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EFFECTS OF THE INHIBITORS OF ENERGY METABOLISM, LONIDAMINE AND LEVAMISOLE, ON 5-AMINOLEVULINIC-ACID-INDUCED PHOTOCHEMOTHERAPY

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The ability of endogenously synthesized protoporphyrin IX (PpIX) to damage Chinese hamster lung fibroblasts of the line V79 by exposure to light was examined. This treatment induced reduction of cellular ATP, GTP, of the NADH/NAD$^+$ ratio and of oxygen consumption. The present results indicate a close relationship between inhibition of respiration of irradiated cells and their ability to survive, e.g. 1 min of light exposure induced 90% inhibition of oxygen consumption and inactivation of approximately 95% of the cells, while the cellular content of ATP was reduced by only 15%. This indicates that the mitochondria are one of the primary targets of 5-aminolevulinic acid (ALA)-mediated photochemotherapy (PCT). In the present study, ALA-PCT was combined with the modulators of the glycolysis and the respiration chain, levamisole (LEV) and lonidamine (LND). A synergistic effect of combining ALA-PCT with non-toxic concentrations of LND was observed when LND was given prior to light exposure. This synergism was observed despite a substantial LND-induced inhibition of PpIX formation. At increasing doses of LND (>0.15 mM) the combination treatment becomes less efficient. This is due to the inhibition of PpIX synthesis induced by LND. A synergistic effect of ALA-PDT and LEV was found when LEV was given prior to light exposure. This was at least partly due to an LEV-stimulated effect on ALA-induced PpIX formation. However, it is not clear from the present results whether LEV may perturb energy metabolism in V79 cells since LEV alone did not reduce the energy charge or the NADH/NAD$^+$ ratio. When LEV or LND were given after ALA-PCT, these 2 treatment modalities acted in an additive or slightly synergistic manner.

The ability of rapidly proliferating transformed mammalian cells to accumulate endogenous porphyrins (mainly protoporphyrin IX (PpIX)) after administration of exogenous 5-aminolevulinic acid (ALA) has been demonstrated. Photoirradiated cell-bound PpIX is capable of inducing an effective destruction of neoplastic cells. Promising data on ALA-induced photochemotherapy (PCT) of different histological types of tumor cells, such as erythrocytic, lymphocytic and myelocytic leukemic cells (Malik *et al.,* 1989; Malik and Lugaci, 1987), B-16 melanoma (Schoenfeld *et al.,* 1994), fibrosarcoma (Rebeiz *et al.,* 1992), human basal-cell carcinoma and superficial squamous-cell carcinoma (Kennedy *et al.,* 1990) have been obtained. Subcutaneously transplanted CaD2 mammary carcinomas in mice were treated more successfully with ALA-PCT than with therapy based on exogenous administration of photosensitizers, such as Photofrin II (Bech *et al.,* 1992). A wide clinical experience of ALA-PCT has been acquired. For example, excellent results, including cosmetic benefits, following clinical treatment of multiple basal-cell carcinoma by ALA-PCT have been reported (Kennedy and Pottier, 1992; Warloe *et al.,* 1992). Even one course of ALA-PCT treatment can lead to the successful eradication of tumors and the cosmetic results are excellent (Kennedy and Pottier, 1992).

The efficiency of ALA-PCT depends first of all on intracellular formation of protoporphyrin IX (PpIX). Thus, several basic investigations have been aimed at finding drugs that activate the biosynthesis of endogenous porphyrins. Promising results have been obtained with 1,10-phenanthroline (Rebeiz *et al.,* 1992), sodium butyrate and hemin (Malik *et al.,* 1989), allyl-isopropyl-acetamide with dimethyl-sulfoxide (DMSO) (Schoenfeld *et al.,* 1994), and desferal (Iinuma *et al.,* 1994; Berg *et al.,* 1996).

Further elaboration of ALA-PCT depends on a detailed knowledge of photoinactivation mechanisms. This aspect of ALA-PCT has so far been insufficiently studied. Since PpIX is a relatively hydrophobic compound (Breitbart *et al.,* 1984) a preferential localization of PpIX and damage to membrane structures of cells under photoirradiation might be expected. Schoenfeld *et al.* (1994) reported that the first effect of ALA-PCT of B-16 melanoma cells is the disintegration of the outer membrane. This was indicated by a sharp increase in ionic efflux (K^+ leakage was accompanied by increased intracellular content of Na^+, Cl^- and Ca^{2+}). However, the subcellular targets after ALA-PCT may depend on the treatment conditions and the cell line.

The final steps in the PpIX biosynthetic pathway take place in mitochondria (Battle, 1993). Damage to these organelles during ALA-PCT may therefore lead to cell inactivation. Indeed, some evidence of mitochondrial damage accompanied by nuclear swelling and suppression of thymidine incorporation was observed *in vitro* after the photoactivation of endogenous porphyrins in erythrocytic leukemic cells (Kennedy and Pottier, 1992). Moreover, numerous studies suggest that mitochondria are critical intracellular targets for hematoporphyrin derivative (HPD) or Photofrin II photosensitization. Damage to mitochondrial functions leads to irreversible cell inactivation. The photosensitization may inhibit a number of mitochondrial enzymes: cytochrome C oxidase, F_0F_1 ATPase, succinate dehydrogenase, NADH dehydrogenase (Gibson *et al.,* 1989; Hilf *et al.,* 1984), adenylate kinase and monoamine oxidase (Murant *et al.,* 1987). Suppression of mitochondrial activity after PCT leads to a significant reduction in the cellular ATP level and, consequently, to a loss of viability (Hilf *et al.,* 1986). Nevertheless, the influence of PCT on the energy metabolism of neoplastic cells may not be limited to inhibition of mitochondrial function. It has been demonstrated that several glycolytic enzymes (lactate dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase, pyruvate kinase, glucose phosphate isomerase), which are located in the cytosol, may be inactivated after HPD-induced PCT (Schoenfeld *et al.,* 1994; Boegheim *et al.,* 1987).

On this basis, it may be assumed that drugs, known as inhibitors of the cellular energy metabolism, are able to potentiate the efficacy of ALA-PCT. Hilf *et al.* (1984) reported an additive effect of iodoacetate (inhibitor of glycolysis) on HPD phototherapy of mammary adenocarcinoma. In contrast, the combination of PCT with oligomycin (an inhibitor of respiration) showed no such additive effects (Kennedy *et al.,* 1990).

