMT6 tumors growing in immunocompetent BALB/c mice, resulted in initial ablation but not permanent cures with EMT6 tumors growing in severe combined immune deficiency (scid) or nude mice. The induction of immunity against a weekly immunogenic murine fibrosarcoma MS-2 by PDT with Al-phthalocyanine was also described (Canti et al., 1994). In this case, the mice that remained tumor free 100 days after PDT were shown to resist a rechallenge with the same tumor.

1.1.5. Photobleaching and role of photoproducts

Most photosensitizing drugs such as porphyrins, chlorins and phthalocyanines, which are used in the PDT of tumors, are not photostable. It was found that in

simple solutions as well as in tumor cells (both in vitro and in vivo) PS(s) undergo light-induced modification resulting in a decrease of their initial absorption and fluorescence intensity. This phenomenon is called photobleaching and includes the following photoprocesses:

1. Photodegradation – the conversion of a PS into products that do not absorb visible light appreciably (Moan and Kessel, 1988);
2. Photorelocalization – the light-induced migration of a PS in target cells from one binding site to another (Moan and Kessel, 1988);
3. Phototransformation – as in case of some porphyrins, is leading to the formation of new red-absorbing photoproducts (Rotomskis et al., 1996; Chekulayev et al., 1998).

The degradation of sensitizers during PDT presents both potential problems and potential advantages. If these compounds are bleached too rapidly during photoirradiation, the tumor may not be destroyed completely (Merlin et al., 1992). Therefore, the photodegradation of HPD has an effect on the PDT clinical dosimetry (Rotomskis et al., 1997). On the other hand, it has been suggested (Boyle and Potter, 1987) that photobleaching could be used as a means to eliminate skin photosensitivity, the main side effect observed in patients undergoing PDT with HPD. Studies showed that the mechanism of HPD photodecay in tumor cells is complex and different from that in water solutions; in the cells this sensitizer is photobleached much faster than in simple solutions (Chekulayev et al., 1998; Chekulayeva et al., 2002). It was also reported that the therapeutic inactivation of molecules of porphyrin PS(s) and their transformation to photoproducts is oxygen dependent and results from the interaction of the PS with activated oxygen species, mainly $^1O_2$ (Moan and Kessel, 1988; König et al., 1990; Chekulayeva et al., 2002). Also, there are indications that the photoproducts are degraded in the same way (Moan and Kessel, 1988; König et al., 1990). When utilizing HPD, a photoproduct absorbing about 640 nm has been observed both in water solutions and in tumor cells (Rotomskis et al., 1996; Chekulayev et al., 1998), while during PDT with ALA-derived PP, an evident photoproduct having an excitation peak at 670 nm and emitting fluorescence nearly the same wavelength has been reported (Ma et al., 2001). Studies on HP-like PS(s) and PP suggest that red absorbing photoproducts are of a chlorin type (König et al., 1993; Rotomskis et al., 1996; Chekulayev et al., 1997; Ma et al., 2001). To that extent these photoproducts can be photoactivate and contribute to the treatment effect is still not clear. In the case of ALA-induced PP sensitization, much interest has been paid to the photoproducts due to the fact that many groups are using broad-banded light sources for PDT of skin cancers. In the case of HPD and PII, there are some indications that arising red-absorbing photoproducts could play a role in the PDT induced eradication of malignancies (Giniunas et al., 1991). Moreover, for HP-like PS(s) it was reported (Rotomskis et al., 1997) that a correction related to the formation of the active photoproduct should be included in the calculations of the appropriate photodynamic dose for PDT when illumination is carried out at wavelengths covered by the absorption region of the photoproduct.

2. OBJECTIVES OF THE STUDY

The main goal of the presented study was to enrich the existing knowledge on the molecular mechanisms of tumor cells death upon photoactivation of HPD, the most widely used sensitizer in PDT of malignancies, or chlorin-e_6 trimethyl ester (E6, a second generation PS). In particular, the work was aimed on elucidation of:

1) the contribution of lipid peroxidation and protein degradation in the photoinactivation of tumor cells by HPD;
2) the role of ROS, other than $^1O_2$, in the antitumor effect of HPD- and E6-based PDT;
3) the significance of some antioxidant systems in the resistance of neoplastic cells to PDT with HPD;
4) the mechanism of the potentiating influence of heating, associated with photoirradiation, on the antitumor efficiency of HPD-PDT; and
5) the influence of a pH value in the sensitivity of tumor cells to the phototoxic action of E6.

This information is needed for further development of more effective PDT protocols. It is important to emphasize that researches in the field of phototoxic influence of tetrapyrrolic compounds towards tumor cells are very topical for our Republic as long as since 2000 PDT is applied for the treatment of malignancies in Estonian Cancer Centre, Tallinn (Tuulik et al., 2002).

3. MATERIALS AND METHODS

3.1. Chemicals

HPD was prepared from HP by the original method of Lipson et al. (1961) as modified by Kessel et al. (1987). E6 was synthesised on the basis of pheophytin-a extracted from nettle leaves according to the procedure of Lötjönen and Hynninen (1980). All other chemicals (of analytical grade or better) were usually purchased from Sigma, St. Louis, USA.

3.2. Animals and cells

Three-month-old mongrel female mice obtained from the National Institute for Health Development (Tallinn, Estonia) were used in all the experiments. The animal experiments were conducted adhering to the Institutional Ethics committee guidelines. Ehrlich ascites carcinoma (EAC) cells obtained from the National Institute of Chemical Physics and Biophysics (Tallinn, Estonia) were maintained by intraperitoneal transplantation of 0.2 ml ascites fluid (~ 2.5×10^7 cells) from mouse to mouse every seven days.

3.3. Light source

In all the experiments, a voltage-regulated 1 kW xenon arc lamp equipped with a focusing optical system and corresponding glass filters to deliver the light at 630 nm (for experiments with HPD) or at 665 nm (for E6) 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. The same irradiation apparatus was utilized for in vivo experiments on mice with subcutaneously transplanted EAC. The intensity of the emitted light at 630 nm and 665 nm was measured by an IMO-2N radiometer (Russian Federation).

3.4. Preparation of cell suspensions and assessment of cellular injury

Briefly, for in vitro experiments, six-to seven-day-old EAC cells were withdrawn from abdominal cavities of the mice, washed and loaded with HPD or E6 in a serum medium. Further, an 8 ml sample of the cell suspension (at a density of 0.5 - 4 x 10^7 cells/mL) was transferred into a 2 x 2 cm thermostatted (by circulating water) quartz cuvette and irradiated under magnetic stirring.
Cytotoxicity was determined immediately after photodynamic treatment of the cells by trypan blue (TB) exclusion assay or by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. More detailed information about the preparation of cell suspensions and assessment of cellular injury is given in the publications included in the thesis.

3.5. Analytical procedures

In this work, the generation of ROS by PDT-treated cells, the activity of cell-bound enzymes, the intracellular content of ATP and other biomolecules were determined by established methods, which were described in detail in the corresponding papers included in the thesis. For unpublished data the information concerning the used analytical procedures is given on figure legends.

3.6. Statistics

Results were analyzed statistically by the Student's t-test. Values of P less than 0.05 were considered statistically significant. Data in the text, tables, and figures are presented as mean ± standard error (SE) of at least 3 separate experiments.

4. RESULTS AND DISCUSSION

4.1. On the mechanism of HPD-PDT induced killing of tumor cells

Studies from several laboratories suggest that lipids in the membranes are the most critical targets upon photoexcitation of HPD. Namely, it was demonstrated that photosensitization of tumor cells by HPD may cause the peroxidative degradation of unsaturated fatty acyl groups and cholesterol (a process commonly referred to as lipid peroxidation) that correlated with alterations in membrane permeability, loss of its fluidity, and finally with cell death (Thomas and Girotti, 1989b; Buettner et al., 1993; Kelley et al., 1997). However, in these published works it has not been proven that such photodamage of lipids is the main reason for cell inactivation, since the effects of PDT with HPD on the intactness of cellular proteins were not examined during the in vitro experiments. Furthermore, an analysis of the literature data showed that in most cases these investigations on HPD-sensitized photooxidation of membrane lipids were performed on leukaemia cells. At the same time, it is well known that the

polyunsaturated fatty acids (PUFA) content of different cell lines can vary greatly. Therefore, it is of great interest to examine the role of lipid peroxidation in HPD-PDT induced cytotoxicity using neoplastic cells of another histological type.

Although $^1O_2$ is generally accepted to attack unsaturated lipids, another oxygenation target could be a protein molecule. There are some indications that cellular proteins are very sensitive to the photodynamic action of HPD. It was reported that photosensitization of tumor cells by HPD may cross-link membranous proteins (Moan and Vistnes, 1986), decrease the activity of various enzymes (Hilf et al., 1986; Gibson et al., 1989; Atlante et al., 1989; Prinsze et al., 1991) and affect the Na^+/K^+-ATPase pump (Penning et al., 1994). Moreover, it was found that in tumor cells a significant fraction of the photodrug is in close contact with proteins (Moan and Vistnes, 1986). Hence, in cell membranes the $^1O_2$ molecule has a much higher probability of reacting with proteins than with surrounding lipids. Hence, it might be expected that at HPD-PDT the photooxidative degradation of proteins, but not lipids, plays a key role in the initiation of cell death.

In this work we, therefore, estimated the importance of damage to lipids compared to proteins in deterioration of the plasma membrane integrity, the mitochondrial function, and cell killing by PDT with HPD.

4.1.1. Peculiarities of the HPD-PDT induced damage of tumor cells

It was found that photosensitization of EAC cells by HPD led to significant alterations in the shape and dimensions of the cells. The following events took place: a two- to three-fold increase in the cell volume, the appearance of numerous small protrusions on the cell surface (also known as “blebs”), the blending of microblebs into large protrusions of cytoplasm. Some of these effects are shown in Figure 4.1.

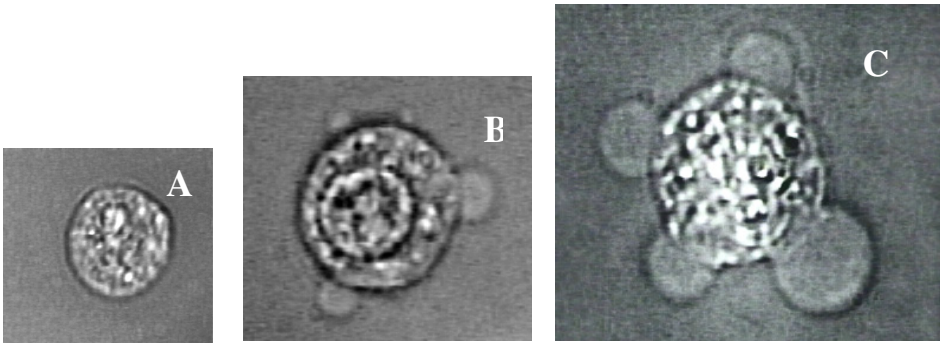


Figure 4.1. Phase-contrast microphotographs of EAC cells before (A) and 45 min after (B and C) their photoirradiation with 5 μg/mL HPD

In addition to morphologic changes, the cells were tested for membrane permeability that can be detected by TB dye uptake (cells whose membranes are intact, exclude TB; when the membrane is damaged, TB can enter the cell). As

shown in *paper I* (Figure 1), the uptake of TB by EAC cells pre-incubated with HPD increased slowly within 20 min of photoirradiation. Longer illumination of the cells induced a burst in the uptake of TB. At a later stage (after a 45 min irradiation) the appearance of ruptures on the outer membrane was also registered.

