First Studies on Preclinical ALA-based Photodynamic Therapy of Cancer

Ž. Lukšienė[1],
L. Griciūtė[1],
R. Gadonas[2],
V. Vaičaitis[2],
V. Sirutkaitis[2]

[1] Lithuanian Oncology Center, Santariškių 1, LT-2007 Vilnius, Lithuania
[2] Laser Research Center, Vilnius University, Saulėtekio 9, LT-2040 Vilnius, Lithuania

ALA-mediated photodynamic therapy is a new and promising cancer treatment modality. Preclinical studies on endogenous photosensitizer PpIX production in two different types of tumors, therapeutic efficiency, the mechanism of tumor damage and induced skin photosensitivity have shown that tumors of different histogenesis are producing different amounts of PpIX, and this is in clear correlation with therapeutic efficiency. Besides, induced mice skin photosensitivity during treatment lasts at least 2–3 days.

Key words: photodynamic therapy, aminolevulinic acid, protoporphyrin, Ehrlich ascites carcinoma, A22 hepatoma, preclinical studies

INTRODUCTION

Photodynamic therapy (PDT) is based on selective destruction of tumor by visible light and a photosensitizing agent mostly accumulated in the tumor cells. One of the most attractive advantages of this treatment modality is its selectivity to tumor tissue: PDT does not damage normal tissues and is also free from the major side effects of surgery, radiotherapy and chemotherapy. Recently photofrin-mediated PDT has been approved by U. S. FDA and the authorities of Canada, Japan, the Netherlands, France and Germany. However, photofrin-mediated PDT has a major side effect of skin phototoxicity for at least 4–6 weeks (1). Newly developed ALA (aminolevulinic acid)-mediated PDT is based on the use of the endogeneous sensitizer protoporphyrin (PpIX), which is synthesized in tumor cells from exogeneously used ALA (2). The benefits of ALA-mediated PDT include reduced skin photosensitivity, easy administration (topical or oral) and fractionation of use if necessary (3).

The aim of this study was to investigate the basic concepts of ALA-based PDT: efficiency of PpIX production in tumors of different histogenesis, tumor growth inhibition after PDT, type of tumor damage and skin photosensitivity.

MATERIALS AND METHODS

Chemicals and photosensitizers. 5-aminolevulinic acid (Merck, Germany) was freshly dissolved in sterile isotonic saline (0.9% NaCl) and was prepared for every new experiment (10^{-3} M, pH 7.0 adapted by 1N NaOH), sterilized and used in various concentrations.

Tumors and experimental apparatus. The experiments were carried out using the BALB/c mice strain. Ehrlich ascites carcinoma was transplanted into female mice aged 6–7 weeks and weighing approximately 21 g. The implantation procedure is summarized as follows.

Tumor is extracted from a donor mouse and E. ascites tumor cells (0.8×10^6) are inoculated intraperitoneally (i. p.) using a 25 G needle to healthy mice.

On the 7th day after tumor inoculation, in its exponential growth phase ALA was injected i. p. 40–800 mg/kg body weight. After 3 h of incubation Ehrlich ascites tumor cells were excluded from the peritoneal cavity and prepared *ex vivo* in the dark as a homogeneous cell suspension with the optical density OD = (3.7×10^6 cells/ml). Irradiation of cells was performed in 2 mm cuvettes. After treatment, 0.2 ml of irradiated cell suspension (0.8×10^6

cells) was inoculated in healthy mice i. p. and tumor growth was measured for 15 and more days. All experiments were done in the dark and repeated 3 times.

The hepatoma A22 implantation procedure could be summarized as follows. Tumor mass was separated from connective tissues, rinsed in physiological saline (0.9% NaCl) and cut into small pieces, passed through a 26-gauge needle and then implanted into the right leg of a recipient mouse (0.3 ml tumor cell suspesion). The tumors grew to a volume of about 200 mm^3 within 6–7 days after inoculation and at this size were used for experiments (4). Every experimental group consisted of 6–8 mice. The control group had no treated tumors of the same day of inoculation. Each experimental point represents the average value of 28–40 mice.