Comprehensive investigations during the last decade have shown that lonidamine (LND), a dichlorinated derivative of

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indazole-3-carboxylic acid, is a non-mutagenic, anti-spermatogenic drug with a significant anti-tumor activity. Clinical studies have reported anti-tumor activity of LND in breast and kidney cancer, soft-tissue sarcoma and lung cancer (Evans *et al.*, 1984; Robustelli della Cuna and Pedrazzoli, 1991). In addition, LND can enhance the anti-tumor and cytotoxic effect of hyperthermia (Kim *et al.*, 1984*b*), of ionizing radiation (Kim *et al.*, 1984*a*), of several anti-neoplastic alkylating agents (Silvestrini *et al.*, 1992) and of adriamycin (Savini *et al.*, 1992). The mechanism of the anti-neoplastic effect of LND is linked to damage to the cellular energy system. Thus, LND inhibits the oxygen consumption of both normal and malignant cells (Floridi *et al.*, 1981*b*) by blocking the electron-transport chain between primary dehydrogenases and the respiration chain of mitochondria (Floridi and Lehninger, 1983). However, LND stimulates the aerobic glycolysis of normal cells, but inhibits that of neoplastic cells by affecting the mitochondrially-bound hexokinase which is not present in normal differentiated cells (Floridi *et al.*, 1981*b*). LND may cause considerable structural damage to mitochondria (Floridi *et al.*, 1981*a*, 1985) as well as to the plasma membrane (Malorni *et al.*, 1988).

We have chosen levamisole (LEV) and lonidamine (LND) as inhibitors of energy metabolism and studied their effect on ALA-PCT. Levamisole was initially discovered as a drug with anthelmintic activity and later it was used as an immunomodulator in human cancer therapy (Amery and Morias, 1977). LEV in therapeutic doses is a specific and strong inhibitor of aerobic glycolysis in Ehrlich ascites tumor (EAT) cells. Metabolic and enzymatic studies have shown that LEV directly inhibits phosphofructokinase in tumor cells, but not in normal ones, and so mediates an essential decrease in the cellular content of 2-phosphoenolpyruvate, pyruvate and ATP (Guminska *et al.*, 1986). By an unknown mechanism, LEV potentiates ionizing radiation-induced destruction of neoplastic cells (Hayostek and Koval, 1992) as well as the anti-proliferative activity of 5-fluorouracil (Grem and Allegra, 1989).

MATERIAL AND METHODS

Chemicals

5-Aminolevulinic acid was purchased as ALA HCl from Porphyrin Products (Logan, UT). ALA was dissolved at 0.1 M in PBS, with the pH adjusted to 7.4 (by means of 5 M KOH) and sterile filtered Levamisole was supplied by Sigma, St. Louis, MO (lot 122H3570) and dissolved in PBS immediately before each experiment. Lonidamine, obtained from F. Angelini Research Institute, Rome, Italy (lot 4/A), was dissolved in DMSO immediately before each experiment and then diluted with fresh medium. Hematoporphyrin derivative was synthesized from hematoporphyrin by the method of Lipson *et al.* (1961) as modified by Kessel *et al.* (1987). All other chemicals used were of the highest purity commercially available.

Cell culture

The V79 Chinese hamster lung fibroblast cell line was used. This cell line has a doubling time of about 8 hr under exponential growth conditions and was subcultured approximately twice a week. V79 cells were grown as monolayer in RPMI 1640 medium containing 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were maintained at 37°C and 5% CO_2/95% air in a humid environment.

Cell survival at low cell density

Cell survival was studied in a clonogenic assay: 500 cells were inoculated in 25-cm^2 plastic flasks (Nunclon, Nunc, Roskilde, Denmark) and incubated for 12 hr. The cells were treated with LND, LEV, ALA-PCT or a combination of LND or LEV with ALA-PCT as described in "Results". Then, 62 mM LND dissolved in DMSO and 0.1 M LEV dissolved in PBS were diluted in culture medium immediately prior to use. Cells treated with ALA-PCT were washed with RPMI 1640 without serum and incubated with 3 ml of fresh medium without serum and with 0.5 mM ALA. After 4 hr, 5 stripes of equal area in each flask were exposed to different light doses from a bank of 4 fluorescent tubes (mod.3026, Applied Photophysics, London, UK) (Berg *et al.*, 1991). The lamp emits light in the wavelength region of 340–420 nm, with a maximum around 405 nm at a fluence rate of 36 W/m^2 (Berg *et al.*, 1988). The lamp and cell flasks were cooled with a fan. After light exposure, the cells were incubated with 4 ml of fresh medium for 5 days at 37°C, then fixed in ethanol and stained with methylene blue, after which the colonies were counted visually. Each stripe contained between 20 and 200 colonies.

Cell survival at high cell density

Some 16 hr before treatment, 1.5 × 10^6 cells were inoculated into 25-cm^2 flasks. During this pre-incubation time, the cells formed a layer that was approximately 80–90% confluent. Then the cells were incubated for 4 hr with 0.5 mM ALA and irradiated as described above. After irradiation, the medium

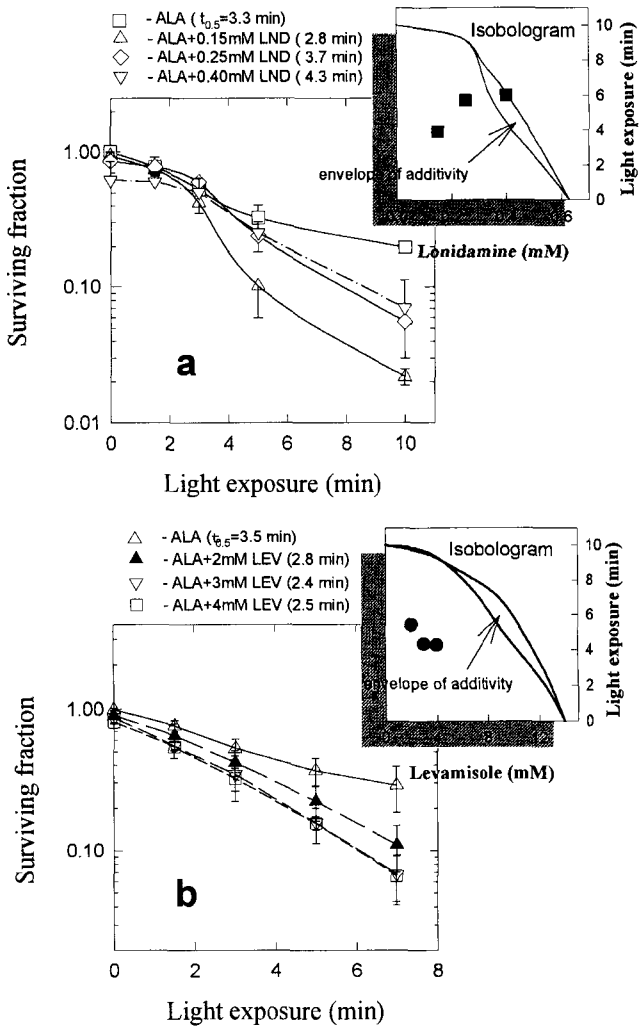


FIGURE 1 – Clonogenicity of V79 cells after photochemotherapy in combination with LND and LEV. Cells (20 cells/cm^2) were pre-incubated for 4 hr with 0.5 mM ALA and different concentrations of LND or LEV as indicated. In parentheses, time in minutes needed for inactivation of 50% of the cells. Bars = SD. Insets: Isobolograms for the combined effects of PDT and LND/LEV on V79 cells. Interaction points were obtained at surviving fractions of 0.2 in the survival curves shown in the main Figure.