We found that irradiation of EAC cells pre-incubated with HPD resulted in a serious damage of their mitochondria; namely, after a 40 min illumination a considerable (~ 90%) decrease in the rate of O_2 consumption by the cells was observed (*paper I*, Figure 1). Besides, the HPD-based PDT caused a substantial inhibition of the glycolytic activity of EAC cells. These events were associated with a dramatic fall in the intracellular content of ATP (*paper I,* Figure 1).

Using the MTT-assay we also estimated the viability of EAC cells subjected to HPD-based PDT in vitro. It was found that irradiation of HPD-loaded EAC cells for 20 min led to a significant (~ 98%) decrease in the number of surviving cells (*paper I*, Figure 2). However, neither HPD nor light alone in the doses used had any effect on the viability of the cells.

Thus, our experiments clearly indicated that photosensitization of EAC cells by HPD causes a strong inhibition of their glycolytic and respiratory activity, and substantially decreases the ATP content. Furthermore, earlier we have shown (Chekulayev et al., 2000) that under the same experimental conditions the photoirradiation of EAC cells with HPD led to a very rapid and drastic decrease in the activity of succinate dehydrogenase, a marker mitochondrial enzyme. Other effects of HPD-PDT, such as an increased permeability of the plasma membrane to TB and the alterations in cell morphology (swelling, appearance of large protrusions on the cell surface) were observed at much larger light doses and could be partly attributed to the depletion of ATP (Gabai et al., 1992). The obtained results indicate a close relationship between HPD-PDT induced disturbances of the energy metabolism of irradiated cells and their ability to survive; for example, 20 min of light exposure caused an 80% decrease in the ATP level and inactivation of approximately 98% of the cells. These findings suggest that after PDT with HPD the carcinoma cells died mainly via the injury of their mitochondria as well as some glycolytic enzymes. Our results are in good agreement with the data of other researchers (Hilf et al., 1986; Atlante et al., 1989; Penning et al., 1994), who consider that inhibition of the mitochondrial function may be a crucial event in the cytotoxicity resulting from HPD-induced photosensitization in vitro. Moreover, in our prior studies we demonstrated that the efficiency of HPD-PDT can be improved by its combination with drugs which inhibit the energy metabolism in tumor cells (Chekulayev et al., 1996).

4.1.2. The role of peroxidation of lipids and degradation of proteins in dying of tumor cells by HPD-PDT

To elucidate the role of damage to membrane lipids in PDT-induced cytotoxicity, the effects of light exposure with HPD on the oxidation of PUFA and cholesterol in EAC cells were examined. Experiments showed that photosensitization of the cells by HPD resulted in an enhancement of lipid peroxidation expressed as a gradual increase in the concentration of conjugated

dienes and malondialdehyde. Nevertheless, only very small amounts of these products of lipid peroxidation were found even after a prolonged (50 min) illumination of the tumor cells (*paper I*, Figure 3). This was surprising as the photodynamic treatment of EAC with HPD caused a strong decrease in the intracellular content of reduced glutathione (GSH) (*paper I,* Figure 4) that, as known, jointly with some GSH-dependent enzymes plays an important role in the detoxification of the formed lipoperoxides. A possible explanation for the phenomenon might be that the membranous phospholipids of EAC cells are characterized by a higher concentration of saturated fatty acids. However, in special experiments we established that the cells contain a sufficient amount of the photooxidizable PUFA.

One of the mechanisms by which tumor cells might be damaged during the HPD-based PDT may include direct oxidation of their proteins by $^1O_2$. Therefore, we evaluated the intactness of proteins in EAC cells after their irradiation with the PS. Studies showed that photosensitization of the cells by HPD resulted in a substantial modification of the amino acid composition of their proteins; namely, the part of photooxidized amino acid residues after 50 min illumination was determined as 8% for tryptophan (Trp), 39% for Histidine (His), and 28% for protein-bound sulfhydryl groups (PrSH) (*paper I,* Figure 4).

In the present study, we found that photosensitization of EAC cells by HPD resulted in an enhancement of lipid peroxidation and caused a serious damage to cellular proteins. These molecular changes were associated with disturbances in the cell morphology, an increased permeability of the outer membrane to TB, an inhibition of the mitochondrial function, and cell photokilling. What is the significance of HPD-sensitized photooxidation of cellular lipids in the phototoxic effects of the photodrug? It is well documented that the oxidative degradation of PUFA and cholesterol is detrimental to membrane structures and functions. Indeed, when the lipids are peroxidized, the membranes, which consist of lipids and proteins, undergo a physical change and are finally destroyed. Moreover, it was also established that in the presence of certain transition metal ions the break-down of lipid hydroperoxides via a radical chain reaction produces ROS, various aldehydes, and other toxic products that can modify proteins and DNA and lead to cell death (Paillous and Forgues-Ferry, 1994; Girotti, 2001). It was suggested that lipid peroxidation may be causally related to cytolethality in HPD-photosensitized cells. Namely, Thomas and Girotti (1989b) reported that photodynamic treatment of murine leukaemia cells by HPD resulted in the formation of large amounts of lipid hydroperoxides. These authors concluded that lipid peroxidation plays an important role in tumor cell eradication. Buettner et al. (1993) using leukaemia cells of the same line (L1210) showed that PDT with PII produces membrane-derived lipid free radicals and that increasing the polyunsaturation of cellular lipids enhances radical production as well as the resulting phototoxicity. By contrast, our experiments on EAC cells indicated that there is a very low probability that the HPD-PDT induced impairment of the mitochondrial function and, as a consequence, cell death was mediated by the peroxidation of membrane lipids.

In fact, after 20 min of light exposure, which caused a substantial (> 2-fold) decrease in the respiratory activity of the cells as well as a 98% decrease in cell survival, only traces of oxidation products of unsaturated fatty acids were detected (*paper I*, Figures 1, 2, and 3, respectively). Furthermore, the photodynamic treatment of EAC with HPD (for 20 min) had no effect on the intracellular content of cholesterol (*paper I*, Figure 4). Nevertheless, we cannot exclude entirely the importance of lipid peroxidation in the PDT-induced deterioration of the plasma membrane integrity in EAC cells.

At the same time, we found that in EAC cells proteins are much more sensitive to the damaging influence of HPD-PDT than their lipid constituents. Namely, in the cells the initial rates of HPD-photosensitized oxidation of PrSH, Trp, and His residues were more than 10 times higher than those for unsaturated lipids (*paper I,* Table). What is the mechanism of the phenomenon? In cell membranes, lipids and proteins provide a competing environment for the formed $^1O_2$. However proteins, as known, are more reactive toward $^1O_2$ than unsaturated lipids. As shown in Table (*paper I*), the typical rate constants reported for unsaturated lipids (PUFA and cholesterol) reacting with $^1O_2$ in organic solvents correspond to the order of 10^5 M^{-1} s^{-1}. In contrast, rate constants for $^1O_2$ reacting with photooxidizable amino acids and proteins are considerably greater, 10^7-10^8 M^{-1} s^{-1}. Moreover, Kanofsky (1991) has shown that only ~ 7% of the $^1O_2$ produced in membranes is quenched by cellular lipids. This is in agreement with our data, which indicate that lipids may not be the primary target for HPD-PDT-induced cytotoxicity in vitro. Taken together, our results suggest that at HPD-PDT the direct photodamage of cellular proteins, but not the peroxidation of membrane lipids, is responsible for cell death.

By the experimental protocol utilized in the work, the mechanism of some cytotoxic effects of HPD-PDT was uncovered to some degree. We found that photodynamic treatment of EAC cells by HPD resulted in the appearance of numerous plasma membrane protrusions (blebs). Similar changes in cell morphology after HPD-induced photosensitization were also registered in other laboratories (Volden et al., 1981). However, the mechanism of blebbing and its significance in cell death remain unclear as yet, especially in the case of PDT-induced cytotoxicity. Literature data suggest that under HPD-PDT the appearance of large blebs on the surface of tumor cells might be associated with cytoskeletal abnormalities, in particular, with detachment of cortical (actin-containing) microfilaments from plasma membrane. Indeed, Fingar and Wieman (1992) revealed that photoirradiation of HPD-loaded endothelial cells of blood vessels results in disruption of their microfilament organization. Our findings, in turn, suggest that a decrease in the intracellular content of ATP and the oxidation of PrSH might be responsible for the cytoskeletal disturbances produced by HPD-PDT. In fact, the formation of blebs was observed in the energy depleted EL-4 thymoma ascites tumor cells after their treatment with rotenone (an inhibitor of respiration) (Gabai et al., 1992). On the other hand, the same morphological changes were found in cells exposed to toxic levels of menadione (2-methyl-1,4-naphthoquinone) (Mirabelli et al., 1988). These researchers

showed that the oxidation of thiol groups in cytoskeletal proteins (particularly, actin) is mainly responsible for the menadione-induced cell surface abnormalities. At the same time, we found that during HPD-PDT the number EAC cells with blebs was inversely related with their PrSH content (*paper I*, Figure 5).

The ability of photoexcited porphyrins to damage proteins is well documented. In tumor cells, such photodamage to proteins, besides the peroxidation of unsaturated lipids, could lead to a severe injury of the cytoplasmic membrane. Our experiments supported this assumption; in EAC cells the HPD-PDT-induced violation of the plasma membrane integrity was directly related with a decrease in the Trp, His, and PrSH content (*paper 1*, Figure 1 and 4, respectively). This observation is in agreement with the suggestion of Dubbelman et al. (1978) that the main photodynamic effect of porphyrins on red cell membranes is caused by the photooxidation of amino acid residues of proteins, mainly His. In the study, we also found that photodynamic treatment of EAC cells with HPD caused a strong reduction in the intracellular level of ATP, which preceded the disintegration of the outer membrane in the cells. Because in mammalian cells practically all vital functions are dependent on the presence of ATP, we believe that upon HPD-PDT the shortage of energy could contribute to the TB-detectable membrane leakage.

