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INCREASING THE EFFICIENCY OF PHOTOSENSITIZED DAMAGE OF EHRLICH ASCITES CARCINOMA CELLS USING HAEMATOPORPHYRIN DERIVATIVE OR CHLORIN-e_6 IN COMBINATION WITH LONIDAMINE

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Abstract. Lonidamine (a dichlorinated derivative of indazole-3-carboxylic acid) is an anti-spermatogenic and anticancer drug, which is believed to act as an inhibitor of energy metabolism. In this study the effect of lonidamine (LND) on the efficacy of photodynamic therapy of tumour was investigated. In in vitro experiments the presence of 0.25 mM LND increases synergistically (up to 2 fold) the intensity of hematoporphyrin derivative (HpD) or chlorin-e_6 trimethylester photosensitized damage of Ehrlich ascites carcinoma cells. Considerable inhibition of Ehrlich carcinoma rate growth in mice (the ascites form of tumour) was observed in vivo when HpD phototherapy (up to 5 mg/kg) was combined with LND (intraperitoneal injection of 0.1 mM/kg, before photoirradiation). The analysis of the interaction between LND and in vivo HpD photodynamic therapy demonstrated an additivity of response. The present effect of LND on the efficacy of phototherapy is mainly caused by the inhibitory effect of LND on the energy production system of tumour cells. Indeed, the increase of photodamage of tumour cells induced by LND was accompanied with an acceleration of the rate of decreasing their glycolytic activity and, at higher extent, oxygen consumption. We assume that the intensification of the reduction of the ATP content under photosensitization in the presence of LND was induced mainly by the inactivation of mitochondrion. Moreover, under hypoglycaemia neoplastic cells are more sensitive toward phototherapy combined with LND. We found that the mechanism of the potentiating effect of LND on HpD-phototherapy probably has a highly complex character. Thus, antineoplastic effect of LND and its combination with HpD-phototherapy is enhanced with increasing extracellular levels of calcium and magnesium cations. The results obtained indicate that the potentiating action of LND on phototherapy may be also explained by the injurious effect of LND on the cytoskeleton structure and cytoplasm membrane of tumour cells.

Key words: photodynamic therapy, tumour, inhibitors of energetic metabolism, lonidamine, calcium, magnesium.

Abbreviations: GSH = glutathione; EAC = Ehrlich ascites carcinoma; E6 = chlorin-e_6 trimethyl-ester; HpD = hematoporphyrin derivative; LND = lonidamine; MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PII = photofrin-II; PDT = photodynamic therapy; TB = trypan blue; PBS = sodium phosphate buffer; $[Ca^{2+}]_i$ = intracellular concentration of calcium cations.

INTRODUCTION

During the last decade the chemistry of tetrapyrrole compounds has made a rapid progress. The field of the application of these compounds is rather wide, including chemical and technological processes, ecology, and medicine. A very interesting field of the use of tetrapyrrole compounds has opened thanks to their photodynamic activity. Under aerobic conditions and presence of light these compounds induce the generation of highly reactive and cytotoxic molecular species, including singlet oxygen and free radicals, which cause damage of biomolecules, cellular components, pathogenic viruses, bacteria, and tumour cells.

Photodynamic therapy (PDT) of tumour is based on the injection of photosensitizers into the organism or directly into the tumour and irradiation of the neoplasm with light specifically absorbed by photosensitizer. When hematoporphyrin derivative (HpD), photofrin-II (PII), or chlorin-e_6 trimethylester (E6) are used as photosensitizer the energetic metabolism of the neoplasm cells is one of the most sensitive targets for the photodynamic damage. In particular, both in vitro (Hilf et al., 1986) and in vivo (Ceckler et al., 1986) the ^{13}P-NMR technique has demonstrated that the earliest stage in biochemical changes after tumour HpD–PDT was the depletion of ATP pool. The latter may in consequence of and in aggregation with other actions (e.g. disturbance of structural integrity of the cell membrane), start up a series of events, which could cause irreversible damage of malignant cells. However, the exact mechanism of cellular damage under depletion of ATP pool is not sufficiently investigated. It has been proposed that the decrease in ATP concentration of the cells leads to an increase in intracellular concentration of calcium ions $[Ca^{2+}]_i$, which activate Ca^{2+}-dependent proteases and phospholipases that destroy cellular components (Nicotera et al., 1989). Deenergization of tumour cells under PDT could be a result of slowing down of glycolysis and respiration activity. Several important glycolytic enzymes (e.g. lactate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase, glucose-6-phosphate isomerase) in tumour cells were shown to be inactivated after photosensitization (Hilf et al., 1984; Prinsze et al., 1989). The prevalent concept is that under HpD–PDT the injury of mitochondria of tumour cells is responsible for the cell death. After dark incubation the active fraction of HpD is localized at nuclear envelope, mitochondria, and the nearby nucleus area that is rich in lysosomes and mitochondria (Berns et al., 1982; Kessel, 1986). Subsequent exposure to the light leads to aggregation of mitochondria and disruption of cristae, swelling of lysosomes and channels of endoplasmic reticulum, damaging of nuclear structures with lysis of chromatin (Ting & Zong-he, 1984). Numerous researchers suggest that mitochondria, in comparison with other cell organelles or structures, are the critical intracellular targets for HpD photosensitization, as the changes in the mitochondrial structure take place at the earliest stage after photoirradiation (Roberts et al., 1989). Moreover, the HpD or PII photosensitization of tumour cells not only decreases the oxygen consumption, but also inhibits the whole group of mitochondrial

enzymes: cytochrome-C-oxidase, F_0F_1-ATPase, succinate dehydrogenase, NADH-dehydrogenase (Hilf et al., 1984; Gibson et al., 1989), adenylate kinase, and monoamine oxidase (Murant et al., 1987). The suppression of mitochondrial activity after PDT leads to a significant decrease in the cellular ATP level and, consequently, to a loss of viability (Hilf et al., 1986).

The purpose of the present work was to investigate the mechanism of and approaches to increasing the efficiency of PDT by means of its combination with a drug, lonidamine (LND), which suppresses the energetic metabolism of tumour cells. Hilf et al. (1986) reported a strong potentiation effect of iodoacetate (inhibitor of glycolysis) on HpD phototherapy of mammary adenocarcinoma. The combination of PDT with oligomycin (an inhibitor of respiration) did not give such effect. Special attention was drawn to anthracycline antibiotics and related drugs. These drugs as well as HpD localized in mitochondria of tumour cells. One possible mechanism of action of anthracycline antibiotics is uncoupling of phosphorylation, inhibition of cytochrome-C-oxidase, increase in permeability of mitochondrion membrane (Bianchi et al., 1987; Muhamed et al., 1982), decrease in the cellular ATP level and, consequently, a loss of viability of tumour cells (Ahmann et al., 1987). Cowled et al. (1987) showed an enhancing effect of 0.5–4 mg/kg adriamycin on HpD-phototherapy when injected into mice with Lewis lung carcinoma. Adriamycin was administered together with HpD and also directly before irradiation. Similar results were obtained when adriamycin was combined with E6-phototherapy (Chekulayev et al., 1991). The increase in photosensitized damage of Ehrlich ascites carcinoma (EAC) cells induced by adriamycin was accompanied by a higher speed of deenergization of cells (the depletion of the intracellular content of ATP). The latter was caused by inhibition of glycolysis and respiration. Cho and co-workers (1992) reported a potentiation effect of adriamycin on PII–PDT of mouse bladder tumour MBT-2. However, the traditional antineoplastic drugs have demonstrated a number of negative side-effects. For example, adriamycin shows strong cardiotoxicity (Klugmann et al., 1982) and can increase the nonspecific phototoxicity of a photosensitizer. These circumstances complicate their clinical use in combination with PDT. In order to increase the photodamage of neoplasm cells, preparations with lower toxicity but still enabling to suppress the energy metabolism of tumour cell should be examined.

Taking into account the mechanism of photosensitized damage of neoplasm cells, we suggest the use of LND. Comprehensive investigations made during the last decade have shown that LND, a dichlorinated derivative of indazole-3-carboxylic acid, is a nonmutagenic antispermatogenic drug with a large profile of antitumour activity. Clinical studies have reported antitumour activity of LND in the cases of breast and kidney cancer, sarcomas of soft tissues, and lung cancer (Follow-up Report, 1987). In addition, LND can enhance the antitumour and cytotoxic effect of hyperthermia (Kim et al., 1984b), radiosensitization (Kim et al., 1984a), several antineoplastic alkylation agents (Silvestrini et al., 1992), and adriamycin (Savini et al., 1992). The mechanism of the antineoplastic

ability of LND is related to the damage of the cellular energy system. It has been shown that LND inhibits the oxygen consumption of both normal and malignant cells (Floridi et al., 1981b) by blocking the electron-transport chain between primary dehydrogenases and the respiration chain of mitochondria (Floridi & Lehninger, 1983). Also, LND stimulates the aerobic glycolysis in normal cells and inhibits that in neoplastic cells by affecting the mitochondrially-bound hexokinase, which is not present in normal differentiated cells (Floridi et al., 1981b). LND may mediate considerable structural damages of mitochondria (swelling, disruption of cristae) (Floridi et al., 1981a, 1985) as well as that of plasma membrane (Malorni et al., 1988).

MATERIALS AND METHODS

Chemicals

Haematoporphyrin derivative was synthesized from haematoporphyrin dihydrochloride (Aldrich) by the method of Lipson et al. (1961) modified by Kessel et al. (1987). Chlorin-e_6 trimethyl ester (E6) was prepared on the basis of pheophytin-a extracted from nettle leaves according to the method of Lötjönen & Hynninen (1980). LND, obtained from F. Angelini Research Institute, Rome, Italy (Lot 4/A), was dissolved in DMSO up to 62 mM. All other chemicals were obtained from Sigma Chemical Co., St. Louis.

Animals and tumours

White mongrel 4-month-old female mice obtained from the Institute of Experimental and Clinical Medicine, Tallinn, were used in experiments. Ehrlich ascites carcinoma (EAC) cells were weekly transplanted intraperitoneally (i.p.) on mice, 3×10^7 cells per animal.

In vitro experiments. To carry out the experiments in vitro, a 6–8 day tumour was withdrawn from the mouse peritoneal cavity, 4 volumes of an isotonic solution (either Hanks' balanced salt solution without phenol red; 154 mM NaCl with 6.2 mM KCl; or 0.9% NaCl) were added, and the cell suspension obtained was centrifuged at 4 °C for 7 min. The precipitated cells were resuspended in one of the isotonic solutions described above and stored on ice-bath until the beginning of experiment. The cell suspension was diluted with a medium to a concentration of 5×10^6 per ml, the buffer (1 M Tris-HCl or HEPES, pH 7.2) was added up to a concentration of 10 mM. Immediately or 5 min after the addition of the photosensitizer that was dissolved in 0.9% NaCl (HpD) or in a minimal volume (5–10 µl) of DMSO (E6), the cells were irradiated at 665 and 630 nm for E6 and HpD, respectively. The irradiation was carried out in a quartz cuvette (layer thickness 2 cm and volume 8 ml) by magnetic stirring at 20 °C. A 1 kW xenon lamp instrumented with a focusing optical system and a light filter served as the radiation source.