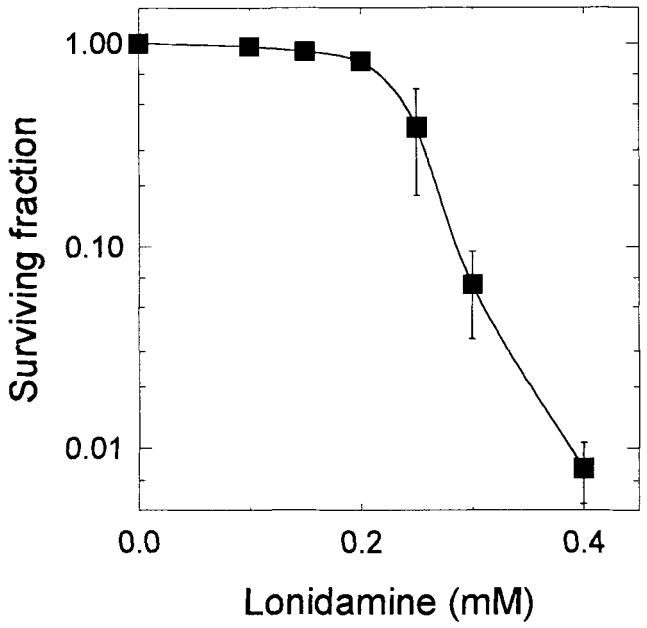
was removed and the cells were incubated with 4 ml of fresh medium with serum for 14 hr. The cultures were then washed with 0.9% NaCl, fixed in absolute ethanol and stained with methylene blue. The relative number of surviving cells was measured spectrophotometrically as a difference between light absorption at 610 nm and 900 nm, *i.e.* the scattering light at 900 nm was subtracted from the absorption maximum of methylene-blue-stained cells (Moan *et al.*, 1984).

Measurements of cellular PpIX content

Cells (0.7×10^6) were inoculated into 9.6-cm^2 plastic multi-well dishes (Nunclon) and left for 16 hr, then treated as described above. The cells were washed with ice-cold PBS, 1 M $HClO_4$ with 50% added methanol and then scraped off the substratum with a cell scraper. After 5 min of incubation the cell debris was removed by centrifugation. The PpIX content of the sample was measured spectrofluorometrically using a Perkin Elmer LS 50B spectrofluorimeter. A standard of known concentration of PpIX was added so that the fluorescence intensity increased by approximately 50%. The excitation wavelength was set at 405 nm and the fluorescence emission was detected at 625 nm. A long pass cut-off filter (530 nm) was used on the emission side.

Measurement of nucleotides

Cells (1.7×10^6) were inoculated into 60-mm plastic dishes and left for 18 hr to obtain a sufficient cell number (5×10^6 cells) and treated with drugs as described above. Nucleotides



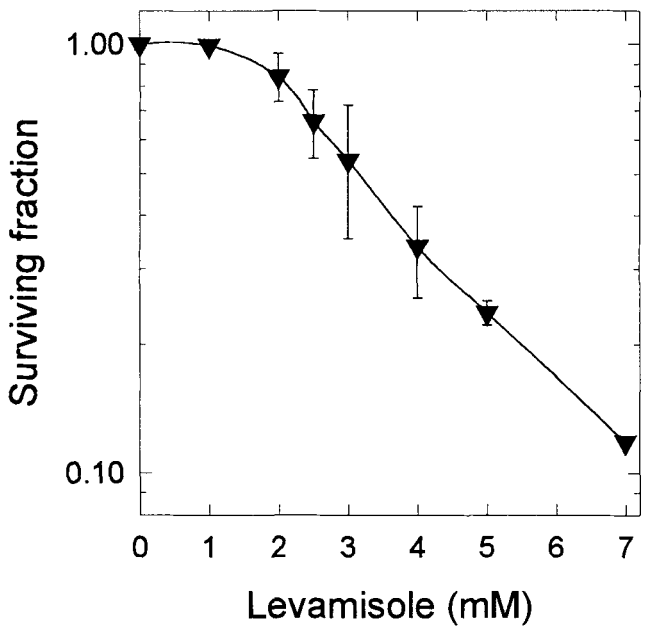
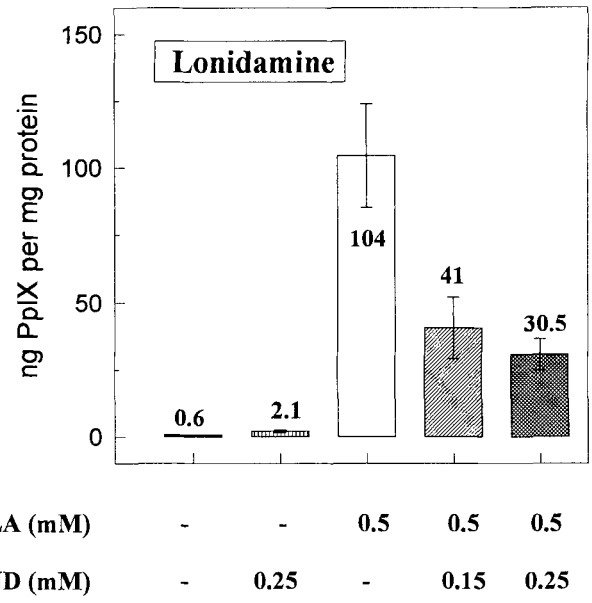


FIGURE 2 – Clonogenicity of V79 cells (20 cells/cm^2) treated for 24 hr with LND or LEV. Bars, SD from 3–5 independent experiments. Bars are shown when larger than symbols.