Our findings suggest that after PDT with HPD the EAC cells died mainly via the inactivation of their energy producing systems; a powerful inhibition of the mitochondrial function as well as of the glycolytic activity of the cells was registered (*paper I,* Figures 1 and 2). The destructive impact of HPD-PDT was associated with oxidation of the PrSH and could be consequently ascribed to the inactivation of some enzymes containing in their active sites SH groups. Indeed, it was reported (Atlante et al., 1989) that oxidation of SH groups in the ADP/ATP translocator is the main cause of oxidative phosphorylation impairment when isolated mitochondria are exposed to HPD + light. Moreover, the strong inhibition of glucose consumption by EAC cells (*paper 1*, Figure 1) may be also explained via the HPD-photosensitized oxidation of PrSH since certain glycolytic enzymes contain, as known, in their active sites thiol groups. In fact, a significant decrease in the activity of glyceraldehyde-3-phosphate dehydrogenase after photosensitization of tumor cells with HPD was registered before by others (Prinsze et al., 1991).

Thus, our studies strongly suggest that proteins, but not lipids, are the primary target of PDT with HPD in tumor cells.

4.2. The role of different ROS in the antitumor effect of HPD-PDT

It was reported (Weishaupt et al., 1976) that $^1O_2$ is the main damaging agent in PDT of tumors. However, some in vitro (Hariharan et al., 1980; Das et al., 1985) and in vivo studies (Athar et al., 1988; Athar et al., 1989) suggest the production of H_2O_2, $O_2^{-\bullet}$, and $OH^\bullet$ radicals in cells and tissues exposed to visible light in the presence of HPD. These oxidizers, especially $OH^\bullet$, can react with most biological molecules, including unsaturated lipids, DNA, and proteins, and cause cell death. However, it has not been proven unequivocally that these ROS are

involved in the phototoxic action of HPD against tumor cells in these published works. For instance Hariharan et al. (1980), 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. It was reported (Davies, 2003) that upon exposure to $^1O_2$ cell proteins can oxidize with the formation of PPO that currently are regarded as a new of ROS; these peroxides can react with metal ion complexes to produce damaging radicals (Headlam and Davies, 2003). As $^1O_2$ involved in the phototoxic action of HPD against tumor cells, we supposed that formation of PPO could occur in tumor tissues subjected to the PDT with the PS.

Thus, the aim of this study was to elucidate the significance of H_2O_2, oxygen radicals and PPO in the phototoxic action of HPD against tumor cells.

4.2.1. Effects of D_2O and traps of different ROS on phototoxicity of HPD

To clarify the significance of ROS, other than $^1O_2$, in the phototoxic action of HPD against tumor cells, the effects of heavy water (D_2O) and some selective scavengers of H_2O_2 as well as oxygen-centered radicals on the yields of HPD-photoinduced inactivation of EAC cells were examined in vitro. In our experiments, HPD-loaded EAC cells were suspended in phosphate buffered saline (PBS, pH 7.3) and then illuminated in air with red light at 630 nm without or in the presence of corresponding scavengers.

Under photoexcitation of HPD, the kinetic of EAC cells inactivation was of the S type; after a lag period (~ 5 min), an avalanche like increase in the number of dead cells was observed (*paper II*, Figure 2). In this work, 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.

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 is approximately 15 times greater than that in H_2O (Bensasson et al., 1993). Our studies showed that HPD-photosensitized inactivation of EAC cells is enhanced, in a synergistic manner, when the cells were suspended in D_2O-PBS (*paper II*, Figure 2). This indicates that $^1O_2$ is involved in the inactivation. However, the potentiating effect of D_2O was relatively small; in PBS made with 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 with H_2O. A plausible explanation for the effect of D_2O would be that besides $^1O_2$, other ROS are involved in the cytotoxicity resulting from photoexcited HPD. Further experiments supported this assumption.

Our data suggest that $O_2^{-\bullet}$ might also participate in HPD-photoinduced killing of neoplastic cells. Indeed (*paper II*, Figure 2), upon illumination of HPD-loaded EAC cells, the presence of 1 mM 4,5-dihydroxy-1,3-benzene-disulfonic acid (Tiron, a cell-permeable trap of $O_2^{-\bullet}$ (Watkins et al., 1999)) suppressed (by 25%) the PDT-induced cytotoxicity. It was also 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 *paper II* (Figure 2), the LD_{50} value of HPD-PDT was increased by 60% after addition of the H_2O_2-scavenging enzyme CAT (2500 units/ml) and by ~ 30% in the presence of 15 mM d-mannitol, a specific trap of $OH^{\bullet}$ (Goldstein and Czapski, 1984). Experiments indicated that the efficiency of HPD-PDT-induced inactivation of EAC cells was substantially (by 40%) decreased at illumination of the cells in the presence of 25 μM deferoxamine mesylate (DEF), a cell-permeable chelator of iron that can inhibit the ferrous ion catalyzed breakdown of H_2O_2 as well as organic peroxides (Headlam and Davies, 2003). This suggests that Fenton-like reactions could be responsible for the generation of $OH^{\bullet}$ in tumor cells subjected to PDT with HPD.
Thus, our experiments support the view that H_2O_2 and oxygen radicals could be involved in the photodestruction of tumor cells under PDT with HPD.

4.2.2. Formation of H_2O_2 and oxygen radicals under HPD-PDT

To provide more convincing evidence the participation of these ROS in the tumor ablating effect of the treatment, our further work was aimed to quantify the formation of H_2O_2, $O_2^{-\bullet}$, and $OH^{\bullet}$ in tumor cells subjected to HPD-based PDT. Using nitro blue tetrazolium (NBT) as an indicator substance, we revealed that photosensitization of EAC cells with HPD caused a very rapid and considerable increase in the intracellular concentration of $O_2^{-\bullet}$ that was strongly inhibited by exogenously added Cu/Zn-SOD (*paper II*, Figure 3A). In separate experiments, we also found that the addition of Cu/Zn-SOD (up to 150 units/mL) led to a substantial (25-30%) inhibition in the rate of HPD-photoinduced inactivation of the cells. By contrast, only negligible levels of $O_2^{-\bullet}$ were detected in EAC cells after their irradiation in the absence of HPD or during incubation of HPD-loaded cells in the dark.
In addition, we estimated the ability of EAC cells treated with HPD-PDT to produce $O_2^{-\bullet}$ using hydroethidine (HE) as an indicator substance. The use of the dye, instead of NBT, was more attractive for the experiments due to higher, in comparison with the NBT assay, sensitivity of the HE method for detection of $O_2^{-\bullet}$. In these studies, HPD-loaded EAC cells were incubated with HE at different times after cessation of photolysis. Experiments showed that photosensitization of EAC cells with HPD strongly increased the ability of the cells to generate $O_2^{-\bullet}$ as compared to control non-irradiated cells (*paper II*, Figure 5B).
In the next experiments, we tested the possibility that photosensitization of tumor cells with HPD could produce H_2O_2 as a cytotoxic agent. Using the ferric-xylenol orange assay, we revealed that photoirradiation of EAC cells loaded with HPD led to the formation of significant (> 2 μM) amounts of the peroxide (*paper II*, Figure 3B). However, only negligible levels of H_2O_2 were detected after irradiation of EAC cells in the absence of HPD, incubation of HPD-loaded cells in the dark or during illumination of the PS in cell-free PBS.
Furthermore, we found that photoirradiation of HPD-loaded EAC cells in the presence of 10 mM 2-deoxy-d-ribose (DOR) was associated with substantial oxidation of the sugar, a scavenger of $OH^{\bullet}$ (*paper II*, Figure 3C). This indicates

that the oxygen radical is continuously formed during photosensitization of the cells with HPD.

Thus, our studies clearly demonstrated that photosensitization of tumor cells by HPD leads to the production of significant amounts of H_2O_2, $O_2^{-\bullet}$, and $OH^{\bullet}$ radicals. Moreover, our experiments with D_2O and traps of different ROS strongly suggest that along with $^1O_2$, H_2O_2, $O_2^{-\bullet}$ and $OH^{\bullet}$ may be involved in the tumor necrotizing effect of the phototherapy. Our findings on the participation of H_2O_2 and $O_2^{-\bullet}$ in cytotoxicity resulting from photoexcited HPD are in good agreement with the data of other researchers (Athar et al., 1988) who studied the mechanism of PII-mediated cutaneous photosensitization (a main drawback of PDT with the PS) in mice and revealed that exogenously administered inhibitors of SOD and CAT augmented the skin photosensitivity; in their work, however, they did not determine the generation of these ROS. Besides, our results on the ability of photoexcited HPD to generate $OH^{\bullet}$ in tumor cells are supported by the data of Hariharan et al. (1980), who reported that photosensitization of V-79 cells by HPD in culture led to the production of $OH^{\bullet}$. However, in their work the mechanism of this radical formation as well as its contribution to HPD-PDT-induced cytotoxicity has remained to be evaluated.

4.2.3. Mechanism(s) of HPD-PDT induced generation of H_2O_2 and oxygen radicals

A comparison of the biochemical changes which were observed in HPD-PDT-treated EAC cells with the data obtained on a model photodynamic system suggest that in cells subjected to the PDT, the generation of H_2O_2, $O_2^{-\bullet}$ and $OH^{\bullet}$ could be largely attributed to the photosensitized oxidation of certain cellular constituents (e.g., proteins and NAD(P)H) and that photoexcited HPD itself, i.e., in the absence of photooxidizable biomolecules, does not generate these ROS as major products. Indeed, we revealed that upon photoirradiation of EAC cells loaded with HPD, the formation of H_2O_2 and oxygen radicals was associated with a serious damage of their proteins; a clearly expressed fall in the levels of tyrosine (Tyr), Trp, PrSH, and especially His was monitored (*paper II,* Figures 1 and 3). At the same time, experiments with bovine serum albumin (BSA), performed under the same irradiation conditions, clearly showed that in PBS, similar photooxidative injuries of the model protein by HPD (at a concentration close to that in EAC cells) were also associated with the generation of sizable (μM) levels of H_2O_2 and $O_2^{-\bullet}$, and significant oxidation of DOR (*paper II*, Figure 4). By contrast, only trace amounts of these ROS were found after photoirradiation of HPD in an aqueous buffer in the absence of BSA. Our conclusion, about the poor ability of photoexcited molecules of HPD to generate H_2O_2 and oxygen-centered radicals in simple water solutions, is consistent with the data from other laboratories (Gibson et al., 1984; Buettner and Hall, 1987). Besides, studies on the mechanism suggested that in tumor cells subjected to PDT with HPD, the formation of H_2O_2 could be ascribed partly to $^1O_2$-mediated reactions. In fact, under the PDT, the yields of H_2O_2 production in EAC cells were markedly decreased by the addition of NaN_3 (a quencher of $^1O_2$) and, on

the contrary, substantially increased when the cells were irradiated in D_2O-PBS (*paper II*, Figure 3B).