The amount of injured cells in the suspension was evaluated by staining the cells with 0.05% trypan blue (TB), additionally an MTT assay was used. The MTT assay is based on the ability of dehydrogenases of viable cells to convert the yellow form of MTT into a purple formazan (Wilson et al., 1990). For the determination of the dehydrogenase activity of EAC cells, 0.5 ml of MTT stock solution (2 mg/ml) dissolved in Hanks' solution with 10 mM HEPES (pH 7.2) was added to 10^6 cells and incubated for 2 h in a water bath at 37 °C. At the end of the incubation period the samples were centrifuged at 4500 rev/min for 10 min. The supernatant was removed and the sediment was dissolved in 2 ml DMSO. The absorbance of the converted dye was measured at a wavelength of 570 nm.

The rate of glycolysis was estimated by lactate formation. Carcinoma cells (1×10^7 cells/ml) were incubated for 1 h in Hanks' solution or the medium containing 154 mM NaCl, 6.2 mM KCl, 5.55 mM glucose, and 10 mM sodium phosphate buffer, PBS (pH 7.2), on a water bath at 37 °C. The lactate was measured enzymatically by lactate dehydrogenase using a Sigma Kit (USA). The respiration was estimated by the consumption of oxygen at 37°C, using a Clark oxygen electrode.

ATP was extracted from cells with trichloroacetic acid/EDTA (5%/4mM) and assayed with a luciferin/luciferase by means of Luminometer 1251 (BioOrbit, Turku, Finland), essentially as described by Kahru et al. (1982).

The total content of the reduced form of glutathione (GSH) in EAC cells was determined by the method of Ellman (1959) as described by Chekulayev et al. (1992a).

Since NADH is responsible for the fluorescence band at 470 nm in normal as well as tumour cells (Andersson-Engels et al., 1991), the content of NADH in EAC cells was measured fluorimetrically. The fluorescence of cell suspension (1×10^7 cells/ml) was measured at 470 nm (excitation at 340 nm) in a 1 cm quartz cuvette under magnetic stirring at 20 °C.

The influence of LND and Ca^{2+} and Mg^{2+} ions on the uptake of HpD by tumour cells was estimated under continuous stirring at 20 °C in the media without serum. HpD was added to EAC cell suspension (5×10^6 cells/ml) (final concentration up to 2 μg/ml), then LND in DMSO and 10% solution of $MgCl_2 \cdot 6H_2O$ or $CaCl_2 \cdot 9H_2O$ was added. After 1 min of incubation 0.4 ml of samples was taken, as the zero point, and immediately centrifuged at 4500 rev/min for 5 min. A supernatant containing nonbound HpD was discarded and cellular pellet was lysed in 1.5 ml of 3% NaOH for 1 h. The content of HpD in the lysate was measured fluorimetrically at 617 nm (excitation at 394 nm). Protein concentration in lysate was assayed according to the method of Lowry et al. (1951).

In vivo experiments. The in vivo experiments were carried out with 25 g white mongrel female mice to whom 0.25 ml of EAC was injected i.p. Three days after the neoplasm inoculation, sterile HpD solution in 0.9% NaCl was injected i.p. at a concentration up to 5 mg/kg. Irradiation was carried out the next day. LND dissolved in DMSO was injected i.p. up to a concentration of 0.1 mM/kg 12 min prior to irradiation. Photoirradiation of mice was carried out under narcosis (sodium thiopental, rectal injection)

with the light (630 nm) isolated from the spectrum of a 1 kW xenon lamp. The flux of light was focused into the stain (D. 2.5 cm) by means of an optical system and directed into the abdominal region of mice. At the irradiation intensity 400 mW, the light exposure time was 12 min (the energetic exposure 288 J). Five days after neoplasm inoculation photo-irradiation was repeated. In this case LND was injected i.p. (0.1 mM/kg) 12 min prior to irradiation. At the irradiation intensity 400 mW, the light exposure time was 6 min (energetic exposure 144 J). One day after the last photoirradiation, LND was injected i.p. (0.1 mM/kg) again but without subsequent irradiation.

3. RESULTS

Lonidamine and photodynamic injury of EAC cells

Figure 1 and 2 demonstrate that the kinetics of the death of tumour cells was S-type and did not depend on the type of the photosensitizer used (HpD or E6). At the same time, after a lag period during which the number of stained cells remained approximately the same as that of control ones, a considerable increase in the rate of inclusion of TB was observed. This indicates an essential injury of carcinoma cells. However, irradiation of EAC cells in the absence of photosensitizer, as well as their incubation with photosensitizer in the dark at 20 °C, did not cause any noticeable increase in the number of injured cells. It should be noted that, despite the test used for the estimation of the viability of cells after PDT (inclusion of TB or of ethidium bromide, release of ^{51}Cr, etc.), the shape of the kinetic curve always had a well expressed lag period. The duration of the lag period depended considerably on the type of cells and photosensitizer, the composition of the irradiated medium, and the time interval after tumour transplantation. That is, the duration of the lag period, followed by an exponential loss of viability, determines the resistance of neoplasm cells to PDT. Then, the efficacy of photodamage of cells in the TB test was expressed as a light dose or irradiation time needed for staining 50% of the tumour cells (τ_{50}). The influence of LND on photodamage of EAC cells was investigated in the presence of HpD and E6. Thanks to its better spectral properties, E6 might be an alternative photosensitizer to HpD (Chekulayev et al., 1991). Moreover, E6 (and not HpD) is able to react in Type I photoreactions (Chekulayev et al., 1992b). The results obtained showed that 0.25 mM of LND acted synergistically increasing HpD or E6 photodamage of cells almost two-fold (Figs. 1, 2). Indeed, photoirradiation of cells in the presence of 0.25 mM of LND without photosensitizer did not increase the amount of stained cells.

The ability of PDT with HpD or E6 to diminish not only the growth of solid tumours, but also the ascites type of tumours was shown (Chekulayev et al., 1992b). Our experimental data have shown a linear relationship (with approximately equal slopes) between the concentration of tumour cells in ascites and the weight of ascites of mice as well as of animals affected by HpD–PDT (Fig. 3). This permits us to estimate the growth of

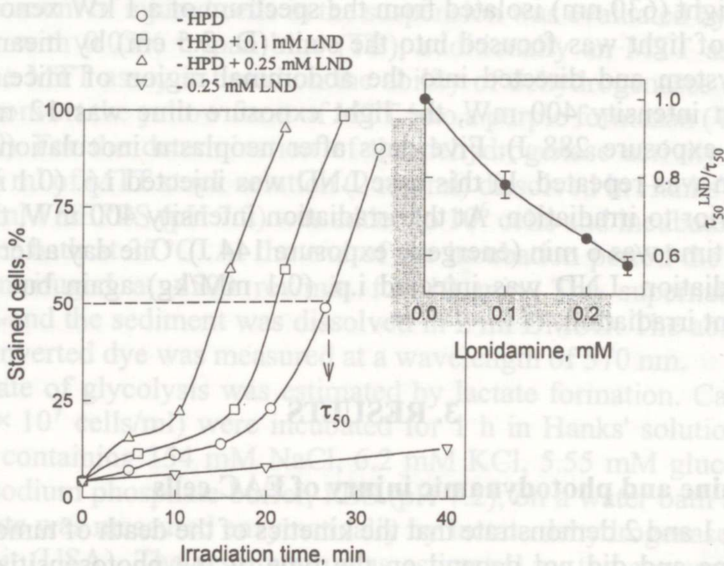

Fig. 1. Influence of LND on 1 μg/ml HpD–PDT (220 mW) injury of EAC cells (5 × 10^{6} cells/ml) in Hanks' medium with 10 mM HEPES, pH 7.2. HpD and LND were incubated for 5 min with cells prior irradiation. Insert: Effect of LND concentration on relative irradiation time needed for the inactivation of 50% of the cells (τ_{50}), measured by the TB test. Bars = SE.

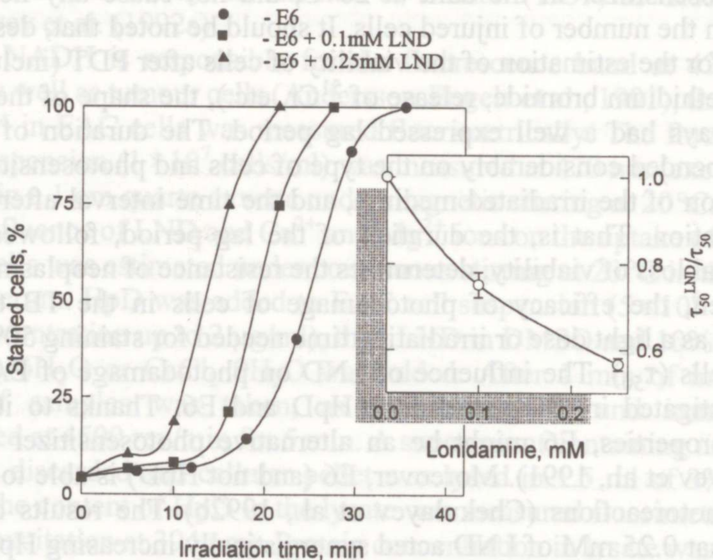

Fig. 2. Effect of LND on 2 μM E6-PDT (120 mW) injury of EAC cells (5 × 10^{6} cells/ml) in Hanks' medium with 10 mM HEPES, pH 7.2. E6 and LND were incubated for 5 min with cells prior irradiation. Insert: Effect of LND concentration on relative irradiation time needed for the inactivation of 50% of the cells (τ_{50}). Bars = SE.

EAC by weighing the tumour-bearing mice. Considerable inhibition of tumour growth in vivo was caused when HpD-phototherapy was combined with the use of LND (Fig. 4) (the effect was obviously additive).

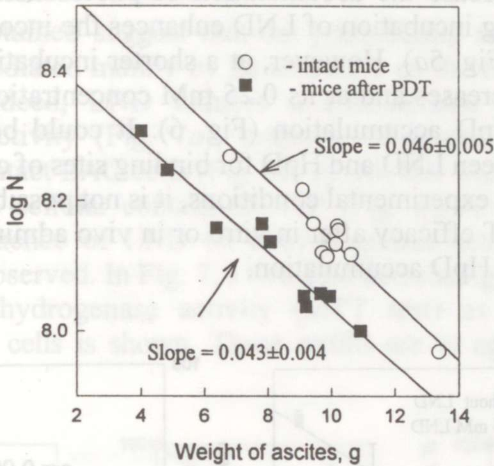

Fig. 3. The relationship between the concentration of tumour cells (N, cells/ml) and weight of ascites in intact ascites bearing mice (8th day after i.p. transplantation of tumour) and animals affected by HpD–PDT. For HpD–PDT 5 mg/kg HpD was i.p. administrated to animals on the third day after of EAC transplantation, on the 4th day animals were irradiated with 630 nm and dose 240 J, and on the 5th day with dose 168 J.