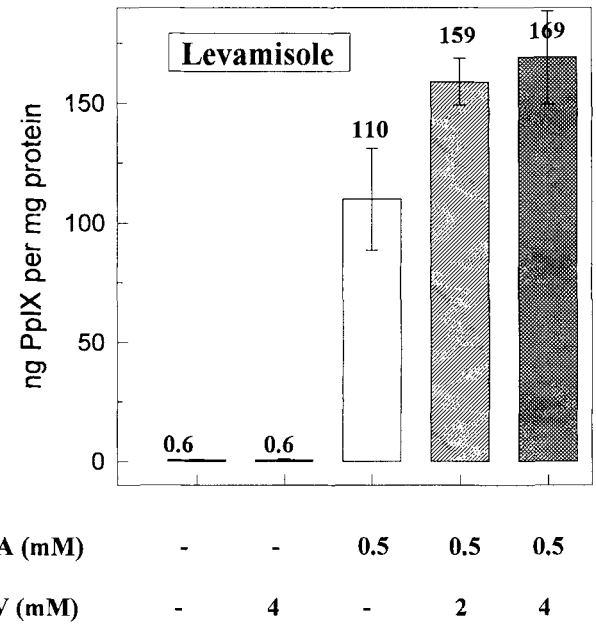


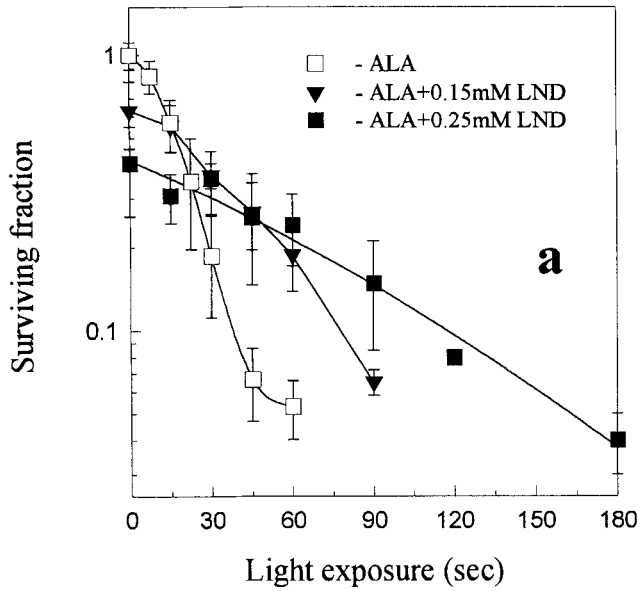
FIGURE 3 – Content of protoporphyrin IX in cells at high cell density (2×10^5 cells/cm^2) after 4 hr incubation in the dark with 0.5 mM ALA or/and LND or LEV as indicated. Bars, SD from 3 independent experiments.

were extracted immediately after irradiation by a method based on the alkaline extraction procedure described by Stocchi *et al.* (1985). The cells were washed with ice-cold PBS and nucleotides were extracted by adding 0.5 ml of ice-cold 0.5 M NaOH to the 60-mm dishes of cells on ice. The dishes were scraped with a rubber policeman, the samples were transferred to centrifuge tubes (2 ml), the dishes were washed once more with 0.5 ml of ice-cold 0.5 M NaOH combined with the first extracts, and 0.2 ml was reserved for protein analysis. The rest of each sample was neutralized by adding 0.8 ml of 1 M KH_2PO_4 per tube, the samples were pelleted and the supernatants stored frozen at −80°C until use for nucleotide determination.

HPLC. The HPLC system consisted of a pump (Spectra Physics 8800, Hempstead, UK), a reversed-phase column (Supelcosil LC-18-T (4.6 × 250 mm), Supelco, Gland, Switzerland), a UV-light detector and an integrator (Spectra Physics Data-jet) connected to a computer. The mobile phase consisted of 2 eluents: (1) start buffer A was a mixture of 1.5% acetonitrile (v/v), 0.1 M KH_2PO_4 and 0.08% tetrabutylammonium bromide, pH 5.0; (2) final buffer B was a mixture of 10% acetonitrile, 0.15 M KH_2PO_4 and 0.08% tetrabutylammonium bromide, pH 5.0. All buffer solutions, as well as standards and sample solutions, were filtered through 0.22 μm Millipore filter. The chromatographic conditions used to obtain the chromatograms were the following: 10 min at 80% of buffer A and 20% of buffer B followed by a 10 min linear gradient to 100% of buffer B, after which chromatography was ended by 10 min of buffer B. The flow rate was 1 ml/min and detection was performed at 254 nm.

Protein analysis

Protein concentrations were determined according to the method of Lowry *et al.* (1951).



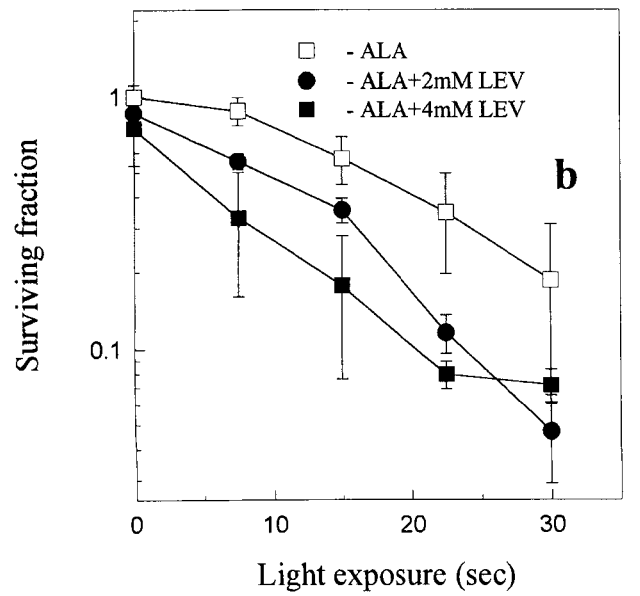
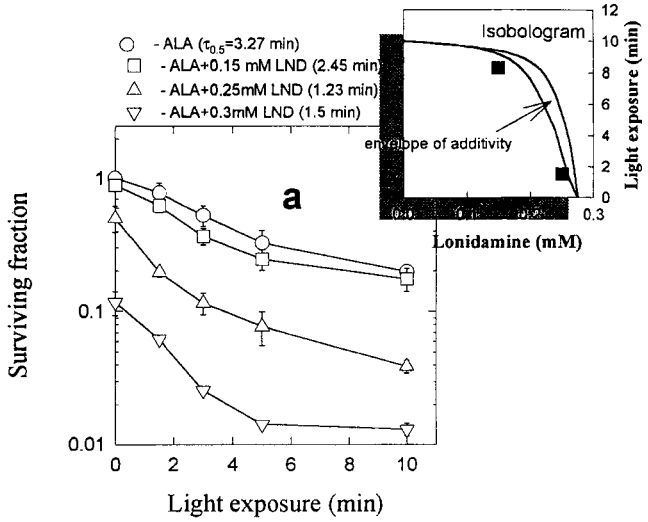


FIGURE 4 – Viability of V79 cells at high cell density (2×10^5 cells/cm^2) after photochemotherapy. Cells were pre-incubated for 4 hr with 0.5 mM ALA and different concentrations of LND or LEV as indicated. Bars = SD.