Our studies point to the possibility that the generation of H_2O_2 and $O_2^{-\bullet}$ in tumor cells could be also mediated by the photoinduced oxidation of NAD(P)H, since upon photosensitization of EAC cells by HPD simultaneously with the formation of these ROS, a substantial (~ 30%) lowering in the intracellular content of these nucleotides was observed (data not shown). This assumption is supported by the data of other researchers (Bodaness and Chan, 1977; Buettner and Hall, 1987), who discovered that HPD-catalysed photooxidation of NAD(P)H in aqueous solution leads to the generation of H_2O_2 and $O_2^{-\bullet}$.

Our data suggest that under HPD-PDT, $O_2^{-\bullet}$ (a precursor of H_2O_2) is derived not only from photooxidation of some cellular constituents (proteins, as indicated above), but also from xanthine oxidase (XOD) activation and that this pathway could play an important role in evoking cellular injury. Indeed, we found that photoirradiation of HPD-loaded EAC cells caused a strong (> 3-fold) increase in the activity of XOD in the cells that correlated well with the production of $O_2^{-\bullet}$ in the cells (*paper II*, Figure 5). It was also established that the production of $O_2^{-\bullet}$ in EAC cells was considerably (by 70%) inhibited by the addition of allopurinol (AP), a potent inhibitor of XOD. Moreover, AP at the concentration studied (50 μM) induced a substantial (25-30%) decrease in the rate of HPD-sensitized photoinactivation of EAC cells. Our results suggest that under HPD-PDT, an increase in the activity of cellular XOD could be mediated via a rise in cytosolic Ca^{2+} that in turn may lead to the activation of calcium-dependent proteases in the cytosol. These proteases convert xanthine dehydrogenase (XDH) into XOD (Amaya et al., 1990). In fact (*paper II*, Figure 5), the HPD-PDT-induced increase in the activity of XOD in EAC cells was inhibited upon their irradiation in the presence of EGTA (a Ca^{2+}-compexing agent) and Leupeptin (an inhibitor of the Ca^{2+} proteases responsible for XDH conversion into the oxidase form (Rami and Krieglstein, 1993)) that was associated with suppression of the $O_2^{-\bullet}$ generation. In the work, we did not examine the influence of HPD-PDT on the level of Ca^{2+} in EAC cells. However, a strong elevation in the intracellular level of Ca^{2+} in HPD-PDT-treated tumor cells is a well-documented event that is attributed to the influx of extracellular Ca^{2+} and/or its release from storage proteins (Penning et al., 1992). Such a mechanism of $O_2^{-\bullet}$ generation connected with a Ca^{2+}-dependent proteolytic conversion of XDH to XOD was uncovered by other researchers (Athar et al., 1989) who studied HPD-mediated cutaneous photosensitization on animal models. Now, we have provided evidence that this process could occur in tumor cells subjected to HPD-PDT. Moreover, our data suggest that under HPD-PDT an increase in the activity of XOD in tumor cells could be causally linked with transformation of XDH to XOD via thiol oxidation, and that this pathway of XDH conversion is dominant. As shown in *paper II* (Figure 1), the photoirradiation of HPD-loaded EAC cells resulted in a substantial fall in the intracellular content of PrSH. In this connection, we assumed that the activation of XOD in HPD-PDT-treated cells could be causally linked with the process, since XDH can be reversibly transformed into XOD by

oxidation of free SH groups (Amaya et al., 1990). In order to elucidate the mechanism of the HPD-photoinduced XOD activation, we compared the activity of XOD in PDT-treated EAC cells from two assay systems, the standard procedure and another containing 50 mM dithiothreitol (DTT) to reverse the disulphide bond oxidation, which could lead to oxidative XDH-XOD conversion. A large difference was observed between the XOD activity yielded by the two assay systems; namely, the 14 min treatment of EAC cells by HPD-PDT resulted in a significant (> 3-fold) increase in the activity of XOD, but the effect was completely abrogated when the activity of XOD in photoirradiated cells was tested in the presence of DTT (*paper II*, Figure 6). Thus, our studies strongly suggest that in tumor cells treated by HPD-PDT, XOD could serve as an important source of $O_2^{-\bullet}$ and, as a consequence, other ROS.

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 less than those after PDT (*paper II*, Figures 3 and 5). The ability of mammalian cells, including transformed cells, to the generation of H_2O_2 and oxygen radicals is well documented, e.g. (Szatrowski and Nathan, 1991). 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 $OH^{\bullet}$ (Chance et al., 1979). Our data suggest that the phenomenon could be also ascribed to the presence in tumor cells of XOD, a ROS-producing enzyme.

Another interesting finding of our studies is that in tumor cells subjected to HPD-PDT, the Fenton-like reactions could play (due to the generation of extremely cytotoxic $OH^{\bullet}$) an important role in cellular injury. Indeed (*paper II*, Figure 2), we found that the phototoxic action of HPD against EAC cells was substantially suppressed upon their irradiation not only with CAT, but also in the presence of DEF (an iron-chelating agent). On the other hand, the strong (~ 80%) inhibition of DOR breakdown by exogenously added CAT (*paper II*, Figure 7) suggested that H_2O_2 is a crucial intermediate in the generation of $OH^{\bullet}$ and that in EAC cells subjected to HPD-PDT the radical might produce 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 ($H_2O_2 + Fe(II) \rightarrow OH^{\bullet} + OH^{-} + Fe(III)$] (Chance et al., 1979). Studies supported this view. Namely (*paper II*, Figure 7), the HPD-photoinduced generation of $OH^{\bullet}$ in EAC cells was markedly (by 30%) decreased at illumination of the cells in the presence of DEF, which can inhibit the decomposition of H_2O_2 in the Fenton-type reactions, preventing thereby the formation of very cytotoxic $OH^{\bullet}$.

We established that photosensitization of tumor cells by HPD leads to the generation 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 was reported (Tarr and Valenzeno, 2003) that the spontaneous dismutation of $O_2^{-\bullet}$ in cells can produce $^1O_2$, a highly reactive oxidant that is involved in the phototoxic action of HPD against tumor cells, including EAC cells. Moreover, the reaction of $O_2^{-\bullet}$ with H_2O_2 can also generate $^1O_2$ (Mao et al., 1995). Secondly, our studies suggest that under HPD PDT, a part of the formed $O_2^{-\bullet}$ may 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 Tiron led to a notable lowering in the rate of DOR oxidation, a specific trap of $OH^\bullet$ (*paper II*, Figure 7). 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 (Harris et al., 1994) 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 $OH^\bullet$.

4.2.4. Formation of protein peroxides (PPO) in HPD-PDT treated cells

To date, it was established that HPD-photoinduced oxidation of unsaturated lipids participates in the initiation of cell death (Thomas and Girotti, 1989b). However, little is known as to the role of PPO, although it was reported that these peroxides can readily degrade (on reaction with transition metals) to reactive free radicals; both the parent hydroperoxides and radicals derived from them may mediate further oxidative damage of adjacent proteins and lipids, inactivate important enzymes, cross-linked DNA, and proteins (Du and Gebicki, 2002; Morgan et al., 2002). Moreover, studies showed that cells do not have efficient enzymatic defences against PPO, with only thiols and ascorbic acid able to remove these peroxides (Morgan et al., 2004).

Our model experiments with BSA as well as on EAC cells loaded with HPD clearly demonstrated the ability of this sensitizer to photooxidative damage of proteins with the formation not only H_2O_2, but also of protein bound peroxides (*paper II*, Figures 1, 3, and 4). It was also found that in the cells the yields of PPO formation considerably exceed those for lipid hydroperoxides suggesting that cellular proteins, but not lipids, are the primary targets of ROS generated by PDT with HPD (*paper II,* Figure 1). This finding is in agreement with our previous studies (*chapter 4.1.2.*). Our results support the view (Silvester et al., 1998) that the oxidation of aromatic acids in proteins induced by photoexcited porphyrins can give rise to PPO; in fact, upon HPD-PDT, the production of these peroxides in EAC cells was associated with a pronounced decrease in the level of Trp, Tyr, and His amino acid residues (*paper II*, Figure 1). Similar events were also registered during HPD-photosensitized oxidation of BSA (*paper II*, Figure 4). Nevertheless, our data suggest that in tumor cells subjected to HPD-PDT, PPO are not the major products of photooxidative transformations of proteins; in fact, in EAC treated by the PDT (for 14 min) the yield of these peroxides formation was calculated (taking into account the part of oxidized PrSH, His, Trp, and Tyr residues) as 0.5%. Studies on the mechanism showed that $^1O_2$ is the main agent responsible for generation of PPO in HPD-PDT-

treated cells; indeed, the yields of this material's formation in EAC cells were greatly (> 4-fold) decreased by NaN_3 and, on the contrary, substantially enhanced when the cells were irradiated in PBS made with D_2O (*paper II,* Figure 8). Besides, we found that H_2O_2, $O_2^{-\bullet}$, and $OH^{\bullet}$ were also involved in the production of PPO in the cells, since upon light exposure the formation of these peroxides in HPD-loaded EAC cells was markedly inhibited by the presence of corresponding scavengers (*paper II*, Figure 8). As H_2O_2 and $O_2^{-\bullet}$ are in themselves weak oxidants, we assume (based on the data presented in *paper II,* Figure 7) that the inhibitory effects of CAT and Tiron on the yields of PPO generation in the cells were largely mediated by suppression of the extremely reactive $OH^{\bullet}$ production. The ability of this radical to damage isolated as well as cellular proteins with the formation of PPO is well documented, e.g. (Du and Gebicki, 2002).

Our data suggest that at photosensitization of EAC cells with HPD, a part of the formed PPO is decomposed via the ferrous ion-catalyzed reactions; namely, during light exposure, the addition of DEF had practically no effect on the yield of PPO generation in the cells, although this iron-chelating agent substantially (by 30%) inhibited the formation of $OH^{\bullet}$ that, as discussed above, is involved in the production of these peroxides upon PDT (*paper II*, Figures 7 and 8). This observation, in turn, suggests that PPO derived radical species could be implicated in the inactivation of EAC cells subjected to HPD-PDT. However, we believe that in our experiments with EAC cells, the formed PPO played, as a source of damaging radicals, a minor role in the direct killing effect of PDT with HPD. Indeed, under HPD-PDT the levels of PPO in EAC cells were found to be considerably (by a factor of about 4.5) lesser than those for H_2O_2 (a source of extremely cytotoxic $OH^{\bullet}$); namely, after 14 min light exposure causing an inactivation of ~ 95% of the cells, the concentrations of these peroxides were assayed as (per 1×10^6 cells): 0.104 nmoles for PPO and 0.45 nmoles for H_2O_2. In other words, these results suggest that the contribution of H_2O_2 in the direct damaging action of HPD-PDT towards EAC cells prevailed over that from PPO.