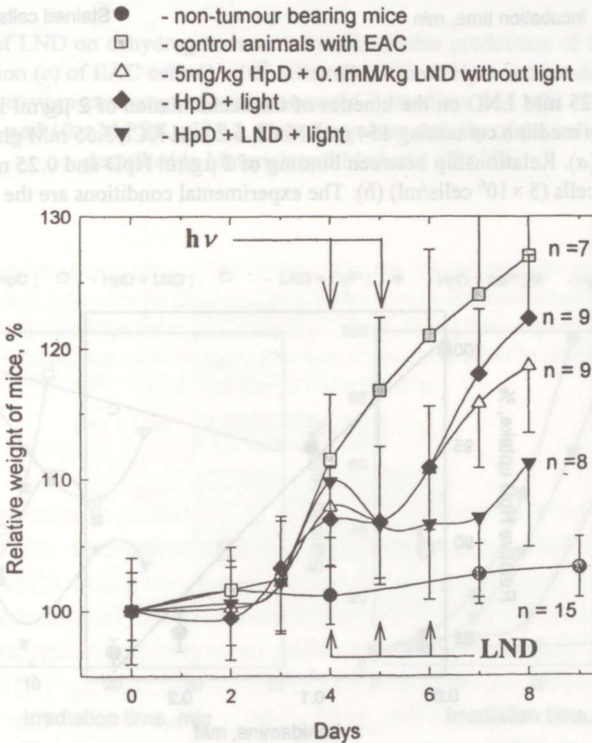

Fig. 4. Change in the weight of tumour-bearing mice before and after PDT. Mice were injected 5 mg/kg HpD on the third day and 0.1 mM/kg LND on the 4th, 5th, and 6th days after EAC inoculation. The dose of the 1st irradiation was 288 J and of the 2nd 144 J. Bars = SE.

LND may increase the accumulation of photosensitizer in neoplasm cells. Indeed, long incubation of LND enhances the incorporation of HpD into EAC cells (Fig. 5*a*). However, at a shorter incubation time (10 min) LND slightly decreases and at its 0.25 mM concentration leads to a 15% deprivation of HpD accumulation (Fig. 6). It could be explained by a competition between LND and HpD for binding sites of cellular structures. So, under present experimental conditions, it is not possible to interpret the enhancing of PDT efficacy after in vitro or in vivo administration of LND as the increase in HpD accumulation.

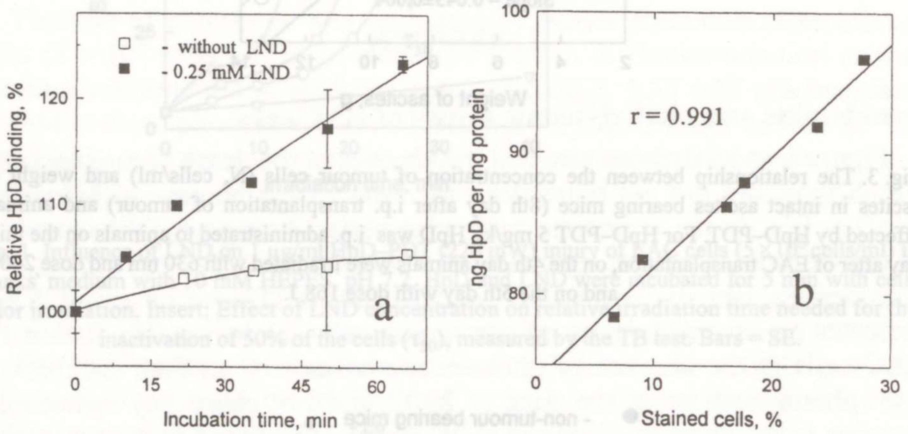

Fig. 5. Effect of 0.25 mM LND on the kinetics of the accumulation of 2 μg/ml HpD in EAC cells (5×10^6 cells/ml) in medium containing 154 mM NaCl, 6.2 mM KCl, 5.55 mM glucose, and 10 mM Tris-HCl, pH 7.4 (*a*). Relationship between binding of 2 μg/ml HpD and 0.25 mM LND induced injury of EAC cells (5×10^6 cells/ml) (*b*). The experimental conditions are the same as in (*a*).

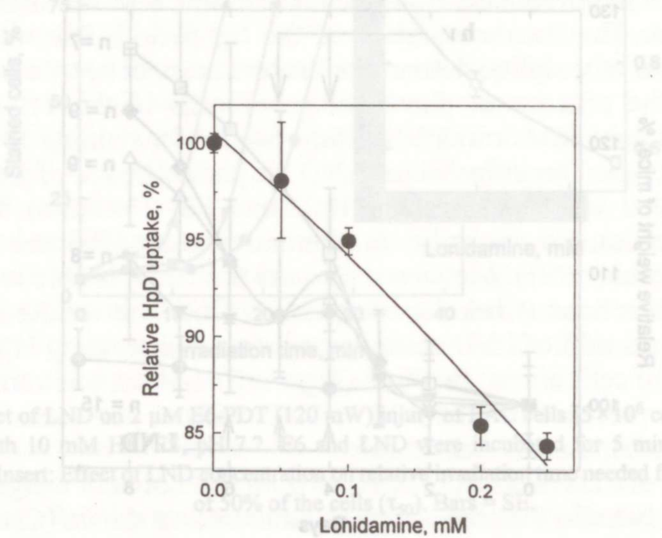

Fig. 6. Influence of LND on 2 μg/ml HpD binding with EAC cells (5×10^6 cells/ml) after 10 min incubation in Hanks' medium with 10 mM Tris-HCl, pH 7.4.

Energy metabolism and lonidamine

The results obtained suggest that the potentiating action of LND on PDT could be mediated mainly by its alteration of the energetic status of tumour cells. Indeed, LND induces a considerable decrease in the dehydrogenase activity (Fig. 7*a*), intensity of glycolysis, and oxygen consumption of intact EAC cells (Fig. 7*b*, *c*). All this was accompanied by a decrease in the cellular content of ATP (Fig. 8). A tight relationship between the influence of LND on dehydrogenase activity and oxygen consumption is observed. In Fig. 7, a two-fold decreasing effect of 0.2 mM LND on the dehydrogenase activity (MTT test) as well as on the respiration of the cells is shown. These results are in agreement with the

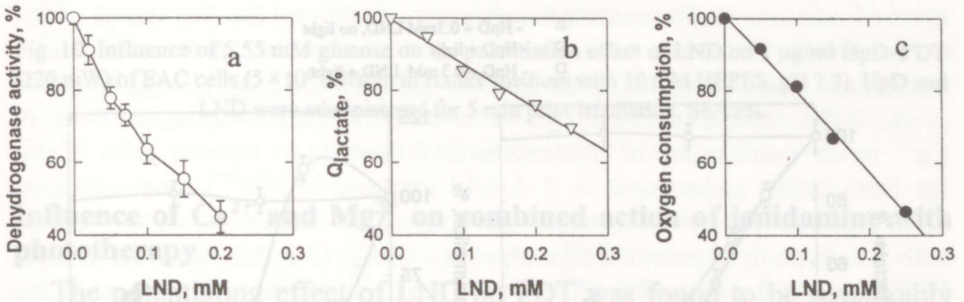

Fig. 7. Influence of LND on dehydrogenase activity (*a*), aerobic production of lactic acid (*b*), and oxygen consumption (*c*) of EAC cells (1×10^7 cells/ml) (without HpD and irradiation). Production of lactic acid and oxygen consumption were measured in medium 154 mM NaCl, 6.2 mM KCl, 5.55 mM glucose, and 10 mM PBS, pH 7.2. Dehydrogenase activity of the cells was measured as described in Materials and Methods. SE<5%.

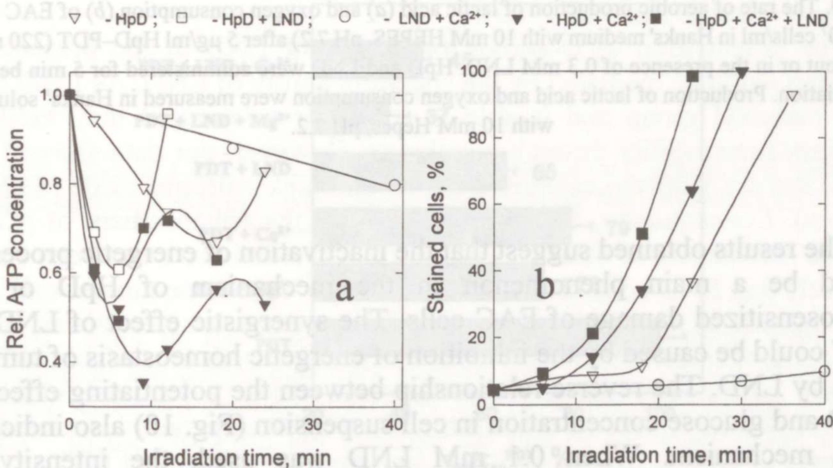

Fig. 8. Contents of ATP in EAC cells (1×10^7 cells/ml) (*a*) and injury of cells after 2 μg/ml HpD–PDT (220 mW) (*b*) in the presence of 0.15 mM LND and/or 1.25 mM $CaCl_2$ in Hanks' medium (without phenol red and $CaCl_2$) with 10 mM HEPES, pH 7.2. SE<7%.

results of other researchers (Floridi & Lehninger, 1983) who postulate that the effect of LND on diminishing the oxygen consumption is mainly caused by the inhibition of mitochondrion dehydrogenases and not the enzymes of electron transport chain.

Induced by LND increase in the EAC cells photodamage was accompanied with an acceleration of the rate of decreasing their glycolytic activity and, at higher extent, the respiration (Fig. 9). The ATP level in cells is mainly maintained by glycolysis and oxidative phosphorylation. We assume that the intensification of the diminishing of the ATP content under photosensitization in the presence of LND was induced mainly by the inactivation of mitochondrion.

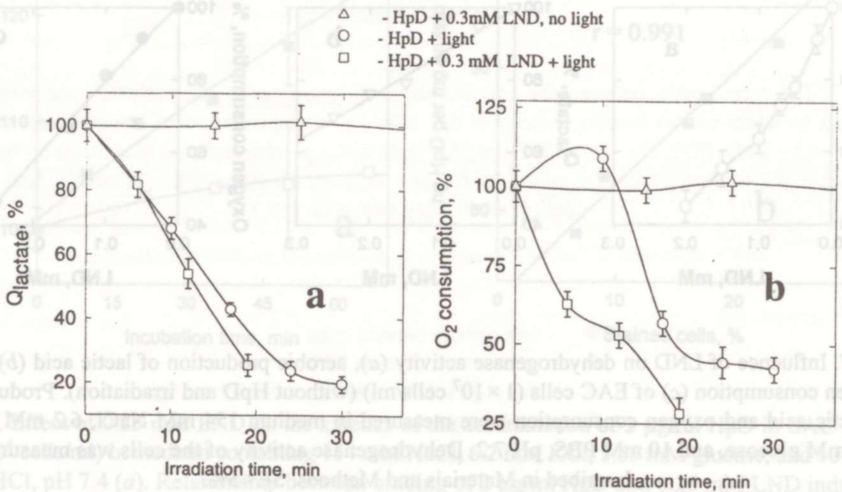

Fig. 9. The rate of aerobic production of lactic acid (*a*) and oxygen consumption (*b*) of EAC cells (4×10^7 cells/ml in Hanks' medium with 10 mM HEPES, pH 7.2) after 5 μg/ml HpD–PDT (220 mW) without or in the presence of 0.3 mM LND. HpD and LND were administered for 5 min before irradiation. Production of lactic acid and oxygen consumption were measured in Hanks' solution with 10 mM Hepes, pH 7.2.