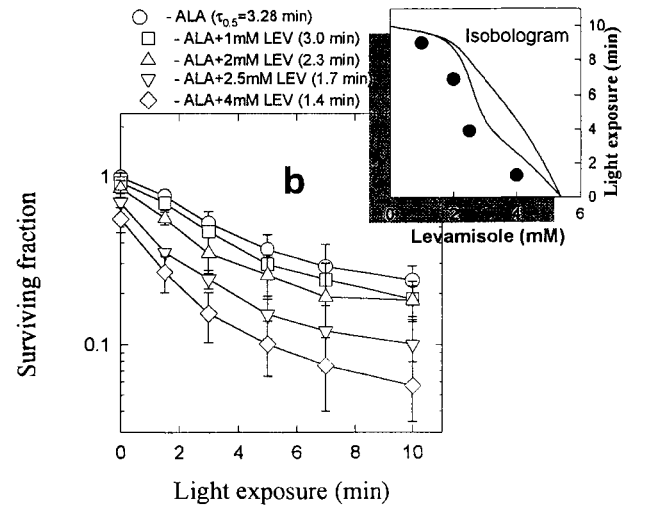


FIGURE 5 – Clonogenicity of V79 cells (20 cells/cm^2) after photochemotherapy (4 hr pre-incubation with 0.5 mM ALA, then irradiation) followed by 24 hr incubation with LND or LEV as indicated. In parentheses, time in minutes needed for inactivation of 50% of the cells. Bars = SD. Insets: Isobolograms for the combined effects of PDT and LND/LEV on V79 cells. Interaction points were obtained at surviving fractions of 0.2 in the survival curves shown in the main Figure.

Oxygen consumption

Oxygen utilization in whole cells was measured using a Clarke oxygen electrode (YSI, Yellow Springs, OH). The oxygen concentration in PBS in the chamber was calibrated at 200 nmol/ml at 37°C. Cell concentration ranged from 2×10^6 to 5×10^6 cells/ml. Linear oxygen consumption was obtained by cell suspensions diluted in PBS containing 5.55 mM glucose. A microchamber (0.6 ml volume) was used to estimate the basal rate of oxygen consumption. Values used to calculate the average oxygen consumption/mg protein/min fell within the linear range of the rate curve for oxygen consumption.

Photochemical oxidation of NADH

Photochemical oxidation of NADH was carried out in the presence of air at 20°C by continuous stirring; 1 μg/ml HPD photosensitized degradation of 0.1 mM NADH was monitored by its absorbance at 340 nm and/or by fluorescence emission at 460 nm and excitation at 340 nm. The fluorescence emission of the photoproduct of NADH was measured at 390 nm with the excitation wavelength set at 300 nm. The irradiation source was a 1-kW Xe lamp, equipped with filters to isolate the spectral region around 580 nm. The amount of NAD^+ formed from 10 μg/ml HPD-sensitized NADH oxidation was measured enzymatically. In particular, to 0.3 ml of irradiated solution, 1 ml of buffer (0.6 M glycine, 0.5 M hydrazine, pH 9.2), 16 U/ml L-lactate dehydrogenase (from bovine heart, Sigma), 0.4 mM L-lactic acid and up to 3 ml distilled water were added. After 30 min incubation at 37°C, the 340-nm absorption band of NADH was measured.

RESULTS

The common inhibitors of energy metabolism, especially those that uncouple oxidative phosphorylation, can act as modulators of porphyrin biosynthesis (Battle, 1993). Thus, the effect of LEV and LND on the ALA-PCT was investigated in 2 ways. The first approach was a 4-hr joint preincubation with ALA, followed by photoirradiation. In the second approach, LEV or LND were administered for 24 hr immediately after ALA-PCT.

LEV and LND exerted only a minor cytotoxic effect when combined with ALA treatment for 4 hr in the dark (Fig. 1). However, both inhibitors, especially LND, mediated complicated concentration-dependent alterations of the ALA-PCT. LND (0.15 mM) was non-toxic in the absence of light even after 24 hr incubation (Fig. 2), but enhanced the sensitivity of the cells to ALA-PDT in a synergistic manner (Fig. 1*a*). In contrast, upon increasing the concentration of LND from 0.15 mM up to 0.4 mM a progressively decreasing efficacy of the combined treatment was observed. The optimal concentration of LND to enhance the sensitivity to ALA-PCT may be below 0.15 mM, while concentrations higher than 0.4 mM might negatively alter the sensitivity of the cells to ALA-PCT. This is confirmed by isobologram analysis as described by Steel and Peckham (1979) (Fig. 1*a*, inset).

LEV (3 mM) induced a 1.5-fold increase in ALA-PCT efficacy (Fig. 1*b*). However, higher concentrations did not further increase the sensitivity to photoinactivation (Fig. 1*b*, inset). The different effects of LEV and LND on the ALA-PCT might be mediated by their alterations of the ALA-induced synthesis of endogenous porphyrins. In fact, increasing the LND concentration up to 0.25 mM decreased the intracellular content of PpIX by a factor of more than 3 (Fig. 3). In contrast, LEV stimulated the ALA-induced synthesis of PpIX within the whole range of concentrations investigated (Fig. 3). This correlates well with the increased rate of photodamage of V79 cells (Fig. 1*b*). It should be pointed out that the efficacy of ALA-PCT increased with increasing cell density in the monolayer. At the high cell density used for measuring cellular PpIX content, the light dose needed to achieve a given level of inactivation was more than 10 times lower than the dose needed at low cell density (Figs. 1, 4). This is probably due to the cells' ability to enhance synthesis of PpIX by increasing the cell density (Berg *et al.*, 1995). The effect of LND or LEV on ALA-PCT seems to be independent of cell density (*cf.* Figs. 1, 4).

Incubation of both LEV and LND for 24 hr subsequent to ALA-phototherapy increased the efficacy of ALA-PCT (Fig. 5). The data indicate that cells pre-treated with phototherapy are more sensitive to the cytotoxic influence of LEV and LND than untreated cells, as seen by the increased slopes of the survival curves when PCT is combined with LEV or LND,

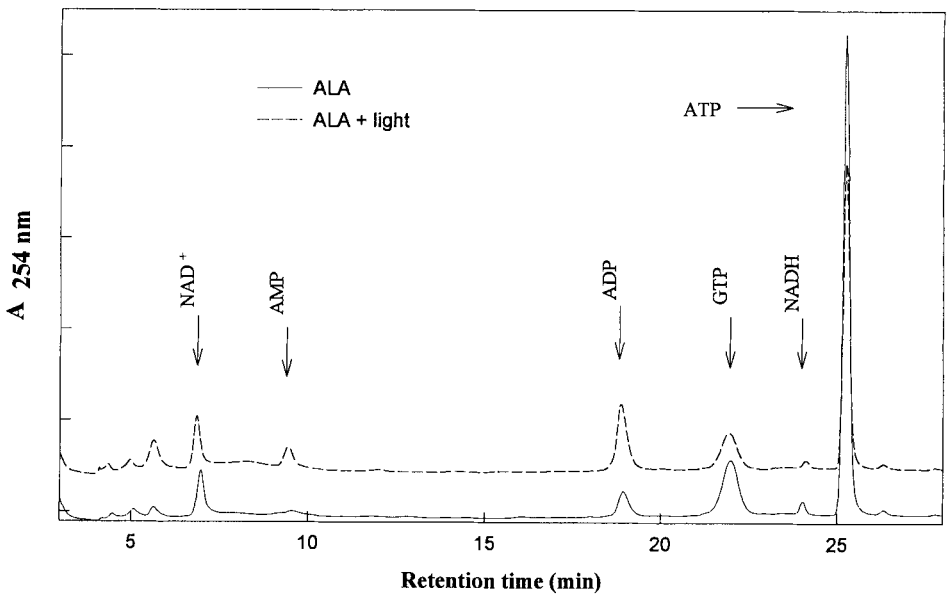


FIGURE 6 – HPLC histograms of nucleotides from cells incubated with ALA only and cells treated with ALA and light as indicated. The cells were incubated with 0.5 mM ALA for 4 hr before light exposure. The nucleotides were extracted from cells immediately after a light dose inactivating 95% of the cells.

respectively (Fig. 5). Isobologram analysis (Steel and Peckham, 1979), also indicates that LEV and LND act synergistically with ALA-PDT (Fig. 5 insets).