Thus, in the presented work we clearly demonstrated the ability of photoexcited HPD to the generation of PPO in tumor cells. However, more detailed studies are needed to elucidate the significance of these peroxides in the tumoricidal effect of PDT with HPD. We assume that PPO could mediate a bystander effect in the PDT-induced dying of tumor cells.

4.3. The role of glutathione redox cycle, CAT and Cu/Zn-SOD in the resistance of tumor cells to PDT with HPD

4.3.1. Glutathione redox cycle

We revealed that the phototoxic action of HPD against tumor cells is mediated by the formation of H_2O_2. Hence, it is of great interest to determine 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; 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 (*Paper II*, Figure 1). The depletion of GSH could be attributed to the known ability of thiol to direct chemical quenching of $^1O_2$ and other ROS (Rougee et al., 1988; Dikalov et al., 1996), and/or its oxidation by Se-dependent glutathione peroxidase (GPX) that detoxifies H_2O_2 and lipid hydroperoxides using GSH as hydrogen donor. This suggests that the glutathione redox cycle could play a role in the protection of tumor cells against HPD-PDT. In this 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 D, L-buthionine-[S,R]-sulfoximine (BSO), a potent inhibitor of glutathione biosynthesis (Bailey, 1998), as well as via inhibiting the activity of glutathione reductase (GR) with 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). First, EAC cells were pre-treated with BSO (in vivo) to lower the level of glutathione and their sensitivity to cytotoxicity resulting from photoexcited HPD was then estimated in vitro. This 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 (*Paper III,* Table 2). It should be pointed out that such a pre-treatment of EAC cells with 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 experiments were conducted on EAC cells pre-treated with BCNU, a selective inhibitor of GR (Nathan et al., 1981); in the cycle, GR is responsible for the regeneration of GSH. Studies indicated that pre-treatment of the cells with 0.1 mM BCNU (which inhibited GR activity by 65% with no effect on the activities of GPX, CAT, or on the number of viable cells) markedly enhanced the phototoxic effect of HPD; the LD_{50} exposure of PDT was decreased by 22% after pre-treatment of EAC cells with the inhibitor (*paper III,* Table 3). It is important to emphasize that despite significantly decreased GR activity, GSH levels were unaffected in the cells after 25 min incubation with 0.1 mM BCNU at 30 °C. These results suggest that lower than normal GR activity was sufficient to maintain GSH levels. However, it was found that under HPD-PDT (at 30 °C) the initial rate of GSH oxidation in BCNU-treated EAC cells (2.26 nmoles GSH/min per 1×10^7 cells) exceeded considerably (> 3-fold) that in control cells (0.71 nmoles GSH/min per 1×10^7 cells). This indicates that in the cells an inhibition of the glutathione cycle activity by BCNU enhanced the PDT-induced oxidative stress.

Thus, our experiments 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 Thomas and Girotti (1989b). 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 these 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.

4.3.2. Catalase

Under PDT, tumor cells could detoxify the formed H_2O_2 not only via the glutathione 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 to the phototoxic influence of HPD. To elucidate the role of the H_2O_2-scavenging enzyme, experiments were performed on EAC cells pre-treated with 3-amino-1,2,4-triazole (ATZ), an irreversible inhibitor of CAT (Margoliash et al., 1962). This treatment with ATZ (25 mM for 1 h) essentially (by 40%) inhibited the activity of cellular CAT (with no 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 (*paper III*, Table 4). Thus, our experiments clearly showed that cell-bound CAT protects tumor cells against HPD-PDT induced cytotoxicity in vitro.

4.3.3. Cu/Zn-SOD

We found that photosensitization of tumor cells by HPD leads to the formation of $O_2^{-\bullet}$ and that this radical is involved in the porphyrin photoinduced killing of the cells in vitro. Therefore, substantial interest has a study on the role of cell-bound SOD(s) in the resistibility of neoplastic cells to HPD-based PDT. For this purpose, experiments were performed on EAC cells pre-treated with sodium diethyldithiocarbamate (DDC), an inhibitor of Cu/Zn-SOD (Heikkila et al., 1976). It is well known that cells can scavenge $O_2^{-\bullet}$ with the help of this constitutive antioxidant enzyme. Studies showed that pre-treatment 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 (*paper III*, Table 1). It is important to note that this treatment of EAC cells with DDC had no effect on the activity of other antioxidant enzymes, such as CAT, GR, and GPX. Thus, our experiments indicated that cell-bound Cu/Zn-SOD can protect tumor cells against HPD-PDT-induced cytotoxicity. This finding is consistent with the recent data of other researchers (Golab et al., 2003), who demonstrated that SOD(s) in tumor cells play an important role in their resistance to PDT with PII.

4.4. On the mechanism of the potentiating influence of heating on the antitumor efficiency of HPD-PDT

Although photochemical reactions of HPD seem to play a major role in the tumoricidal action of PDT, clinical trials (Berns et al., 1984) and the data obtained on animal models (Mattiello et al., 1987) showed that during a standard regime 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 41-46 °C (Hildebrandt et al., 2002). 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-catalysed photodestruction of tumor cells (Mang, 1990; Leunig et al., 1994; Uehara et al., 1996). However, until now the mechanism of the synergism remains unclear.

Several assumptions have been advanced to explain the potentiating effect of photoirradiation-induced heating on the efficiency of HPD-PDT. It was suggested that in PDT the hyperthermia produced by a laser irradiation could promote tumor destruction via an increase in the reactivity of the formed $^1O_2$ (Gottfried and Kimel, 1991). Further, Prinsze et al. (1991) based on the model experiments with glyceraldehyde-3-phosphate dehydrogenase, 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 a principal target of PDT with HPD (*chapter 4.1.2.*). 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 oxygen, causing acute hypoxia and limiting treatment effectiveness (Henderson et al., 2000). 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 (Hetzel et al., 1994).

However together with the 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 the hyperthermia, associated with photoirradiation, could promote the formation of $^1O_2$ and/or other toxic ROS by activated molecules of the PS. Indeed, components of HPD are known to aggregate readily in certain solvents; in aqueous solution the PS exists as a complex mixture of non-aggregated or self-aggregated monoporphyrinic and oligomeric species (Tanielian et al., 2001). At the same time, in prior studies (Chekulayeva et al., 2002) we found 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 Tanielian et al. (2001), who revealed that HPD in the aggregation state is a poor source of $^1O_2$. Furthermore, a likely explanation for the potentiating action of light induced heating on the efficiency of HPD-PDT 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 demonstrated that upon illumination HPD undergoes photochemical modifications resulting in the formation of a photoproduct with peak absorption around 640 nm and peak fluorescence emission around 644 nm (Rotomskis et al., 1996; Chekulayev et al., 1998). 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. Moreover, it was reported (Giniunas et al., 1991) 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. So, if the heating of tumors during PDT will promote the formation of PhP-640, it might be expected that this event could be also responsible for a heat-induced increase in the efficiency of HPD-based PDT. However, these assumptions require an experimental checking.
Thus, the aim of this study was to clarify the mechanism of the potentiating effect of photoirradiation-induced heating on the efficiency of PDT with HPD.

4.4.1. Influence of temperature on the response of tumor cells to HPD-PDT

In our studies, HPD-loaded EAC cells were irradiated with red light at 630 nm or incubated in the dark at five different temperatures (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 (Jones et al., 1984) 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 (*paper III*, Figure 4a). 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 (Hildebrandt et al., 2002), we examined the influence of temperature on HPD-photoinduced damage of proteins in EAC cells. As shown in *paper III* (Figure 4), the photodynamic treatment of EAC cells by HPD led to a substantial modification of the amino acid composition of their proteins; 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-catalysed photoinjury of proteins in EAC cells. Namely, upon raising the temperature from 30 to 44 °C a substantial (~ 55%) growth in the rates of Trp residues and PrSH photooxidation was registered that well correlated with an

increase in the rate of HPD-PDT induced inactivation of the cells. Lowering the irradiation temperature from 30 to 10 °C, on the contrary, made cell proteins less sensitive to the photooxidative injuries, resulting in the 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 revealed 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 (*paper III*, Figure 4d). 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 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 consistent with the results of other researchers (Orenstein et al., 1999).

4.4.2. The generation of ROS during HPD-PDT at elevated temperatures

In these studies, we concentrated our efforts on elucidation of the mechanism of pro-oxidative effects of heat stress in PDT with HPD, because this 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, as the event must, in turn, lead to an enhanced production of $^1O_2$ (Tanielian et al., 2001; Chekulayeva et al., 2002). 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. Figure 5 (*paper III*) 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; indeed, the heating of EAC cells from 10 to 44 °C resulted in a 25% fall in the fluorescence intensity of HPD at 630 nm (without any shifts in the position of its main fluorescence peaks at 630 and 677 nm). Studies showed that this 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 rising the temperature from 10 to 44 °C a substantial (1.5-2.0-fold) decrease in the

volume of EAC cells was registered microscopically. Thus, we cannot explain the potentiating action of photoirradiation-induced 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 not only via an increase in the reactivity of $^1O_2$ (Gottfried and Kimel, 1991), but also through the simulation of other ROS formation. In fact, we found that upon HPD-PDT a rise in the temperature from 30 to 44 °C strongly (by ~ 2.5-fold) enhanced the generation of $O_2^{-\bullet}$ (Figure 4.2.) as well as H_2O_2 and $OH^\bullet$ in EAC cells that directly correlated with an increase in the rate of HPD-photoinduced inactivation of the cells (*paper III*, Figures 3 and 4, respectively).