All animals were under general anesthesia (ketamine hydrochloride, i. p.) during all experimental procedures.

Irradiation sources. The light source used for irradiation of Ehrlich ascites carcinoma cell suspension consisted of tungsten lamp (500 W), optical system for light focusing and optical filter for UV and infrared light elimination (370 nm < λ < 680 nm). Light intensity at the position of the cells was 50 mW/cm^2. The inradiation time was 90 s.

The non-coherent light source for irradiation of solid tumours A22 was constructed at the Vilnius University Laser Research Center. As a primary light source a 400 W power incandescent lamp was used for this device. The emitted light was collimated with the help of short focal length lens and subsequently twice-reflected from the dielectric narrowband optical mirrors. Additional optical filters were used to cut off the radiation with the wavelengths far from a selected wavelength region, because dielectric filters are effective only in a limited spectral region. The ambient air was used for cooling the system. This device had two outputs: one delivering the radiation with a fixed central wavelength of 630 nm, and the second one, delivering radiation with 5 changeable central wavelengths: 522, 630, 650, 670, and 690 nm. The radiation in each spectral region had the bandwidth of about 50 nm. The power of produced radiation in each case was more than 120 mW and the intensity of light in focus (assuming the diameter of the spot as 10 mm) in all cases exceeded 90 mW/cm^2.

The wavelength chosen for the excitation of ALA was 630 nm. Irradiation time to reach tumor necrosis in this case was 35 min.

Tumor growth determination. Relative Ehrlich ascites tumor growth was measured daily up to day 15 of its growth according to the equation:

$$S = (S_1 - S_0)/S_0,$$

where S_1 is the final weight of mouse with tumor, S_0 is the initial weight of intact mouse, S is the relative tumor growth.

Moreover, Ehrlich ascites tumor growth was measured in two other ways: 1) absolute tumor volume growth during 15 days; 2) tumor cell number during 15 days.

The correlation between absolute tumor weight and relative tumor growth was found very strong (r = 0.98). In order to simplify the experimental protocol, we usually measured just the relative tumor growth.

The volume of A22 hepatoma was measured *in vivo* and calculated (5):

$$V = \tfrac{1}{2}(4\pi/3) \times (l/2) \times (w/2) \times h,$$

where V is the volume of tumor, *l* is the length of tumor, w is the width of tumor, h is the height of tumor.

Measurements of intracellular concentration of photosensitizer. Ehrlich ascite was collected from the mice 3 h after treatment with photosensitizer. Tumor cells were suspended in phosphate-buffer solution (PBS) to an optical density OD = 0.6 (3.7 mill/ml).

The fluorescent spectra of the suspension were measured with a unique spectrofluorometer СФР-1 (Moscow, Russia), the sample being excited through an interference filter with $\lambda_{exc.}$ = 405 nm and an epiobjective. The fluorescence was registered from the front surface of the sample. The constructional features of the device made it possible to measure the fluorescence of a thin layer (less than 1 mm) of the solution without spectrum distortions due to the effects of the intrinsic filter and light scattering. The fluorescence was excited with the radiation of a mercury lamp through an interference filter with $\lambda_{exc.}$ = 405 nm and was registered at λ = 600–680 nm with an emission slit of 10 nm. The measurements were made at room temperature.

A22 hepatoma tumor was separated from the mice 24 h after treatment with photosensitizer and tumor cells were suspended in PBS to an OD = 0.8 (3.7 mill./ml).

PpIX pharmacokinetic studies

The tissue fluorescence in experimental animals was measured using a spectrofluorimetric setup. It consisted of a light source (halogen lamp 150 W), fiber optic probe, and registration system (flat field grating polychromator (Solar TII S380) with a cryogenically cooled CCD camera (Princeton Instruments)). A quartz fiber optic probe was used to deliver excitation light to the brain surface and to collect the

emitted fluorescence. The spectrum of the light source was filtered to provide the violet-blue light for excitation (375–500 nm, BGG22 and BG15). Seven quartz fibers were used to guide the light to the site of investigation. The fluorescence was collected by separate fiber and guided to a polychromator entrance slit. To eliminate stray light at the wavelengths below 500 nm, an OG13 long-pass filter was placed in front of the polychromator slit. During all measurements the same exposure time was used for accumulation of fluorescence spectra. All fluorescence measurements were made in a darkened room with the probe hold approximately at a distance of 0.8–1 mm from the tissue surface at a perpendicular angle.