The intracellular levels of several nucleotides (ATP, ADP, AMP and GTP) and pyridine dinucleotides (NADH and NAD^+) were assessed by HPLC (Fig. 6). In accordance with results from treatment with HpD and light (Boegheim *et al.*, 1988) the level of ATP was decreased and that of ADP increased immediately and transiently after ALA-PCT (Fig. 7). Energy charge and the NADH/NAD^+ ratio were therefore determined immediately after ALA-PCT. V79 cells treated with ALA show a considerable decrease in their intracellular ATP and GTP levels after photoirradiation (Figs. 6, 7, 8*a, h*). This was accompanied by a significant increase in levels of ADP and, to a lesser extent, of AMP (Fig. 8*b, c*). The energy charge [(ATP + 0.5 ADP)/(ATP + ADP + AMP)] decreased significantly up for to 120 sec following light exposure but increased thereafter (Fig. 8*d*). The decrease in ATP and GTP levels depended upon the endogenous porphyrin level and dose of light. The alterations in ATP and GTP were faster and more striking in the presence of LEV (Fig. 8*a, h*), which caused an increase in the PpIX content in cells (Fig. 3). LND, which in turn inhibited the synthesis of ALA-induced endogenous porphyrins, reduced the effect of photoirradiation on energy charge and the NAD^+/NADH ratio (Fig. 9). LND (0.25 mM) alone or in the presence of light caused a 20–30% depletion in the levels of ATP and GTP, while administration of LEV (± light) or ALA in the absence of light did not induce significant changes in the levels of ATP or GTP.

The ratio of NADH to NAD^+ in cells plays an important role in controlling the rate of energy production. As previously shown (Chekulaev *et al.*, 1991; Kirveliene *et al.*, 1989; Bodaness and Chan, 1977), several photosensitizers (hematoporphyrin and its derivative, chlorin e_6 and others) are able to oxidize the reduced, but not the oxidized form of pyridine dinucleotides. The rate of porphyrin-photosensitized oxidation of NADH considerably exceeds (4–5 times) that of L-tryptophan (Hilf *et al.*, 1984; Kirveliene *et al.*, 1989). Since NADH is the main electron donor for the mitochondrial respiration chain, we assumed that the depletion of intracellular ATP and GTP after ALA-PCT (Fig. 8) could be induced by photochemical oxidation of NAD(P)H, especially if the photoproducts differ from enzymatically active forms (NAD^+, $NADP^+$). It was found that the decrease in the absorption of the 340-nm band of NADH during HPD-photosensitized oxidation was accompanied by a reduction in the characteristic NADH fluorescence emission (Fig. 10). An emission band at 390 nm was simultanously formed. Since NAD^+ has no fluorescence, the appearance of this emission band may be explained by the formation of photoproducts which are different from NAD^+. Photoproducts with the above-mentioned properties were detected during photo-induced oxidation of NADH (Czochralska *et al.*, 1984). However, enzymatic measurements indicate that the main product of NADH oxidation is enzymatically active NAD^+ (Table I). Similar results have been presented for

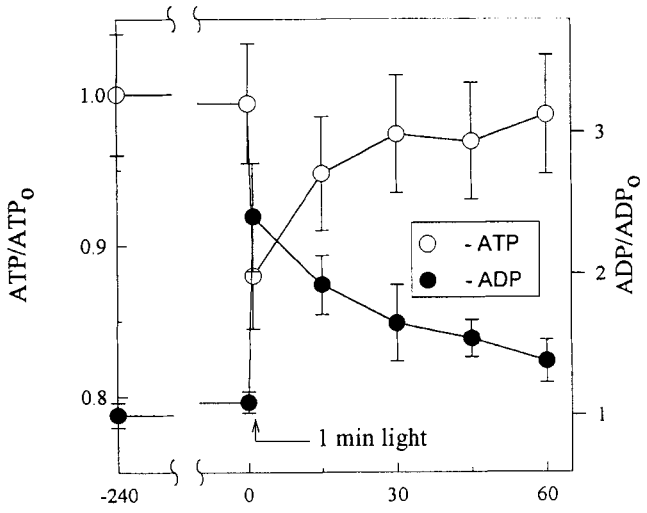


FIGURE 7 – Effects on cellular ATP (○) and ADP (●) levels during the first hour after ALA-PCT. Cells at high density were exposed to 1 min of light after 4 hr pre-incubation with 0.5 mM ALA. Cells were left on the monolayer and re-fed with fresh medium immediately after irradiation.