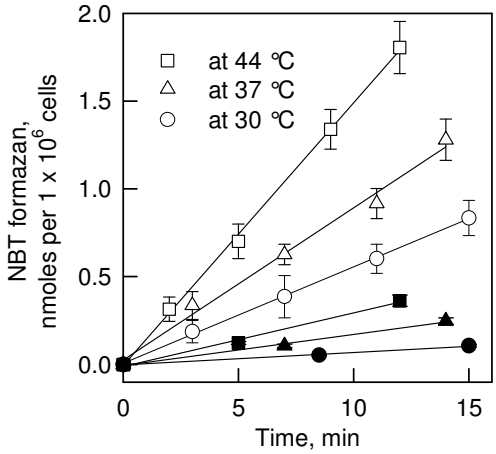


Figure 4.2. The generation of $O_2^{-\bullet}$ by HPD-loaded EAC cells (5 x 10^6 cells/mL in PBS) during their irradiation (opened symbols) with red light at 630 nm (102 mW/cm^2) or incubation in the dark (filled symbols) at different temperatures. The formation of $O_2^{-\bullet}$ was monitored by the nitro blue tetrazolium (NBT) assay detailed as described in Paper II. Bars designate standard errors (SE)

The mechanism of the heat-induced activation of H_2O_2 and oxygen radicals formation upon photosensitization of EAC cells with HPD could be attributed to the stimulatory effects of heating on primary photophysical processes as well as on the photooxidation of certain cellular constituents, e.g., proteins (*paper II,* Figure 4 as well as *paper III,* Figures 3 and 4) by photoexcited molecules of the porphyrin both via Type 1 and 2 photochemistry. Further, using HE as an indicator substance we revealed that EAC cells pre-treated with HPD-PDT are characterized by an elevated production of $O_2^{-\bullet}$ and that the ability of the cells to the generation of the anion-radical strongly enhanced with a rise in the irradiation temperature (*paper III*, Figure 3b). To clarify the phenomenon, we examined the effect of heating on the activity of XOD in EAC cells during their photosensitization with HPD; as stated above (*chapter 4.2.3.*), the photodynamic treatment of the cells with HPD results in a substantial increase in the activity of XOD and that under the PDT a part of the formed $O_2^{-\bullet}$ may be formed by the

ROS producing enzyme. It was established that heating catalysed the HPD-PDT induced increase in the activity of XOD in EAC cells; namely, the 12 min treatment of EAC cells by HPD-PDT at a control (30 °C) temperature caused an approximately 40% growth in the activity of XOD whereas the same light exposure at 44 °C led to almost 2-fold increase in the activity of the enzyme in the cells (Figure 4.3.). It is important to emphasise that incubation of HPD-loaded EAC cells in the dark even at hyperthermic (44 °C) temperature had no effect on the activity of XOD in the cells. In the work, we discovered that under HPD-PDT an increase in the activity of XOD in tumor cells may be causally linked with transformation of XDH to XOD via thiol oxidation (*chapter 4.2.3.*). Since heat shock promotes the HPD-photosensitized oxidation of PrSH in EAC cells (*paper III*, Figure 4), we assume that under HPD-PDT at elevated temperatures a rise in the activity of XOD in EAC cells could be largely mediated by a heat-catalysed photooxidation of SH groups in XDH.

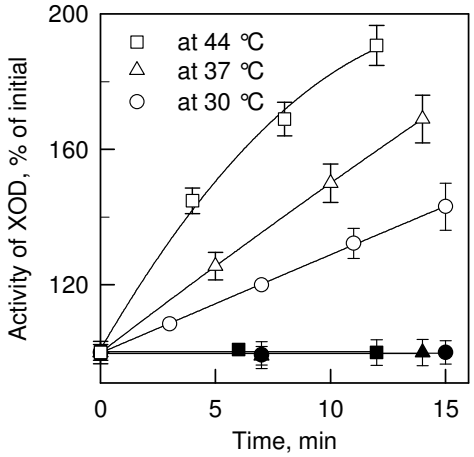


Figure 4.3. The activity of xanthine oxidase (XOD) in EAC cells (5 x 10^6 cells/mL in PBS) after their photodynamic treatment with HPD (opened symbols) or incubation in the dark (filled symbols) at various temperatures. The experimental conditions as well as the assay for XOD activity were the same as described in *paper II* and *III*, respectively. The initial activity of XOD in EAC cells not exposed to the PDT was measured as 13.1 ± 0.7 pmoles isoxanthopterin formed/min per mg cell protein. Bars, SE

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 (see Figure 3.2., and in *paper III* Figure 3). The finding is in a good agreement with the observations of other researchers (Flanagan et al., 1998), who discovered that heating (from 37 to 43–45 °C) of non-transformed as well as tumor cells strongly enhances the generation of oxygen radicals in the cells. Until now, the precise location and mechanisms of increased formation oxygen-derived free 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 (Salo et al., 1991). Our data are consistent with these findings; it was found that within the temperature range of 10-44 °C, the ability of EAC to produce H_2O_2 is directly related to the respiratory activity of the cells (*paper III*, Figure 6). We revealed that XOD in tumor cells may serve as another of ROS. Heat stress could enhance the activity of the enzyme in the cells and thereby oxidative stress. In this relation, we explored the influence of heating on the activity of XOD in intact, not exposed to HPD-PDT, EAC cells. It was found that rising the temperature of a medium from 30 to 44 °C caused more than 2-fold increase in the activity of XOD in the cells, however, further heating from 44 to 60 °C resulted in suppression of the enzyme activity (Figure 4.4.). Thus, our studies suggest that XOD, along with the respiratory chain of mitochondria, can be responsible for an increased formation of H_2O_2 and oxygen radicals in tumor cells at hyperthermic (up to 44-45 °C) temperatures.

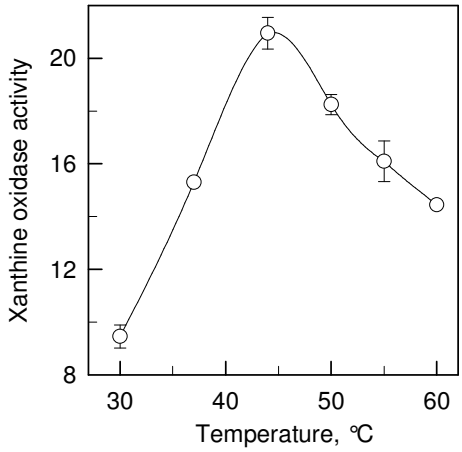


Figure 4.4. Influence of temperature on the activity of xanthine oxidase (XOD) in intact EAC cells. The activity of XOD in the cells was expressed as pmoles isoxanthopterin formed/min per mg cell protein. Bars, SE

It was suggested that the cellular damage and/or toxicity associated with hyperthermia could be mediated in part by an increased flux of oxygen-free radicals (Li and Oberley, 1997). 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 can 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 (Issels et al., 1986).

It was also reported that hyperthermia (42 °C) can enhance the cytotoxicity of H_2O_2 (Lord-Fontaine and Averill, 1999).

4.4.3. 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 defences. 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 the work, we already demonstrated that these H_2O_2-detoxifying systems play an important role in the protection of the cells against the phototoxic action of HPD (*chapter 4.3.*). 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 this H_2O_2-destroying 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 (*paper III*, Figure 7a). 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 enzyme is inactivated during photoirradiation of HPD-loaded EAC cells and that heating sensitizes CAT to HPD-photoinduced inactivation. Namely, 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 (*paper III*, Figure 7b). 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, at light doses that 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% (*paper III*, Figure 7). 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. Also, they suggest that during PDT with HPD the heating associated with the absorption of optical radiation could promote the phototherapy-induced oxidative stress and,

as a consequence, the tumor eradication via increasing the rates of HPD-photosensitized inactivation of cellular CAT and GPX.

4.4.4. 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 these aspects of PDT remain undetermined. At the same time, this information could clarify some discordance in the literature data concerning the 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 (Leunig et al., 1994). However, it was reported (Jones et al., 1984) that the cooling of tumors during interstitial phototherapy with HPD also improves the tumor response to the photochemotherapy 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 an 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, we found 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 the cooling of the cells from 30 to 10 °C caused a substantial (more than 4-fold) inhibition in the rate of the sensitizer photodegradation, as found by measuring a slope on the kinetic curves of HPD photobleaching (*paper III*, Figure 8a). 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 photostabile drug; indeed, 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 (*paper III*, Figure 8a). Furthermore, at the light dose 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 cooling 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 PS 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, other 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 light-induced heating on HPD-PDT-induced tumor eradication could be mediated by a heat-catalysed 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 (Giniunas et al., 1991). In other words, the light sources used in PDT must effectively excite not only HPD but also the PhP-640, because the absorption band of the photoproduct is not significantly red shifted (Rotomskis et al., 1996). Using fluorescence spectroscopy, we found that photoirradiation of HPD-loaded EAC cells 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 (*paper III*, Figure 8b). 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 favour 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 towards 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 does not absorb appreciably in this spectral region (Rotomskis et al., 1996)). 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, 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 a substantially lesser (by 26%) growth in the rate was observed when the cells were exposed to green light at 510 nm (*paper III*, Table 5). It was reported (Karu, 1999) 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 ATP, Ca^{2+}, and etc.) and that these light-induced alterations in cellular homeostasis are largely dependent 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 effect of heating on the formation of PhP-640 as well as the temperature dependence of photosensitising activity of HPD on the wavelength of radiation using the simplest photochemical system - Trp and HPD dissolved in PBS. It was found that when the system was exposed to red light at 630 nm, raising the temperature from 30 to 44 °C led to a considerable (67%) increase in the rate of HPD-sensitized photooxidation of Trp, which correlated well with a heat-induced increase in the rate of PhP-640 formation (*paper III*, Table 5 and Figure 9, respectively). However, the same heating of a reaction mixture produced a 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 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, may enhance the tumoricidal effect of the phototherapy via the stimulation of PhP-640 formation.

4.5. Influence of pH on the antitumor efficiency of PDT with chlorin-e_6

As known the pH value in malignant tumors is lower that of normal tissues and can be decreased selectively by glucose administration (Thistlethwaite et al., 1987; Thomas and Girotti, 1989a). This phenomenon could be exploited to enhance the antitumor efficiency of PDT. A low pH value might make tumor cells more vulnerable to the phototoxic of tetrapyrrolic compounds, e.g., by increasing their susceptibility to free radical toxicity due to an inhibition of the cellular repair enzymes activity. An additional tumor-selective effect might be expected for PS(s) of the porphyrin type, whose lipophilicity and cell uptake increase with decreasing pH (Thomas and Girotti, 1989a; Pottier and Kennedy, 1990). In this relation, we examined the influence of pH and artificial hyperglycaemia on the antitumor efficiency of PDT with E6, using both in vitro and in vivo protocols.

4.5.1. On the mechanism of cellular destruction under E6-based PDT

The tumoricidal effect of PDT with E6 depends largely on the presence of O_2 (Chekulayev et al., 1992). However, until now the identity of the ROS mediating the PDT-induced tumor eradication has remained unclear. Studies performed in other laboratories (Bachor et al., 1991) indicated that $^1O_2$ plays an important role in the phototoxicity of chlorin-e_6 against malignant cells. However, our data suggest that under E6-based PDT besides $^1O_2$, $OH^{\bullet}$ is involved in cell killing. In fact, upon addition of DEF, a well-known scavenger of $OH^{\bullet}$, the LD_{50} value of E6-PDT was increased by a factor of about 1.6 (*paper IV*, Figure 1). Moreover, using the HE assay we established (*paper IV*, Figure 2) that photoirradiation of E6-loaded cells leads to the production of significant amounts of $O_2^{-\bullet}$, a precursor of H_2O_2 and very cytotoxic $OH^{\bullet}$. The generation of $O_2^{-\bullet}$ during E6-PDT could be explained in two ways. The first could be associated with oxidation of some biomolecules by $^1O_2$ (Buettner and Hall, 1987). The other way might involve direct electron transfer from photoexcited molecules E6 to

molecular O_2. In prior studies we already demonstrated that E6, in comparison with other sensitizers proposed for the use in PDT of tumors, has an increased inclination to photoprocesses with charge transfer (Chekulayev et al., 1992).
Thus, the obtained results indicate that upon E6-based PDT along with $^1O_2$, other ROS (such as $O_2^{-\bullet}$ and $OH^\bullet$) are involved in cell killing.