The results obtained suggest that the inactivation of energetic processes could be a main phenomenon in the mechanism of HpD or E6 photosensitized damage of EAC cells. The synergistic effect of LND on PDT could be caused by the inhibition of energetic homeostasis of tumour cells by LND. The reverse relationship between the potentiating effect of LND and glucose concentration in cell suspension (Fig. 10) also indicates such mechanism. When 0.1 mM LND was used, the intensity of photodamage of EAC cells was 1.5 times as high in the experiments without glucose as that in the presence of 5.55 mM glucose. Consequently, at hypoglycaemia neoplasm cells are more sensitive towards PDT combined with LND.

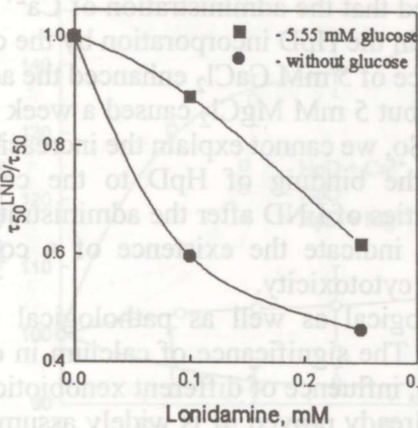

Fig. 10. Influence of 5.55 mM glucose on the potentiation effect of LND on 1 μg/ml HpD–PDT (220 mW) of EAC cells (5×10^6 cells/ml in Hanks' medium with 10 mM HEPES, pH 7.2). HpD and LND were administered for 5 min prior irradiation. SE<5%.

Influence of Ca^{2+} and Mg^{2+} on combined action of lonidamine with phototherapy

The potentiating effect of LND on PDT was found to be remarkably dependent on the extracellular level of calcium and magnesium ions. Both cations increased the injuring effect of LND on EAC cells photosensitization. The photosensitized damage of tumour cells in the presence of 0.2 mM LND was remarkably increased (1.5 times) when Ca^{2+} and Mg^{2+} at concentrations up to 2.5 mM were added to the cells (Fig. 11). At least an additive effect of LND and both cations was observed.

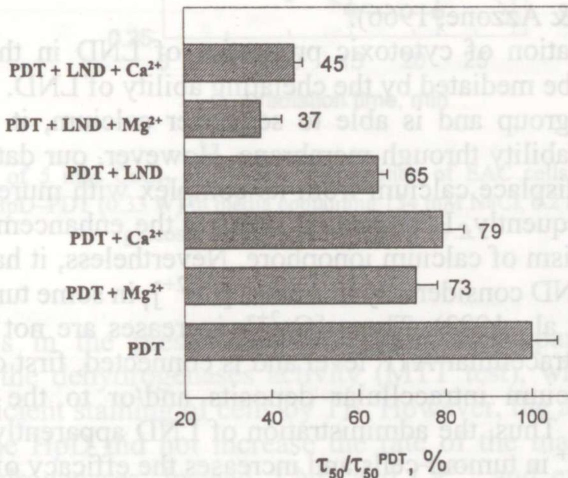

Fig. 11. Effect of $MgCl_2$ or $CaCl_2$ (both 2.5 mM) in combination with 0.2 mM LND on 1 μg/ml HpD–PDT (220 mW) of EAC cells (5×10^6 cells/ml in media with 154 mM NaCl, 6.2 mM KCl, 5.55 mM glucose, and 10 mM HEPES, pH 7.2).

It can be supposed that the administration of Ca^{2+} or Mg^{2+} to the cells leads to an increase in the HpD incorporation by the cells. The incubation of cells in the presence of 5 mM $CaCl_2$ enhanced the accumulation of HpD up to 30% (Fig. 12) but 5 mM $MgCl_2$ caused a week (up to 7%) decrease in the HpD content. So, we cannot explain the increasing effect of Mg^{2+} on PDT by affecting the binding of HpD to the cells. An increase in antineoplastic properties of LND after the administration of Ca^{2+} or Mg^{2+} to the media might indicate the existence of a common stage in the mechanisms causing cytotoxicity.

A major physiological as well as pathological role in the cells is assigned to calcium. The significance of calcium in cytotoxicity induced by anoxia, starvation, influence of different xenobiotics, and immunologic reactions has been already proved. It is widely assumed that an increased level of calcium might mediate the damage of cells by the disruption of the cytoskeleton structure, activation of phospholipases, proteases, endonucleases, and damage of mitochondria (Azzi & Azzone, 1966; Nicotera et al., 1989; Pounds, 1990; Agarwal et al., 1991). However, the role of Ca^{2+} in the mechanism of photosensitized damage of tumour cells is still far from being understood. A 3–5-fold increase in $[Ca^{2+}]_i$ concentration after HpD or Al-phthalocyanine photodynamic treatment of carcinoma cells has been demonstrated (Rasch et al., 1992). Our data showed that the PDT induced damage of the EAC cells was considerably increased in the presence of physiological concentration of calcium. This was expressed by an increase in the cytoplasm membrane permeability for vital dyes (Fig. 8), by the enhancement of intracellular content of free fatty acids (data not shown), by the increase in ATP depletion (Fig. 8), and by the decrease in the oxygen consumption by EAC cells (Fig. 13). The same happened under photosensibilization of cells in the presence of LND. The increasing effect of calcium cations on the photodamage in EAC cells appears to be to some extent due to the increase in the mitochondrion damage (Azzi & Azzone, 1966).

The potentiation of cytotoxic properties of LND in the presence of calcium could be mediated by the chelating ability of LND. As LND has a free carboxyl group and is able to sequester calcium, it may facilitate calcium permeability through membrane. However, our data showed that LND did not displace calcium from its complex with murexide (data not shown). Consequently, LND cannot mediate the enhancement of $[Ca^{2+}]_i$ by the mechanism of calcium ionophore. Nevertheless, it has been shown recently that LND considerably increases $[Ca^{2+}]_i$ in some tumour cell lines (Castiglione et al., 1993). These $[Ca^{2+}]_i$ increases are not related to the depletion of intracellular ATP level and is connected, first of all, with the release of calcium intracellular deposits and/or to the inhibition of Ca^{2+}-ATPases. Thus, the administration of LND apparently mediates the increase in Ca^{2+} in tumour cells and increases the efficacy of PDT.

However, some unexpected results also became evident during the investigation of the influence of LND and exogenic Ca^{2+} on the photodamage of neoplasm cells. Figure 14 shows that the photoirradiation

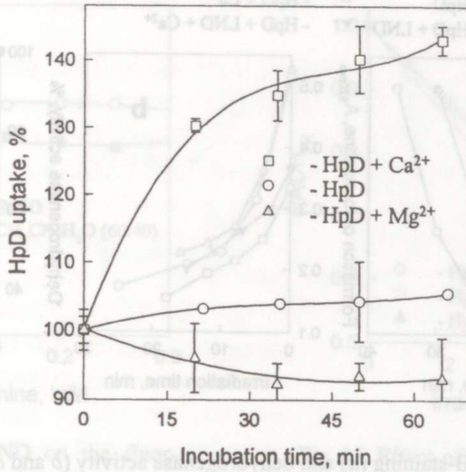

Fig. 12. Effect of $MgCl_2$ and $CaCl_2$ (both 5 mM) on the uptake of 2 μg/ml HpD to EAC cells (5×10^6 cells/ml) under dark incubation in media with 154 mM NaCl, 6.2 mM KCl, 5.55 mM glucose, and 10 mM Tris-HCl, pH 7.2.

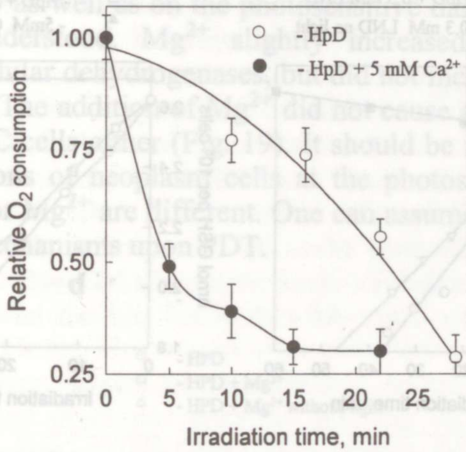

Fig. 13. Effect of 5 mM $CaCl_2$ on oxygen consumption of EAC cells (4×10^7 cells/ml) under 5 μg/ml HpD–PDT (0.33 W) in media containing 154 mM NaCl, 6.2 mM KCl, 5.55 mM glucose, and 10 mM HEPES, pH 7.2.

of EAC cells in the presence of HpD was accompanied with 70% reduction in the dehydrogenases activity (MTT test), which earlier had revealed sufficient staining of cells by TB. However, the addition of LND or Ca^{2+} to the HpD did not increase the rate of the inactivation of the cellular dehydrogenases. Instead, LND and Ca^{2+}, and even to a higher extent their combination, inhibit the destruction of dehydrogenases (Fig. 14*c*), increasing the content of GSH (Fig. 15). GSH plays an important role in the detoxification of endogenously formed peroxides. It is important

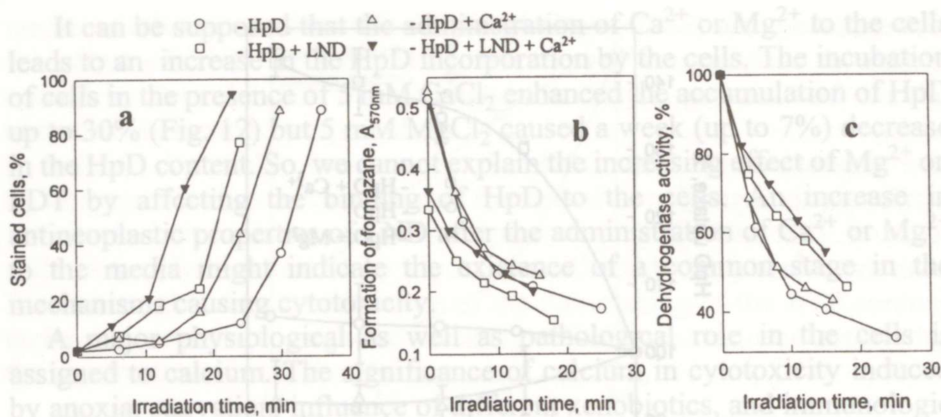

Fig. 14. The kinetics of TB-staining (*a*) and dehydrogenase activity (*b* and *c*) of EAC cells (5×10^6 cells/ml) after 1 µg/ml HpD–PDT (220 mW) in the presence of 2.5 mM $CaCl_2$ or/and 0.2 mM LND. Composition of the incubation medium: 154 mM NaCl, 6.2 mM KCl, 5.55 mM glucose, and 10 mM HEPES, pH 7.2.