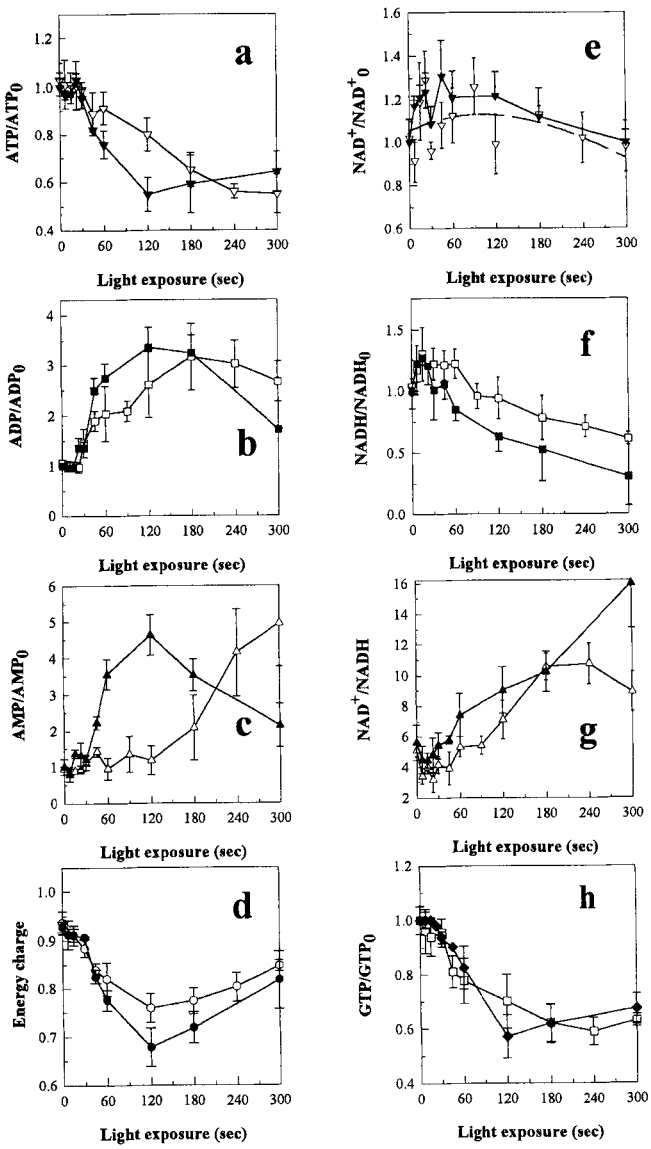


FIGURE 8 – Contents of cellular nucleotides after photochemotherapy with or without co-treatment with LEV. The cells at high density were exposed to light after 4 hr of pre-incubation with 0.5 mM ALA in the presence (filled symbols) or absence (open symbols) of 4 mM LEV in the medium. Cell viability at the same cell density and under the same conditions is presented in Figure 4. Bars = SD. No changes were observed in the contents of nucleotides in the controls left in the dark or in the content of cellular proteins after irradiation.

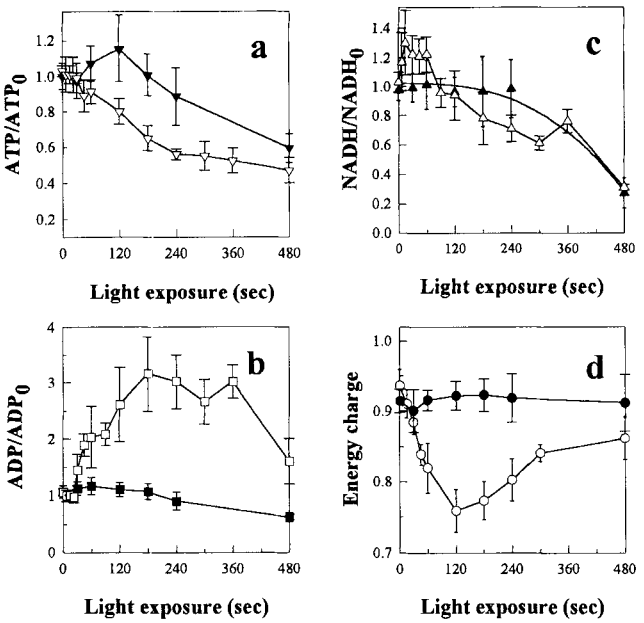


FIGURE 9 – Influence of LND on the contents of cellular nucleotides after ALA-photochemotherapy. The cells at high density were exposed to light after 4 hr of pre-incubation with 0.5 mM ALA in the presence (filled symbols) or absence (open symbols) of 0.25 mM lonidamine in the medium. LND (0.25 mM) alone or in the presence of light caused a 20–30% depletion in the levels of ATP and GTP which is not shown in the Figure.

HPD-sensitized photooxidation of NADPH (Bodaness and Chan, 1977).

In contrast to the *in vitro* results, photoactivation of endogenous PpIX in V79 cells mediated an approximately 30% increase in the content of NADH after low doses of light (Fig. 8*f*). This was accompanied by a decreased NAD^+/NADH ratio (Fig. 8*g*). After higher doses of light, a depletion in the cellular redox potential was observed. An increased NAD^+/NADH ratio and a slight enhancement of the NAD^+ level were observed only at very high light doses, inactivating more than 90% of the cells (Figs. 4, 8). Treatment of the cells with LEV or LND in the absence or presence of light, in combination with ALA, or with ALA alone, did not significantly change the NAD^+/NADH ratio.

Light alone did not perturb the cellular rate of oxygen consumption, while a substantial reduction in oxygen consumption was observed upon ALA-PCT (Fig. 11). It should be pointed out that the ALA-PCT-induced reduction in O_2 consumption followed the inactivation of cells, while higher light doses were necessary for reduction of the levels of ATP, GTP and NADH (Figs. 5, 8, 11).

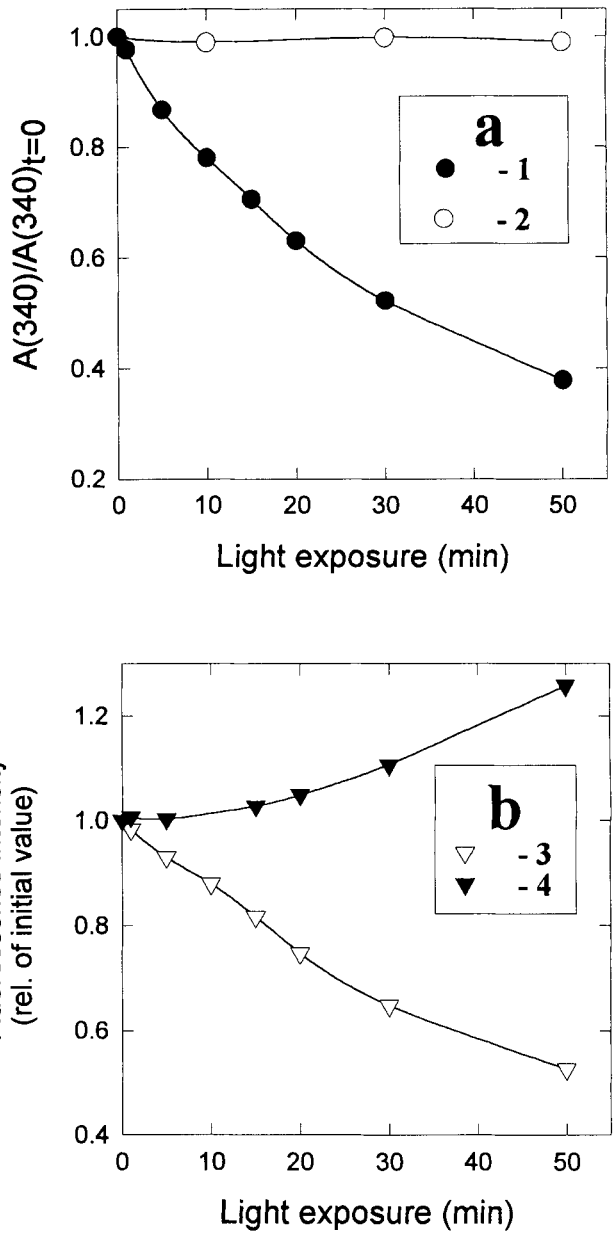


FIGURE 10 – Relative absorbance and fluorescence of 0.1 mM NADH during 1 μg/ml HPD-photosensitized (irradiation at 580 nm) oxidation. Relative absorbance at λ = 340 nm was measured in the presence (2) or the absence (1) of HPD in the solution. The NADH fluorescence was measured at 460 nm and excitation at 340 nm (3). The fluorescence of the NADH photoproduct was measured at 390 nm and excitation 300 nm (4). Means of 2–3 experiments. Error limits approximately 5%.