4.5.2. Effects of pH on E6-based PDT in vitro

Figure 1 (*paper IV*) depicts the dose-response curves for EAC cells subjected to E6-PDT after their pre-incubation with the PS in PBS at pH 7.2 or 6.2. These data show that at pH 6.2 the cell killing effect of PDT was significantly higher than at pH 7.2; the acidification caused (in a synergistic manner) a more than 1.5-fold increase in the rate of EAC cells inactivation, as found by measuring the LD_{50} value of PDT.
Several ways can be envisioned in which a decrease in the pH value might enhance cellular photosensitivity in vitro. It could be assumed that the cellular uptake of the PS is more efficient at a low pH than at pH values found in normal tissues, although E6 has no side groups whose protonation might increase its lipophilicity. Surprisingly, lowering the PBS pH from 7.2 to 6.2 decreased (by about 25%) the accumulation of E6 in EAC cells (*paper IV*, Figure 3). Thus, we cannot explain the increased phototoxicity of E6 at a low pH value by binding the PS to the cells.
It was reported (Spikes and Bommer, 1993) that derivatives of chlorin-e_6 proposed for PDT of tumors are progressively destroyed during illumination and the process may potentially result in a reduced yield of inactivation of tumor cells per incident photon. Hence, another explanation for the pH dependence of E6-based PDT might be that photobleaching of the PS is less efficient at acidic pH than at pH values close to neutral. Our studies supported the hypothesis. It was found that upon illumination of EAC cells the photodrug undergoes a very rapid decay; however, lowering the pH value of PBS from 7.2 to 6.2 caused a substantial (~ 1.5-fold) decrease in the initial rate of E6 photodestruction in the cells (*paper IV*, Figure 4). These results could partly explain the elevated phototoxicity of E6 against the cells at a low pH value.
To achieve a more precise understanding of the influence of pH on the antitumor efficiency of E6-PDT, we measured the intracellular content of $O_2^{-\bullet}$ and GSH. The latter was chosen as a biomarker of oxidative stress because the peptide reacts directly with some ROS, such as $^1O_2$ and $OH^\bullet$ (Rougee et al., 1988). It was established (*paper IV*, Figure 2) that the photoirradiation of E6-loaded EAC cells at a low pH value (6.2) did not mediate a more efficient formation of $O_2^{-\bullet}$, a known precursor of very reactive $OH^\bullet$, which is found (*paper IV*, Figure 1) to be involved in the phototoxic action of the sensitizer. In addition, studies revealed that EAC cells subjected to E6-PDT at pH 6.2 had higher levels of GSH in comparison with those treated at pH 7.2 (*paper IV*, Figure 5). Thus, it seems to be unlikely that the increased phototoxicity of E6 toward the cells at acidic pH is mediated by a more pronounced oxidative stress.
At the same time, we found that the acidification of EAC cells causes a serious inhibition of their mitochondrial activity; namely, lowering the PBS pH from 7.2

to 6.2 induced a strong (~ 3-fold) decrease in the dehydrogenase activity of the cells in the MTT-assay (*paper IV*, Figure 6). Our results on the effect of pH on the energy metabolism of tumor cells are in good agreement with the data from other laboratories. For instance, Gabai and Mosin (1991) showed that the oxygen consumption of EAC cells is strongly (by 4-fold) inhibited after a decrease in pH from 7.3 to 6.0. In addition, these authors revealed that at pH 6.0 the cells have a low level of glycolysis. On this basis, we supposed that these acidic pH-induced disturbances of energy metabolism must cause a substantial decrease in the ATP content of neoplastic cells and thereby increase their sensitivity to E6-PDT. This assumption is very probable, because in our previous studies (Chekulayev et al., 1991) we established that suppression of mitochondrial oxidative phosphorylation, which coincided in time with a drastic decrease in the ATP level, is a crucial event in the cytotoxicity resulting from E6-induced photosensitization in vitro.

4.5.3. Influence of glucose administration on the efficiency of PDT with E6

It was reported that induction of experimental hyperglycaemia in tumor-bearing animals (Thomas and Girotti, 1989a) and humans (Thistlethwaite et al., 1987) results in a significant lowering of the tumor pH. In an attempt at exploiting this phenomenon to enhance the antitumor efficiency of E6-PDT, we, first of all, evaluated the effect of glucose administration on the pH of the interstitial fluid in Ehrlich carcinomas. It was found that a single intraperitoneal injection of glucose (2 g/kg) resulted in a substantial decrease in the pH from an initial value of ~ 7.2 to ~ 6.3 after 2 h (*paper IV*, Table 1). Similar effects of glucose administration on the pH in experimental tumors were observed in other laboratories (Thomas and Girotti, 1989a). It is important to note that the glucose-induced tumor pH drop can persist for 24 h (Osinsky et al., 1987). In this work, the mechanism of the effect of glucose dose on pH depression was not explored; however, previous studies (Hiraoka and Hahn, 1990) clearly indicated that increased acidity is a consequence of elevated lactic acid production, although reduced blood flow in the tumor vasculature could be a contributing factor.

Further we explored the effect of glucose administration on the antitumor efficiency of E6-PDT of mice bearing subcutaneously transplanted EAC cells. Since the cellular uptake of the PS was slightly depressed in acidic conditions (*paper IV*, Figure 3), in the in vivo experiments the injection of glucose (up to 2 g/kg) to the tumor-bearing animals was carried out on the next day after the administration of E6 and 2 h before light exposure. An apparent delay in the tumor growth was found by combining PDT with a single dose of glucose (*paper IV*, Table 2). It is important to emphasize that the same injection of glucose, but without E6–PDT, had no effect on the rate of the tumor growth. As the PDT-induced death of EAC cells is sensitive to the pH within the range 6.2–7.2 (*paper IV*, Figure 1), the data presented *in paper IV* (Table 1) suggest that the potentiating effect of glucose on the antitumor efficiency of E6-PDT in vivo could be ascribed to a decrease in the pH value of the tumors. It is important to note that tumor cells are sensitive to hyperthermia and that glucose can potentiate this effect (van den Berg et al., 2001). Hyperthermia has been

considered to be a possible contributory factor in tumor eradication by PDT (Leunig et al., 1994). However, under the relatively mild irradiation conditions used in this study, it is unlikely that local heating played any significant role.
Thus, our experiments clearly demonstrated that the efficiency of PDT with chlorin-e_6-type PS(s) may be substantially improved by administration of glucose causing a decrease in the pH value of tumor tissues.

5. CONCLUSIONS

On the basis of the results obtained we made a few general conclusions:

- Cellular proteins, but not membrane lipids, are a principal target of photodynamic therapy (PDT) with hematoporphyrin derivative (HPD) as photosensitizer (PS).
- Following PDT with HPD tumor cells die via necrosis due mainly to the inactivation of their energy producing systems.
- The HPD-PDT induced alterations in morphology of tumor cells (swelling, appearance of numerous protrusions "blebs" on the cell surface) are mediated by the depletion of adenosine triphosphate as well as photooxidative damage of cytoskeletal proteins.
- H_2O_2, superoxide ($O_2^{-\bullet}$) and hydroxyl ($OH^{\bullet}$) radicals are involved, along with singlet oxygen ($^1O_2$), in the tumoricidal effect of HPD-based PDT.
- In tumor cells subjected to HPD-PDT, the formation of H_2O_2 and oxygen radicals is induced by the photosensitized oxidation of cellular constituents (proteins, NAD(P)H) as well as an increase in the activity of xanthine oxidase (XOD), due to the conversion of xanthine dehydrogenase (XDH) to XOD via a Ca^{2+}-dependent proteolytic process as well as oxidation of SH groups in XDH. Besides, our studies showed that in tumor cells treated with HPD-PDT, the Fenton-like reactions play an important role in the generation of $OH^{\bullet}$.
- In tumor cells, the photoexcited HPD oxidizes proteins with the formation of semistable protein peroxides. However, further work is needed to clarify the role of these peroxides in the tumoricidal effect of PDT with the PS.
- Besides $^1O_2$, $O_2^{-\bullet}$ and $OH^{\bullet}$ radicals are involved in the phototoxic action of chlorin-e_6 trimethyl ester (E6, a second generation PS) against tumor cells.
- Cell-bound Cu/Zn-superoxide dismutase, catalase and the glutathione redox cycle protect tumor cells against the phototoxic action of HPD. On this basis, we suppose that the antitumor efficiency of HPD-PDT may be improved by its combination with inhibitors of these antioxidant barriers.
- Our studies support the view that in laser photochemotherapy the mild hyperthermia (around 44 °C) produced by irradiation can enhance synergistically the HPD-photoinduced tumor eradication. Experiments indicated that the potentiating effect of heating on the efficiency of PDT with

HPD may be largely explained via a heat-induced increase in the formation of cytotoxic reactive oxygen species (ROS), such as H_2O_2, $O_2^{-\bullet}$, and $OH^{\bullet}$. We found that the activation of H_2O_2 and oxygen radicals formation is mediated by the stimulatory effects of heating on HPD-photosensitized oxidation of cellular proteins as well as an increase in the activity of XOD.

- Heat shock itself promotes the generation of ROS in tumor cells. Our studies showed that XOD, along with the respiratory chain of mitochondria, are the main factors responsible for an increased formation of H_2O_2 and oxygen radicals in tumor cells at hyperthermic (44-45 °C) temperatures. They, together with photochemically generated oxidants, could take part in the HPD-PDT-induced destruction of tumor tissues.
- We established that photosensitization of tumor cells by HPD causes an inactivation of catalase and glutathione peroxidase, and that heat stress sensitized the H_2O_2-detoxifying enzymes to HPD-photoinduced inactivation. Upon HPD-PDT these events must result in loss of protection against accumulating H_2O_2, providing an enhancement of phototoxicity.
- During clinical PDT, employing a 630 nm laser light to activate HPD, an increase in the temperature of tumor tissues may enhance the tumoricidal effect of the therapy via the stimulation of a chlorin-type photoproduct formation that possesses photosensitizing activity.
- There is the very low probability (due to high photostability of HPD) that at a sufficient concentration of the PS 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.
- A tight control of tumor temperature during HPD-PDT (especially when it is combined with simultaneous local hyperthermia) is needed to obtain the maximal value of tumor necrosis, since severe hyperthermia (at temperatures above 45 ºC) may cause, contrary to mild-hyperthermia (about 42-44 ºC), a reduction in the tumoricidal action of the therapy. Indeed, we found that heating (from 30 to 45 ºC) of non-irradiated Ehrlich ascites carcinoma cells caused a substantial (> 2-fold) increase in the activity of XOD, however, further elevation of temperature (up to 60 ºC) led to a decrease in the activity of the ROS producing enzyme.
- Tumor cells in an acidic milieu (both in vitro and in vivo) are more sensitive to E6-induced photosensitization than at pH values found in normal tissues. Experiments showed that upon acidification the impairment of mitochondrial function as well as the inhibition of E6 photobleaching in the cells are the main factors underlying the discovered effect of pH on the antitumor efficiency of PDT with the PS. Our findings suggest a new strategy for improving of the selectivity and the cure rate of PDT with chlorin-e_6-type PS(s), namely, the induction of transient hyperglycaemia that causes a substantial fall in the pH value of malignant tumors.