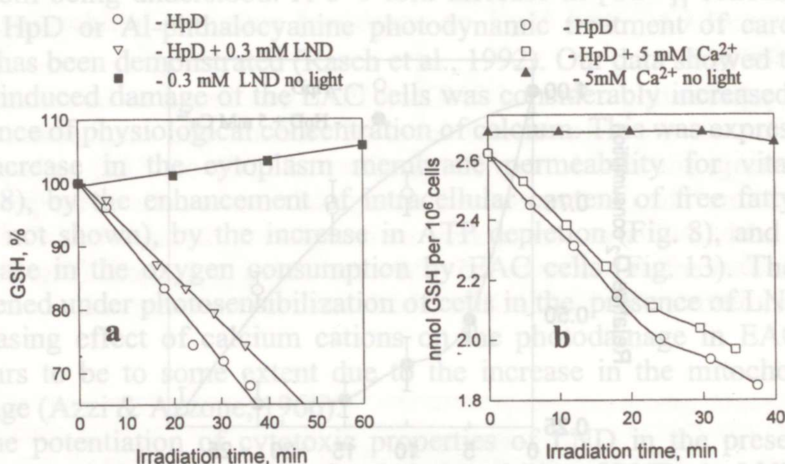

Fig. 15. Influence of 0.3 mM LND (*a*) and 5 mM $CaCl_2$ (*b*) on glutathione content in EAC cells (4×10^7 cells/ml) under 5 µg/ml HpD-PDT (220 mW) in the incubation medium containing 154 mM NaCl, 6.2 mM KCl, 5.55 mM glucose, and 10 mM HEPES, pH 7.2.

to note that during PDT photosensitized oxidation of some biomolecules (tryptophan, NAD(P)H, unsaturated fatty acids, etc.) yields organic peroxides and H_2O_2. One can assume that the higher level of GSH and the lower level of peroxides in EAC cells after irradiation in the presence of LND could be due to the antioxidant properties of LND and/or its ability to quench the excited states of photosensitizer. However, our experiments did not reveal the above-mentioned properties of LND and Ca^{2+} (data not shown). LND did not alter the fluorescence of HpD cell free solution (Fig. 16) or the rate of HpD-photosensitized oxidation of NADH (Fig. 17).

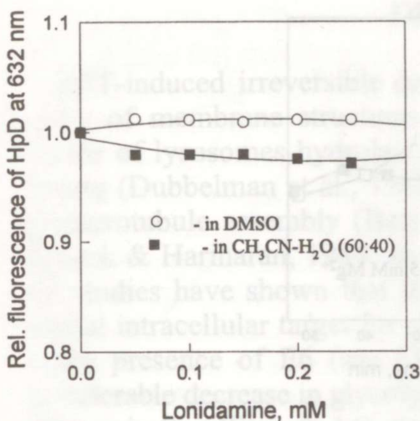

Fig. 16. Influence of LND on the fluorescence of 1 µg/ml HpD ($\lambda_{ex}=405\pm5$ nm, $\lambda_{em}=632\pm5$ nm) in DMSO and acetonitrile-water (60:40, v/v).

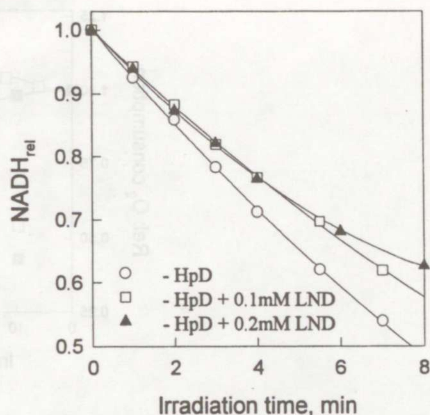

Fig. 17. Effect of LND on 5 µg/ml HpD-photosensitized (330 mW) oxidation of 0.2 mM NADH (λ_{ex} = 630 nm) in acetonitrile-water (60:40, v/v).

The mechanisms of the influence of Mg^{2+} on the antineoplastic properties of LND as well as on the photosensitive damage of EAC cells are not fully understood. Mg^{2+} slightly increased the rate of the inactivation of cellular dehydrogenases, but did not increase the depletion of ATP (Fig. 18). The addition of Mg^{2+} did not cause any decrease in the respiration of EAC cells either (Fig. 19). It should be mentioned that the registered alterations of neoplasm cells at the photosensitization in the presence of Ca^{2+} or Mg^{2+} are different. One can assume that these cations act by different mechanisms upon PDT.

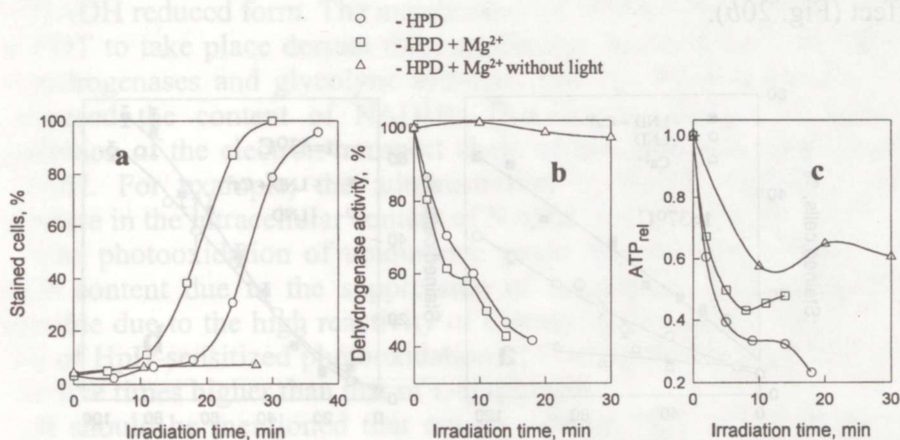

Fig. 18. Influence of 5 mM $MgCl_2$ on the injury of EAC cells (1×10^7 cells/ml) (*a*), dehydrogenase activity (*b*), and intracellular content of ATP (*c*) under 2 µg/ml HpD–PDT (630 nm, 220 mW) in media containing 154 mM NaCl, 6.2 mM KCl, 5.55 mM glucose, and 10 mM PBS, pH 7.2. SE<7%.

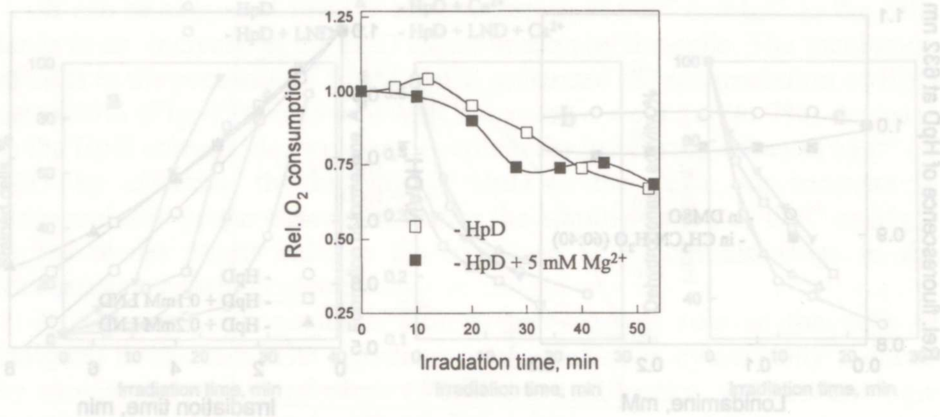

Fig. 19. Influence of 5 mM $MgCl_2$ on oxygen consumption of EAC cells (4×10^7 cells/ml) under 5 μg/ml HpD-PDT (630 nm, 220 mW) in media containing 154 mM NaCl, 6.2 mM KCl, 5.55 mM glucose, and 10 mM PBS, pH 7.2.

Hyperthermia and lonidamine

A combination of PDT with local hyperthermia increases considerably the injury of neoplasm cells (Henderson et al., 1985). At laser PDT a remarkable warming of tissue takes place. The use of thermosensitizers together with PDT could enhance the injury of cells. It has been shown that LND could increase the cytotoxic effect of hyperthermia (Kim et al., 1984b). Our data also show that LND has thermosensitizing properties. Dark incubation of the cells with 0.2 mM LND at 37 °C increased the injury of EAC cells four times as compared to these observed in the absence of LND; moreover, combination of LND with Ca^{2+} also enhanced its cytotoxicity (Fig. 20*a*). At 43 °C Ca^{2+} did not have any potentiation effect (Fig. 20*b*).

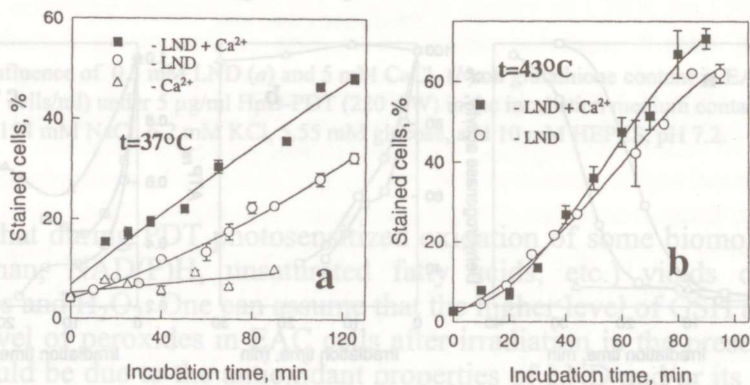

Fig. 20. The intensity of injury of EAC cells (5×10^6 cells/ml in Hanks' medium without glucose and Ca^{2+} but with 10 mM Hepes, pH 7.2) in the presence of 0.2 mM LND and/or 1.26 mM $CaCl_2$ at dark incubation at 37 °C (*a*) and at 43 °C (*b*).