DISCUSSION

It has been suggested that PpIX accumulated in ALA-treated cells is located, at least partly, in the mitochondria (Iinuma *et al.*, 1994; Sandberg and Romslo, 1980). The purpose of the current studies has therefore been to evaluate the effects of ALA-PCT on the energy metabolism of V79 cells and the effects of combining ALA-PCT with the modulators of energy metabolism LEV and LND. The present study shows that ALA-PCT treatment of V79 cells induces an inhibition of respiration which is correlated with the sensitivity of the cells to photoinactivation (Figs. 4, 11). Higher light doses are needed for perturbation of the energy charge, *e.g.* 1 min of light exposure induced 90% inhibition of oxygen consumption, 15% decrease in ATP and inactivation of approximately 95% of the cells (Figs. 4, 8, 11). This might suggest that mitochondria are primary targets of ALA-PCT. Indeed, the photodestruction of other cellular structures, particularly the plasma membrane, was observed at much higher (2–3 times) light doses. This is indicated by a reduction in the sum of all the nucleotides (Figs. 8, 9). Our data are in good agreement with those of other investigators. Sandberg and Romslo (1980) demonstrated, using isolated rat liver mitochondria, the ability of PpIX to mediate photodamage to these organelles (uncoupling, decreasing of membrane potential, diminishing of succinate dehydrogenase activity). Iinuma *et al.* (1994), using fluorescence and electron microscopy, showed that exogenous ALA-induced PpIX accumulated in mito-

TABLE I – ESTIMATION OF THE ENZYMATICALLY ACTIVE NAD^+ FORMED FROM NADH HPD-PHOTOSENSITIZED OXIDATION[1]

Irradiation time (min)	% of photooxidized NADH	Reformation of NADH from photoproducts (% of unirradiated solution)
0	0.0	100.0 ± 4.6
3	35.4 ± 0.9	85.4 ± 4.3
7	65.8 ± 1.9	73.1 ± 3.6

[1]The HPD-photosensitized (10 μg/ml) oxidation of 1 mM NADH was carried out in PBS under filtered light with the maximum at 580 nm. The content of formed NAD^+ was measured enzymatically as described in the text.

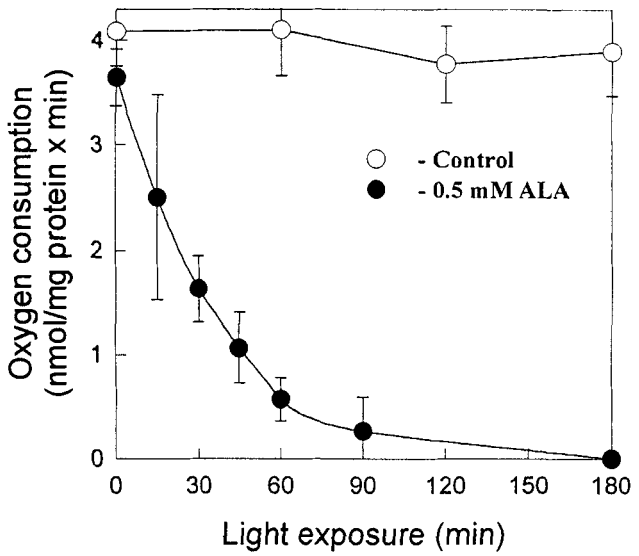


FIGURE 11 – Influence of ALA-PDT on the oxygen consumption of V79 cells. The cells at high density were exposed to light after pre-incubation with 0.5 mM ALA as indicated. Bars = SD. Cell viability at the same cell density and under the same conditions is presented in Figure 4.

chondria. The primary site of photodamage was suggested to be the mitochondria (swelling, disruption of the cristae) since other organelles (*e.g.* lysosomes, Golgi apparatus) appeared to be unaffected. However, it has been suggested that ALA-induced PpIX may also be located in other subcellular organelles (Gaullier *et al.*, 1995; Berg *et al.*, 1996).

The observed reduction in energy charge was most pronounced immediately after light treatment. After low doses of light, the energy charge reverted to control values 30 min after light exposure (Fig. 11). The intracellular content of ATP was also found to revert to control values after PCT with HpD in L 929 cells (Boegheim *et al.*, 1988). Thus, despite the obvious signs of early injury to mitochondria, no persistent decrease in the ATP level was observed at doses sufficient to inhibit respiration. Maintenance of the energetic homeostasis of tumor cells is provided by glycolysis and oxidative phosphorylation (Pedersen, 1978). Therefore, the maintenance of a high content of ATP in V79 cells may be provided by glycolysis. The present data indicate that the inactivation of cells after ALA-PCT is not caused by energy deprivation.

In the present study, ALA-PCT was combined with LEV and LND since these compounds are inhibitors of the glycolysis and the respiration chain (Guminska *et al.*, 1986; Floridi *et al.*, 1981*b*; Floridi and Lehninger, 1983). In the present study, LEV and LND were also found to influence the 5-ALA-induced porphyrin synthesis (Fig. 3). A synergistic effect of combining ALA-PCT and non-toxic concentrations of LND was found when LND was given prior to light exposure (Figs. 1, 4). This synergism was observed despite the substantial LND-induced inhibition of PpIX synthesis (Fig. 3), *i.e.* the quantum yield for photoinactivation of cells can be substantially increased by pre-treatment with LND. This potentiating effect of LND might be due to its effect on either glycolysis or the respiration chain, although only a small effect on the energy charge and the NAD^+/NADH ratio was observed after LND treatment alone. At high doses of LND (0.4 mM), the combination treatment became less efficient. This was most likely due to the inhibition of PpIX synthesis induced by LND (Fig. 3).

LEV given prior to ALA-PDT acted in a synergistic manner when survival, energy charge and the NAD^+/NADH ratio were used as end points (Figs. 1, 4, 8). This was at least partly due to the effect of LEV on ALA-induced PpIX formation (Fig. 3). Thus, the sensitivity of the cells to photoinactivation per cell-bound PpIX molecule is lower than indicated in Figures 1 and 4. It is not clear from the present results whether LEV may perturb the energy metabolism since LEV alone reduced neither the energy charge nor the NAD^+/NADH ratio.

When LEV or LND were given after phototherapy, these 2 treatment modalities acted in an additive or slightly synergistic manner (Fig. 5). This is in accordance with the results of combining PCT and mitomycin C, where mitomycin C was found to be more efficient when given before than after PCT (Ma *et al.*, 1993).

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