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ABSTRACT

Photodynamic therapy (PDT) is a relatively new and very promising approach for the treatment of cancer. The main goal of the study was to enrich the existing knowledge on the molecular mechanisms of tumor cells death at photoexcitation of hematoporphyrin derivative (HPD, the most widely used sensitizer in PDT of malignancies) or chlorin-e_6 trimethyl ester (E6), a second generation photosensitizer (PS). Namely, the work was aimed on elucidation of: a) the significance of H_2O_2 and oxygen radicals in the antitumor effect of PDT with these PS(s); b) the role of damage of lipids and proteins in the deterioration of plasma membrane integrity, the mitochondrial function, and cell killing by PDT with HPD; c) the influence of pH and temperature on photodynamically induced killing of tumor cells; and d) the importance of some antioxidant barriers of tumor cells in their sensitivity to the phototoxic influence of PDT with HPD.

The primary results of this thesis can be summarised as follows:

- There is the very low probability that HPD-photosensitized impairment of mitochondria and, as a consequence, killing of tumor cells is mediated by the injury of membrane lipids. Our data suggest that cellular proteins, but not lipids, are the most critical targets upon PDT with HPD.
- Besides singlet oxygen ($^1O_2$), other reactive oxygen species (ROS), such as H_2O_2, superoxide ($O_2^{-\bullet}$) and hydroxyl ($OH^\bullet$) radicals, could be involved in the tumoricidal action of PDT with HPD; namely, we found that photosensitization of Ehrlich ascites carcinoma cells with HPD led to the formation of significant amounts of H_2O_2, $O_2^{-\bullet}$, and $OH^\bullet$ radicals, which along with $^1O_2$ were involved in photoinactivation of the cells in vitro. Our data suggest that in tumor cells subjected to HPD-PDT, the generation H_2O_2, $O_2^{-\bullet}$, and very reactive $OH^\bullet$ may be largely mediated by: (**i**) an increase in the activity of xanthine oxidase (XOD), due most probably to the conversion of xanthine dehydrogenase (XDH) to XOD via a Ca^{2+}-dependent proteolytic process as well as oxidation of SH groups in XDH; and (**ii**) photooxidation of some cellular constituents (NAD(P)H, proteins). Furthermore, it was found that in tumor cells subjected to HPD-PDT the Fenton-like reactions play an important role in the formation of $OH^\bullet$.
- We clearly demonstrated the ability of photoexcited HPD to the generation of protein peroxides in tumor cells, which currently are regarded as a new form of ROS. However, further work is needed to clarify the significance of these peroxides in the antitumor effect of PDT with HPD.
- H_2O_2, $O_2^{-\bullet}$, and $OH^\bullet$ radicals may participate in the antitumor effect of PDT with chlorin-e_6-type PS(s).
- It was established that cell-bound Cu/Zn-superoxide dismutase, catalase, and the glutathione redox cycle protect tumor cells against the phototoxic action of HPD.

- Our experiments support the view that in PDT the mild hyperthermia (around 44 °C) induced by photoirradiation can enhance synergistically the HPD-photoinduced tumor eradication. The obtained results suggest that the potentiating effect of light-induced heating on the antitumor efficiency of HPD-PDT could be largely mediated by a heat-induced increase in the formation of cytotoxic ROS, such as H_2O_2, $O_2^{-\bullet}$, and $OH^{\bullet}$. Also, studies indicated that photosensitization of tumor cells by HPD induces a strong decrease in the activity of catalase and glutathione peroxidase, and that heat stress sensitizes the H_2O_2-detoxifying enzymes to HPD-photoinduced inactivation; upon HPD-PDT, these events may result in loss of protection against accumulating H_2O_2. Moreover, our findings suggest that during PDT with HPD, an increase in the temperature of tumor tissues may enhance the efficiency of this therapy via the stimulation of a chlorin-type photoproduct formation that possesses photosensitizing activity.
- Tumor cells in an acidic environment (both in vitro and in vivo) are more sensitive to the phototoxic influence of E6 than at pH values found in normal tissues. Studies on the mechanism of the phenomenon showed that under acidification the impairment of mitochondrial function as well as the inhibition of E6 photobleaching in tumor cells are the main factors as to the revealed effect of pH on the antitumor efficiency of PDT with the sensitizer. Our findings suggest a new strategy for improving of the selectivity and the cure rate of PDT with chlorin-e_6-type PS(s); namely, via the induction of transient hyperglycaemia that causes a significant drop in the pH value of interstitial fluid of malignant tumors.

KASVAJARAKKUDE FOTOSENSIBILISEERITUD HÄVIMINE PORFÜRIINIDE JA KLORIINIDE TOIMEL

KOKKUVÕTE

Fotodünaamiline teraapia (FDT) on suhteliselt uus ning kiiresti arenev tõhus pahaloomuliste kasvajate ravimeetod. Töö eesmärgiks oli laiendada olemasolevaid teadmisi kasvajarakkude hävimise molekulaarsete mehhanismide mõistmiseks hematoporfüriini derivaadi (HPD, enim kasutatavam sensibilisaator pahaloomuliste kasvajate FDT-s) ja kloriin-e_6 trimetüülestri (E6, teise generatsiooni fotosensibilisaator (FS)) fotoergastamisel. Nimelt, töö ülesandeks oli hinnata: a) H_2O_2 ja hapniku radikaalide osalust ülalmainitud FS-te kasvajavastases toimes; b) kuidas HPD poolt fotosensibiliseeritud lipiidide ja proteiinide kahjustamine mõjub plasmamembraani terviklikkusele, mitokondrite funktsioonile ja rakkude hävimisele; c) pH ja temperatuuri mõju fotodünaamiliselt indutseeritud kasvajarakkude hävimisele ning d) kasvajarakkude mõningate antioksüdantsete kaitsesüsteemide osatähtsust HPD fototoksilisusele.

Doktoritöö olulisemad tulemused on järgmised:

- On vähetõenäone, et mitokondrite HPD fotosensibiliseeritud vigastamine, mis viib kasvajarakkude hävitamisele, on tingitud membraani lipiidide kahjustamisest. Saadud tulemuste kohaselt on raku proteiinid, kuid mitte lipiidid, kõige olulisemateks sihtmärkideks HPD-FDT mõjustusel.
- Singletsest hapnikust ($^1O_2$) erinevad hapniku reaktiivsed osakesed (HRO), nagu H_2O_2, superoksiidi ($O_2^{-\bullet}$) ja hüdroksüülradikaalid ($OH^\bullet$), võivad osaleda HPD manulusel FDT vähivastases toimes. Nimelt, Ehrlichi astsiidse kartsinoomi rakkude kiiritamine HPD manulusel tingib olulistes kogustes H_2O_2, $O_2^{-\bullet}$ ja $OH^\bullet$ radikaalide teket, mis nagu $^1O_2$ on kaasatud rakkude fotoinaktivatsiooni protsessi in vitro. H_2O_2, $O_2^{-\bullet}$ ning väga reaktiivse $OH^\bullet$ teke kasvajarakkudes HPD-FDT mõjutusel võib olla tingitud: (**i**) ksantiinoksüdaasi (XOD) aktiivsuse kasvust tänu ksantiindehüdrogenaasi (XDH) konversioonist XOD-iks läbi Ca^{2+} sõltuvate proteolüütiliste protsesside kui ka SH-rühmade oksüdatsioonist XDH-s; (**ii**) mõningate raku koostisosade (valgud, NAD(P)H) fotooksüdatsioonist. Leiti, et kasvajarakkudes HPD-FDT toimel Fenton tüüpi reaktsioonid etendavad olulist rolli $OH^\bullet$ tekkel.
- Leiti, et fotoergastatud HPD on võimeline genereerima kasvajarakkudes proteiinide peroksiide, mida käsitletakse kui uut HRO-te vormi. Proteiinide peroksiidide osatähtsust HPD-FDT kasvajavastases toimes oleks vaja uurida edaspidi.
- Kloriin-e_6 tüüpi FS-te manulusel FDT kasvajavastane toime võib olla seotud H_2O_2, $O_2^{-\bullet}$ ja $OH^\bullet$ tekkega.
- Cu/Zn-superoksiidi dismutaas, katalaas ja glutatiooni redokstsükkel on võimelised kaitsma transformeerunud rakke HPD fototoksilise toime eest.

- Eksperimentide andmed toetavad seisukohta, et kiiritamise käigus esinev pehme hüpertermia (44 °C) võib sünergistlikult HPD kasvajavastast toimet suurendada. Saadud tulemused annavad alust arvata, et kiiritamisel võib kudede soojenemise soodustav mõju HPD-FDT vähivastasele toimele olla seotud tsütotoksiliste HRO H_2O_2, $O_2^{-\bullet}$ ja $OH^{\bullet}$ tekkega. Lisaks langes katsetes oluliselt katalaasi ja glutatiooni peroksüdaasi aktiivsus ning termiline stress suurendas H_2O_2 lagundavate ensüümide tundlikkust HPD fotoindutseeritud inaktivatsioonile. Seega võivad need nähtused vähendada rakkude kaitset HPD-FDT käigus tekkiva H_2O_2 vastu. Uuringust tuleneb, et HPD-FDT toimel võib kasvajakoe temperatuuri tõus tõhustada teraapia efektiivsust tänu kloriin-tüüpi fotoprodukti tekke stimulatsioonile. Seejuures tekkiv fotoprodukt võib ise täita FS rolli.
- Kasvajarakud on happelises keskkonnas (in vitro kui ka in vivo) tundlikumad E6 fototoksilisele toimele võrreldes normaalsete kudedega. Nähtuse toimemehhanismi uurimine näitas, et arvatavasti on mitokondrite vigastamine ja E6 fotolagunemise inhibeerimine rakkudes põhilisteks faktoriteks, mis tingivad pH mõju E6-FDT kasvajavastases toimes. Seda võiks ära kasutada FDT tõhustamise strateegia arendamisel kloriin-e_6 tüüpi sensibilisaatorite manuluse puhul, tekitades ajutise hüperglükeemia, mille tulemusena oluliselt alaneb pH kasvajasiseses koes.

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