DISCUSSION

PDT-induced irreversible damage of tumour cells could be caused by injury of membrane structures (Dubbelman & Van Steveninck, 1984), release of lysosomes hydrolases (Gèze et al., 1993), DNA-protein cross-linking (Dubbelman et al., 1982; Blazek & Hariharan, 1984), destruction of microtubule assembly (Berg et al., 1992), brakes in DNA molecules (Blazek & Hariharan, 1984; Agarwal et al., 1991), etc. At the same time, our studies have shown that the energy generation system could be the critical intracellular target for photosensitization: irradiation of EAC cells in the presence of E6 (see Chekulayev et al., 1991) or HpD led to a considerable decrease in glycolysis and inhibition of dehydrogenase activity and respiration (Figs. 9, 14). Respiration inhibition was accompanied by a remarkable decrease in the intracellular level of ATP (Fig. 8). The above-mentioned alterations take place before the increase in the permeability of the cytoplasm membrane of EAC cells. The decrease in the ATP level at HpD-photosensitization of tumour cells could be due to the inactivation of glycolytic enzymes (Hilf et al., 1984; Prinsze et al., 1989), enzymes of Krebs cycle and the respiration chain (Hilf et al., 1984; Gibson et al., 1989). However, other mechanisms may exist that explain such a fast and dramatic decrease in the ATP pool. So, porphyrin compounds (hematoporphyrin and its derivatives, chlorin-e_6, etc.) can mediate the oxidation of NADH at photoexcitation (Fig. 17) (Bodaness & Chan, 1977). The rate of porphyrin-photosensitized oxidation of NADH considerably exceeds (4–5 times) that of L-tryptophan. As NADH is the main electron donor for the mitochondrial respiration chain, we assumed that the depletion of intracellular ATP during PDT was induced by the photochemical oxidation of NADH. On EAC cells HpD-sensitization (Fig. 21) showed that the intracellular content of NADH did not change much. Hence, the depletion of ATP content could not be due to the insufficiency of NADH reduced form. The maintenance of NADH content in EAC cells in PDT to take place despite the considerable decrease in the activity of dehydrogenases and glycolytic enzymes (i.e. the biochemical pathways increased the content of NADH). This occurs due to the powerful inhibition of the electron transport chain of mitochondria, which oxidize NADH. For example, the administration of NaCN induced a sharp increase in the intracellular content of NADH in EAC cells (Fig. 21).

The photooxidation of ubiquinone might also cause the depletion of ATP content due to the suppression of respiration. This seems to be possible due to the high reactivity of Coenzyme Q. Indeed (Fig. 22), the rate of HpD-sensitized photooxidation of Coenzyme Q_{10} (oxidized form) was five times higher than that of L-tryptophan.

It should be mentioned that not all data on PDT of neoplasm cells have shown that a decrease occurs in the intracellular content of ATP (Dubbelman et al., 1982, 1992). Moreover, some researchers even claim that the injury of the functional activity of the mitochondrion as well as the depletion of ATP level do not affect the viability of cells influenced by PDT (Boegheim et al., 1988). Our data showed that the HpD–PDT of EAC

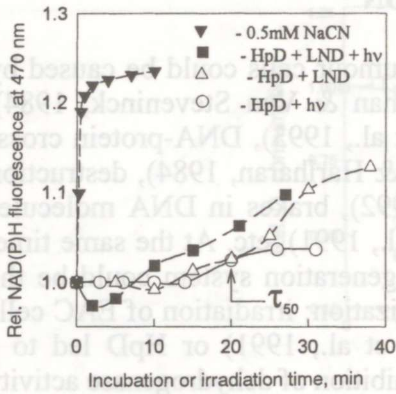

Fig. 21. The kinetics of the NAD(P)H relative fluorescence (λ_{ex}=340±5 nm, λ_{em}=470±5 nm) of EAC cells (1×10^7 cells/ml in media containing 154 mM NaCl, 6.2 mM KCl, 5.55 mM glucose, and 10 mM HEPES, pH 7.2) at 2 μg/ml HpD-photosensitization (630 nm, 220 mW) without or in the presence of 0.25 mM LND.

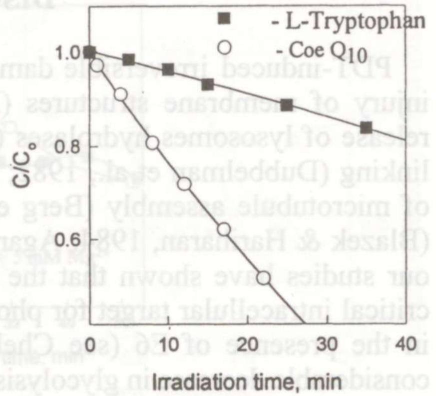

Fig. 22. The kinetics of 5 μg/ml HpD-photosensitized oxidation of 0.1 mM L-tryptophan and 0.1 mM Coenzyme Q_{10} in methanol. Irradiated at 405 nm, 70 mW, 20°C, and magnetic stirring.

cells sharply decreased the amount of ATP in cells (Fig. 8). However, a remarkable increase in the ATP level after a long exposure to light was unexpected. The latter took place when the glycolytic, dehydrogenase, and respiration activities of cells were inhibited. The increase of the ATP level could be explained by the inhibition of metabolic pathways of ATP consumption (functioning of ATPases, nucleic acids, and protein synthesis). First of all, it concerns the membrane ATPases that use for the maintenance of ionic balance 50–60% of the total amount of the ATP produced by glycolysis and oxidative phosphorylation (Schmidt et al., 1989). This suggestion is based on experimental data that have shown a strong inactivation of Na^+/K^+-ATPases and a low extent of Mg^{2+}-ATPases after HpD–PDT of tumour cells (Dubbelman et al., 1992; Gibson et al., 1988). The time needed for increasing the ATP level coincides with an intense destruction of plasma membrane (Fig. 8) (the sharp enhancement of TB incorporation in cells). So, only the determination of the ATP amount in the cells could be not sufficient for the estimation of the effect of PDT injury on the energy system of tumour cells.

The results of the present work show that the combination of PDT with LND (an inhibitor of glycolysis and respiration of tumour cells) leads to a significant therapeutical profit. The present investigation demonstrated that the enhancing effect of LND on the efficacy of PDT did not depend on the nature of photosensitizer (HpD or E6) and was most probably caused by the inhibitory effect of LND on the energy production system of tumour cells. The mechanism of LND influence on PDT seems to be more complicated. Figure 8 illustrates the LND induced acceleration of the

depletion of the ATP pool at the beginning of light exposure but later the ATP content fell sharply almost to the initial value. We assumed that LND induced not only the inactivation of the ATP production but also a metabolic pathway of energy consumption. It was shown by Malorni et al. (1988) that LND can induce a remarkable structural damage of the plasma membrane. It would be reasonable to suppose that the effect of LND on PDT was mediated by the destruction of the plasma membrane, especially by inhibiting the membrane ATPases. Inhibition of membrane ATPases promotes the swelling of tumour cells as a result of an osmotic shock. A significant alteration of cell shape and size was observed after HpD or E6 photoirradiation of EAC cells. A cascade of events could be expected to follow: a 2–3 fold increase in the cell volume, the appearance of multiple small bumps on the surface (also called "blebs"), the blending of microblebs into large bumps of cytoplasm. If irradiation goes on, an intensive staining of cells with TB is observed. The observed morphological changes of photodamaged cells started earlier when LND was present. The appearance of multiple surface protrusions is usually associated with the disintegration of binds of the plasma membrane with cytoskeleton. HpD–PDT mediates remarkable damages of cytoskeleton of endothelial cells (Fingar & Wieman, 1992). Also Malorni et al. (1992) showed the disappearance of stress fibres at the administration of LND to tumour cells. They assumed that cytoskeleton elements could be additional targets for the cytotoxic action of LND. We suppose that the enhancing effect of LND on PDT might be induced by its effect on the cytoskeleton and plasma membrane.

Our data showed that LND increased the incorporation of HpD into the tumour cells (Fig. 5*a*.). This could be the way how LND potentiates the efficacy of PDT. Figure 5*b* shows that the enhanced binding of HpD in the presence of LND at a prolonged incubation time with EAC cells is directly related to the degree of TB staining. This suggests that LND promotes HpD binding by enhancing the plasma membrane permeability. Also, the ability of LND to induce a sufficient decrease in intracellular pH was demonstrated earlier (Ben Horin et al., 1993). The authors suggested it occurred with the increase in the intracellular content of lactate. The decrease in intracellular pH is known to promote accumulation of HpD in tumour cells (Böhmer & Morstyn, 1985). So, we suggest that the enhancing of the binding of HpD in the presence of LND could be mediated also by intracellular acidification. Consequently, it would be useful to accompany the first administration of LND into an organism with HpD.

The effect of calcium on the enhancement of the antineoplastic ability of LND seems to be mediated via damaging the mitochondria of EAC cells. Indeed, Ca^{2+} considerably decreased the oxygen consumption of photoirradiated EAC cells (Fig. 13). However, the increase in both the efficacy of PDT and cytotoxic properties of LND by Ca^{2+} could be explained by the disintegration of the cell cytoskeleton. For instance, Kuhne et al. (1993) showed the disintegration of the cytoskeleton structure of actin filaments in endothelial cells after the administration of KCN

together with 2-desoxy-D-glucose, which was induced by the enhancement of $[Ca^{2+}]_i$. Since PDT and the administration of LND increase the content of $[Ca^{2+}]_i$ in tumour cells, the above-mentioned mechanism seems to be quite possible (Rasch et al., 1992; Castiglione et al., 1993).

Our experiments showed no increase in the inactivation rate of dehydrogenases under PDT action when LND and/or Ca^{2+} were added (Fig. 14). In such experiments the cellular content of GSH was also at higher level (Fig. 15). The presence of LND and Ca^{2+} had no negative effect on the photophysical properties of the photosensitizer (Fig. 16) and on the rate of photochemical reactions (Fig. 17). The decrease in the rate of the dehydrogenase inactivation at LND photoirradiation can diminish the binding of HpD with cells a little (Fig. 6). However, a decrease in the rate of the dehydrogenase inactivation was measured at the administration of Ca^{2+}, which vice versa increases the accumulation of HpD in tumour cells (Fig. 12). We suppose the effect of LND and Ca^{2+} on the dehydrogenase activity to appear as a consequence of the disturbance of the functional activity of mitochondria and the deprivation of free radical reactions. It is well known that the respiration chain of mitochondria is the main source of oxygen superoxide anion-radicals ($O_2^{\dot{-}}$) (Konstantinov et al., 1987). Non-enzymatic decomposition of hydrogen peroxide by the Fenton–Haber–Weiss reaction (Haber & Weiss, 1934) leads to the generation of very cytotoxic hydroxyl radicals ($HO^{\cdot}$): $Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + HO^{\cdot}$. Moreover, spontaneous recombination of $O_2^{\dot{-}}$ (Khan, 1970) and its reaction with H_2O_2 may generate singlet oxygen (Kellogg & Fridovich, 1975). However, the generation of $O_2^{\dot{-}}$, which is able to catalyze the decomposition of H_2O_2 ($Fe^{3+} + O_2^{\dot{-}} \longrightarrow Fe^{2+} + O_2$), is appropriate under intensive cellular respiration. The potentiating effect of LND and Ca^{2+} was accompanied with a diminished activity of the respiration chain. This, to all appearances, decreases the production of $O_2^{\dot{-}}$ and consequently the formation of cytotoxic $OH^{\cdot}$ radicals.

The results obtained show that extreme caution is needed in the estimation of the efficacy of PDT when the MTT-test is used. Only a slight relationship with the results derived from the TB-test was observed.

There is no literature data concerning the influence of Mg^{2+} on photosensitized damage of tumour cells. The present investigation has demonstrated the potentiating effect of Mg^{2+} on PDT and the antitumour activity of LND, which could be explained by the influence of Mg^{2+} on the energetic status and cytoskeleton structure of a cell. A considerable depletion of the ATP content is observed at the incubation of EAC cells with 5 mM $MgCl_2$ (Fig. 18). The effect of Mg^{2+} is related with the sharp stimulation of the phosphofructokinase activity and will be eliminated at the administration of cytochalasin B (Bossi et al., 1989).

The low toxicity of LND (LD_{50} in mice and rats (mg/kg): 900, 1700 orally; 435, 525 i.p.) (Heywood et al., 1981) and its non-mutagenic properties (Forster et al., 1990) make LND very attractive for the treatment of some kinds of tumours. At the same time an oral daily dose of LND cannot exceed 450 mg (or 0.02 mM/kg) as shown clinically (Robustelli & Pedrazzoli, 1991). A significant positive effect on PDT of

EAC cells was reached when the concentrations of LND used exceeded the permissible therapeutic dose 5–10 times. This means that a combined use of LND with PDT is possible only for the therapy of superficial tumours (e.g. basalioma). Nevertheless, these doses do not exclude the possibility of a systemic use (administration to an organism) of LND at PDT. An analysis of literature data shows that the sensitivity of tumour cells to cytotoxic effects of LND is variable (dependent upon the histologic type of tumour) in a very large concentration range. A significant reduction of the human glioma cells was reached already at the therapeutic doses (Floridi et al., 1990).

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REFERENCES

Agarwal, M. L., Clay, M. E., Harvey, E. J., Evans, H. H., Antunez, A. R. & Oleinick, N. L. 1991. Photodynamic therapy induced rapid cell death by apoptosis in L5178Y mouse lymphoma cells. – Cancer Res., **51**, 5910–5914.

Ahmann, F. R., Garewal, H. S., Schifman, R., Celniker, A. & Rodney, S. 1987. Intracellular adenosine triphosphate as a measure of human tumor cell viability and drug modulated growth. – In Vitro Cellular and Developmental Biology, **23**, 474–480.

Andersson-Engels, S., Baert, L., Berg, R., D'Hallewin, M. A., Johansson, J., Stenram, U., Svanberg, K. & Svanberg, S. 1991. Fluorescence characteristics of atherosclerotic plaque and malignant tumors. – Optical Methods for Tumor Treatment and Early Diagnosis: Mechanisms and Techniques (Dougherty,T. J. (ed.)), Proc. SPIE, **1426**, 31–43.

Azzi, A. & Azzone, G. F. 1966. Swelling and shrinkage phenomena in liver mitochondria. III. Irreversible swelling induced by inorganic phosphate and Ca^{2+}. – Biochim. Biophys. Acta, **113**, 438–444.

Ben Horin, H., Kaplan, O. & Navon, G. 1993. The mechanism of action of the antineoplastic drug lonidamine – nuclear magnetic resonance spectroscopy studies. – Proc. Annu. Meet. Am. Assoc. Cancer Res., **34**, A2067.

Berg, K., Steen, H. B., Winkelman, J. W. & Moan, J. 1992. Synergistic effects of photoactivated tetra(4-sulfonatophenyl)porphine and nocodazole on microtubule assembly, accumulation of cells in mitosis and cell survival. – J. Photochem. Photobiol. B: Biol., **13**, 59–70.

Berns, M. W., Dahlmann, A., Johnson, F. M., Burns, R., Sperling, D., Guiltinan, M., Siemens, A., Walter, R., Wright, W., Hammer-Wilson, M. & Wile, A. 1982. In vitro cellular effects of hematoporphyrin derivative. – Cancer Res., **42**, 2325–2329.

Bianchi, C., Bagnato, A., Paggi, M. G. & Floridi, A. 1987. Effect of adriamycin on electron transport in rat heart, liver, and tumor mitochondria. – Exp. Mol. Pathol., **45**, 123–135.

Blazek, E. R. & Hariharan, P. V. 1984. Alkaline elution studies of hematoporphyrin-derivative photosensitized DNA damage and repair in Chinese hamster ovary cells. – Photochem. Photobiol., **40**, 5–13.

Bodaness, R. S. & Chan, P. G. 1977. Singlet oxygen as a mediator in the hematoporphyrin-catalyzed photooxidation of NADPH to $NADP^+$ in deuterium oxide. – J. Biol. Chem., **252**, 8554–8560.

Boegheim, J. P. J., Lagerberg, J. W. M., Dubbelman, T. M. A. R., Tijssen, K., Tanke, H. J., Van der Meulen, J. & Van Steveninck, J. 1988. Photodynamic effects of hematoporphyrin derivative on the uptake of rhodamine 123 by mitochondria of intact murine L929 fibroblasts and Chinese hamster ovary K1 cells. – Photochem. Photobiol., **48**, 613–620.

Böhmer, R. M. & Morstyn, G. 1985. Uptake of hematoporphyrin derivative by normal and malignant cells: Effect of serum, pH, temperature, and cell size. – Cancer Res., **45**, 11, 5328–5334.

Bossi, D., Wolf, F. I., Calviello, G. & Cittadini, A. 1989. The effect of Mg^{2+} upon 6-phosphofructokinase activity in Ehrlich ascites tumor cells in vivo. – Arch. Biochem. Biophys., **275**, 174–180.

Castiglione, S., Kennedy, K. A., Floridi, A. & Fiskum, G. 1993. Non-ionophoretic elevation of intracellular Ca^{2+} by lonidamine. – Biochem. Pharmacol., **46**, 330–332.

Ceckler, T. L., Bryant, R. G., Penney, D. P., Gibson, S. L. & Hilf, R. 1986. ^{31}P-NMR spectroscopy demonstrates decreased ATP levels in vivo as an early response to photodynamic therapy. – Biochem. Biophys. Res. Commun., **140**, 273–279.

Chekulayev, V., Shevchuk, I., Kahru, A., Mihkelsoo, V. & Kallikorm, A. 1991. Investigation of the photodynamic properties of some chlorophyll *a* derivatives. The effect of doxorubicin on the chlorin e_6 photosensitized death of Ehrlich carcinoma cells. – Optical Methods for Tumor Treatment and Early Diagnosis: Mechanisms and Techniques (Dougherty, T. J. (ed.)), Proc. SPIE, **1426**, 367–377.

Chekulayev, V., Shevchuk, I., Mihkelsoo, V. & Kallikorm, A. 1992a. A change in the sulfhydryl status of Ehrlich carcinoma cells by exposure to light in the presence of chlorin-e_6 trimethyl ester. The effect of L-buthionine-[S, R]-sulfoximine on the photosensitized damage of tumor cells. – Proc. Estonian Acad. Sci. Chem., **41**, 2, 52–61.

Chekulayev, V., Shevchuk, I., Mihkelsoo, V. & Kallikorm, A. 1992b. The effect of metronidazole on the chlorin e_6 photosensitized death of Ehrlich carcinoma cells. – Proc. Estonian Acad. Sci. Chem., **41**, 1, 18–25.

Cho, Y.-H., Straight, R. C. & Smith, J. A. 1992. Effects of photodynamic therapy in combination with intravesical drugs in a murine bladder tumor model. – J. Urology, **147**, 743–746.

Cowled, P. A., Mackenzie, L. & Forbes, I. J. 1987. Pharmacological modulation of photodynamic therapy with hematoporphyrin derivative and light. – Cancer Res., **47**, 971–974.

Dubbelman, T. M. A. R. & Van Steveninck, J. 1984. Photodynamic effects of hematoporphyrin-derivative on transmembrane transport systems of murine L929 fibroblasts. – Biochim. Biophys. Acta, **771**, 201–207.

Dubbelman, T. M. A. R., Van Steveninck, A. L. & Van Steveninck, J. 1982. Hematoporphyrin-induced photooxydation and photodynamic cross-linking of nucleic acid and their constituents. – Biochim. Biophys. Acta, **719**, 47–52.

Dubbelman, T. M. A. R., Penning, L. C., Tijssen, K. & Van Steveninck, J. 1992. Influence of glucose on HpD-induced photodynamic inhibition of Na^+/K^+-ATPase of L929 fibroblasts, CHO-K1 epithelial cells and T24 human bladder transitional carcinoma cells. – In: Photodynamic Therapy and Biomedical Lasers (Spinelli, P., Dal Fante, M. and Marchesini, R. (eds.)), Excerpta Medica, Amsterdam, 137–141.

Ellman, G. L. 1959. Tissue sulfhydryl groups. – Arch. Biochem. Biophys., **82**, 70–77.

Fingar, V. H. & Wieman, T. J. 1992. Mechanisms of vessel damage in photodynamic therapy. – Optical Methods for Tumor Treatment and Detection: Mechanisms and Techniques in Photodynamic Therapy (Dougherty, T. J. (ed.)), SPIE, **1645**, 98–103.

Floridi, A. & Lehninger, A. 1983. Action of the antitumor and antispermatogenic agent lonidamine on electron transport in Ehrlich ascites tumor mitochondria. – Archives of Biochemistry and Biophysics, **226**, 73–83.

Floridi, A., Paggi, M. G., D'Atri, S., Martino, C., Marcante, M. L., Silvestrini, B. & Caputo, A. 1981a. Effect of lonidamine on the energy metabolism of Ehrlich ascites tumor cells. – Cancer Res., **41**, 4661–4666.

Floridi, A., Paggi, M. G., Marcante, M. L., Silvestrini, B., Caputo, A. & Martino, C. 1981b. Lonidamine, a selective inhibitor of aerobic glycolysis of murine tumor cells. – JNCI, **66**, 497–499.

Floridi, A., Alexandre, A., Paggi, M. G., Pellegrini, L., Marcante, M. L., Silvestrini, B. & Lehninger, A. L. 1985. Mechanisms of mitochondrial swelling induced by lonidamine. – In: Cell Membranes and Cancer (Galeotti, T. et al. (eds.)), Elsevier Science Publishers B.V., Amsterdam, 217–221.

Floridi, A., Gentile, F. P., Bruno, T., Castiglione, S., Zeuli, M. & Benassi, M. 1990. Growth inhibition by rhein and lonidamine of human glioma cells in vitro. – Anticancer Res., **10**, 1633–1636.

Follow-up Report. Profile L DLO/L1042 anticancer marketed, Drug License Opport, 9 Nov. 1987, 653.

Forster, R., Campana, A., Onofrio, E. D., Henderson, L., Mosesso, P. & Barcellona, P. S. 1990. Lonidamine: A non-mutagenic antitumor agent. – Carcinogenesis, **11**, 1509–1515.

Gèze, M., Morlière, P., Mazière, J. C., Smith, K. M. & Santus, R. 1993. Lysosomes, a key target of hydrophobic photosensitizers proposed for photochemotherapeutic applications. – J. Photochem. Photobiol. B: Biol., **20**, 23–35.

Gibson, S. L., Murant, R. S. & Hilf, R. 1988. Photosensitizing effects of hematoporphyrin derivative and photofrin II on the plasma membrane enzymes 5'-nucleotidase, Na^+/K^+-ATPase, and Mg^{2+}-ATPase in R3230AC mammary adenocarcinomas. – Cancer Res., **48**, 3360–3366.

Gibson, S. L., Murant, R. S., Chazen, M. D., Kelly, M. E. & Hilf, R. 1989. In vitro photosensitization of tumor cell enzymes by photofrin II administered in vivo. – Br. J. Cancer, **59**, 47–53.

Haber, F. & Weiss, J. J. 1934. The catalytic decomposition of hydrogen peroxide by iron salts. – Proc. R. Soc. London, A147, 332–351.

Henderson, B. W., Waldow, S. M., Potter, W. R. & Dougherty, T. J. 1985. Interaction of photodynamic therapy and hyperthermia: Tumor response and cell survival studies after treatment of mice in vivo. – Cancer Res., **45**, 6071–6077.

Heywood, R., James, R. W., Barcellona, P. S., Campana, A. & Cioli, V. 1981. Toxicological studies of 1-substituted-indazol-3-carboxylic acids. – Chemotherapy, **27**, Suppl. 2, 91–97.

Hilf, R., Smail, D. B., Murant, R. S., Leakey, P. B. & Gibson, S. L. 1984. Hematoporphyrin derivative-induced photosensitivity of mitochondrial succinate dehydrogenase and selected cytosolic enzymes of R3230AC mammary adenocarcinomas of rats. – Cancer Res., **44**, 1483–1488.

Hilf, R., Murant, R. S., Narayanan, U. & Gibson, S. L. 1986. Relationship of mitochondrial function and cellular adenosine triphosphate levels to hematoporphyrin derivative-induced photosensitization in R3230AC mammary tumors. – Cancer Res., **46**, 211–217.

Kahru, A., Liiders, M., Vanatalu, K. & Vilu, R. 1982. Adenylate energy charge during batch culture of *Thermoactinomyces vulgaris*. – Arch. Microbiol., **133**, 2, 142–144.

Kellogg, E. W. & Fridovich, I. 1975. Superoxide, hydrogen peroxide, and singlet oxygen in lipid peroxidation by a xanthine oxidase system. – J. Biol. Chem., **250**, 8812–8817.

Kessel, D. 1986. Sites of photosensitization by derivatives of hematoporphyrin. – Photochem. Photobiol., **44**, 489–493.

Kessel, D., Thompson, P., Musselman, B. & Chang, C. K. 1987. Chemistry of hematoporphyrin-derived photosensitizers. – Photochem. Photobiol., **46**, 5, 563–568.

Khan, A. U. 1970. Singlet molecular oxygen from superoxide anion and sensitized fluorescence of organic molecules. – Science, **168**, 3930, 476–477.

Kim, J. H., Alfieri, A., Kim, S. H., Young, C. W. & Silvestrini, B. 1984a. Radiosensitization of Meth-A fibrosarcoma in mice by Lonidamine. – Oncology, **41**, Suppl. 1, 36–38.

Kim, J. H., Kim, S. H., Alfieri, A., Young, C. W. & Silvestrini, B. 1984b. Lonidamine: A hyperthermic sensitizer of HeLa cells in culture and the Meth-A tumor in vivo. – Oncology, **41**, Suppl. 1, 30–35.

Klugmann, E. B., Decarti, G., Klugman, S., Marchetti, M., Benussi, B. & Baldini, L. 1982. Acute and chronic cardiotoxicity of adriamycin in the mouse: Electrocardiographic, morphologic and biochemical investigations. – Riv. Farmacol. Ther., **12**, 269–276.

Konstantinov, A. A., Peskin, A. V., Popova, E. Yu., Khomutov, G. B. & Ruuge, E. K. 1987. Superoxide generation by the respiratory chain of tumor mitochondria. – Biochim. Biophys. Acta, **894**, 1–10.

Kuhne, W., Besselmann, M., Noll, T., Muhs, A., Watanabe, H. & Piper, H. M. 1993. Desintegration of cytoskeletal structure of actin filaments in energy-depleted endothelial cells. – Am. J. Physiol., **264**, 5, 1599–1608.

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

Lötjönen, S. & Hynninen, P. H. 1980. A convenient method for the preparation of chlorin e_6 and rhodin g_7 trimethyl esters. – Synthesis, **7**, 541–543.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. 1951. Protein measurement with the Folin phenol reagent. – J. Biol. Chem., **193**, 265–275.

Malorni, W., Arancia, G., Martino, C., Nista, A., Accinni, L., Masella, R. & Cantafora, A. 1988. On the mechanism of action of lonidamine. A study on human erythrocyte membrane. – Exp. Mol. Pathol., **43**, 361–372.

Malorni, W., Meschini, S., Matarrese, P. & Arancia, G. 1992. The cytoskeleton as a subcellular target of the antineoplastic drug lonidamine. – Anticancer Res., **12**, 2037–2046.

Muhamed, H., Ramasarma, T. & Kurup, C. K. R. 1982. Inhibition of mitochondrial oxidative phosphorylation by adriamycin. – Biochim. Biophys. Acta, **722**, 43–50.

Murant, R. S., Gibson, S. L. & Hilf, R. 1987. Photosensitizing effects of photofrin II on the side-selected mitochondrial enzymes adenylate kinase and monoamine oxidase. – Cancer Res., **47**, 4323–4328.

Nicotera, P., Hartzell, P., Davis, G. & Orrenius, S. 1989. The formation of plasma membrane blebs in hepatocytes exposed to agents that increase cytosolic Ca^{2+} is mediated by the activation of a non-lysosomal proteolytic system. – FEBS Lett., **209**, 139–144.

Pounds, J. G. 1990. The role of cell calcium in current approaches to toxicology. – Environ. Health Perspect., **84**, 7–15.

Prinsze, C., Dubbelman, T. M. A. R. & Van Steveninck, J. 1989. Interaction of photodynamic treatment with haematoporphyrin derivative and hyperthermia in various cells and models. – In: Third Congress of the Europe. Soc. for Photobiology, 27 Aug.–2 Sept. 1989, Budapest, Book of Abstracts, 133.

Rasch, M. H., Penning, L. C., Ben-Hur, E., Havlaar, A. C., Dubbelman, T. M. A. R. & Van Steveninck, J. 1992. A protective role for the transient increase of cytoplasmic free calcium after photodynamic treatment of CHO-K1 cells and T24 human bladder transitional carcinoma cells. – In: Photodynamic Therapy and Biomedical Lasers (Spinelli, P., Dal Fante, M. and Marchesini, R. (eds.)), Elsevier Science Publishers B.V., 142–146.

Robustelli, C. G. & Pedrazzoli, P. 1991. Toxicity and clinical tolerance of lonidamine. – Seminars in Oncology, **18**, Suppl. 4, 18–22.

Roberts, W. G, Liaw, L.-H. L. & Berns, M. W. 1989. In vitro photosensitization. II. An electron microscopy study of destruction with mono-L-aspartyl chlorin e_6 and photofrin II. – Lasers Surg. Med., **9**, 102–108.

Savini, S., Zoli, W., Nanni, O., Volpi, A., Frassineti, G. L., Magni, E., Flamigni, A., Amadori, A. & Amadori, D. 1992. In vitro potentiation by lonidamine of the cytotoxic effect of adriamycin on primary and established breast cancer cell lines. – Breast Cancer Res. Treatment, **24**, 27–34.

Schmidt, H., Siems, W., Müller, M., Dumdey, R., Jakstadt, M. & Rapoport, S. M. 1989. Balancing of mitochondrial and glycolytic ATP production and of the ATP-consuming processes of Ehrlich mouse ascites tumour cells in a high phosphate medium. – Biochem. Int., **19**, 985–992.

Silvestrini, B., Zaffaroni, N., Villa, R., Orlandi, L. & Costa, A. 1992. Enhancement of cisplatin activity by lonidamine in human ovarian cancer cells. – Int. J. Cancer, **52**, 813–817.

Ting, Ch. I. & Zong-he, G. 1984. Photodynamics effect of the He-Ne laser with HpD on the ultrastructure of rhabdomyosarcoma cell. – In: Porphyrins in Tumor Phototherapy. Plenum Press, New York, London, 193–200.

Wilson, J. K., Sargent, J. M., Elgie, A. W., Hill, J. G. & Taylor, C. G. 1990. A feasibility study of the MTT assay for chemosensitivity testing in ovarian malignancy. – Br. J. Cancer, **62**, 189–194.

HEMATOPORFÜRIINI DERIVAADI VÕI KLORIIN-e_6 TOIMEL EHRLICHI ASTSIITSE KARTSINOOMI RAKKUDE FOTOSENSIBILISEERITUD KAHJUSTAMISE SUURENDAMINE LONIDAMIINI MANUSTAMISEGA

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Lonidamiin (LND) on antispermatogeenne ja antineoplastiline agent, mis mõjub pärssivalt rakkude energeetilisele metabolismile. On uuritud LND (dikloreeritud indasool-3-karboksüülhappe derivaat) mõju pahaloomulise kasvaja fotodünaamilisele teraapiale. *In vitro* katsetes on leitud, et 0,25 mM kontsentratsioonis suurendas LND sünergistlikult (kuni 2 korda) hematoporfüriini derivaadi (HpD) või kloriin-e_6 poolt Ehrlichi astsiitse kartsinoomi (EAK) rakkude fotosensibiliseeritud kahjustamise kiirust. Oluline EAK kasvu pärssiv toime leiti *in vivo* katsetes, kus HpD-fototeraapiat (5 mg/kg) kombineeriti LND-ga (i.p. manustus hiirtele doosis 0,1 mM/kg enne kiiritust). Seejuures oli *in vivo* katsetes tuvastatud aditiivne efekt HpD ja LND vahel. LND põhjustatud efekt on seotud tema inhibeeriva mõjuga kasvaja raku energeetilisele metabolismile. LND suurendab fotodünaamiliselt kahjustatud kasvaja rakkude glükolüütilist aktiivsust ning vähendab oluliselt hapniku tarbimise intensiivsuse langust. Võib arvata, et täheldatud ATP kontsentratsiooni kiirendatud langus fototeraapia ja LND manustamise puhul on seotud põhiliselt mitokondrite inaktivatsiooniga. Katseliselt on tuvastatud, et kasvaja rakud olid hüpoglükeemia tingimustes veelgi tundlikumad fototeraapia ja LND kombinatsiooni suhtes. Seejuures LND potentseeriv efekt HpD fototeraapiale on arvatavasti keerulisem. Nii näiteks on kindlaks tehtud kaltsiumi ja magneesiumi katioonide stimuleeriv mõju fototeraapia ja LND kombinatsiooni korral. LND potentseeriv efekt fototeraapia puhul võib olla seotud aine mõjuga kasvaja rakkude tsütoskeletile ja tsütoplasma membraanile.