The measured fluorescence spectra were stored in the computer memory for further analysis. At each side, three measurements were taken and their values averaged. Every spectrum was corrected to eliminate the distortion provided by the long-pass filter, and a separately measured background of the CCD camera was taken off.

Protein quantitation determination. The quantitation of protein was determined by the Bradford method in cell suspension.

Pathohistological examination. The tumor tissue was fixed in 10% formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin.

Statistical evaluations. All experiments were repeated at least three times. Averaged values and standard deviations were calculated.

All animals were kept according to requirements for the use of Laboratory Animals in Scientific Experiments in Lithuania (1999).

RESULTS

PpIX producton in EAT and A22 hepatoma. In order to prognosticate the ALA-mediated PDT efficiency, PpIX production was investigated in 2 different types of tumors, using a broad range of ALA concentrations (20–800 mg/kg body weight). The time between ALA injection and tumor irradiation in all cases was 4 hours. Data presented in Fig. 1 clearly indicate that PpIX production significantly differed in EAC and A22 hepatoma tumors. It seemed that EAC tumor cells seemed to be weakly accumulating ALA or hardly synthetizing PpIX, because only a small amount of PpIX was detected in these cells.

Quite another picture was observed in a A22 hepatoma tumor model. In this case the increasing ALA concentration produced increased amounts of intracellular PpIX.

Tumor growth Inhibition after ALA-mediated photodynamic treatment. Tumor growth inhibition is

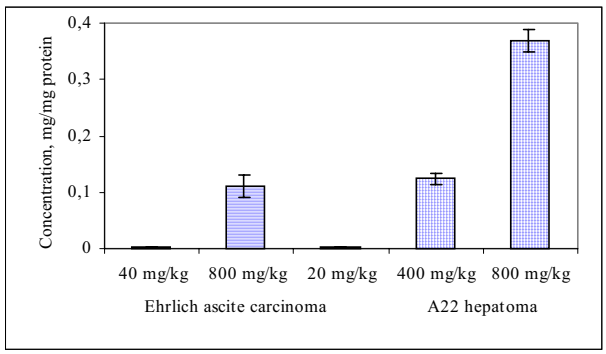

Fig. 1. PpIX production in Ehrlich ascite carcinoma and A22 hepatoma

one of the most important parameters in evaluation of PDT efficiency. Figure 2 shows A22 hepatoma tumor growth in control and 800 mg/kg ALA-PDT-treated mice groups. Observations that lasted 10 days after treatment indicated that ALA-based PDT inhibited tumor growth by 82% in comparison with control. Figure 3 shows EAT growth in control and 800 mg/kg ALA-PDT-treated mice groups. According to the data obtained, no statistically significant tumor growth inhibition was observed in this case during 15 days after treatment.

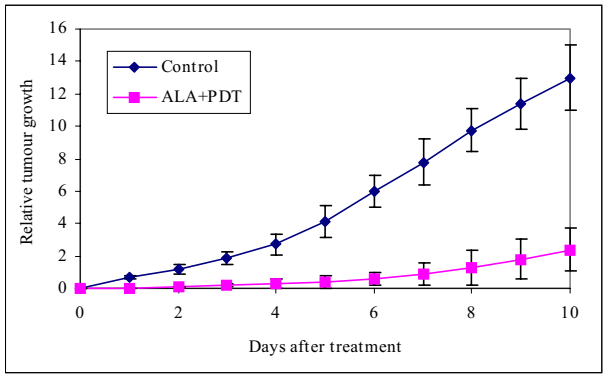

Fig. 2. A22 hepatoma growth inhibition after 800 mg/kg ALA-based photodynamic treatment

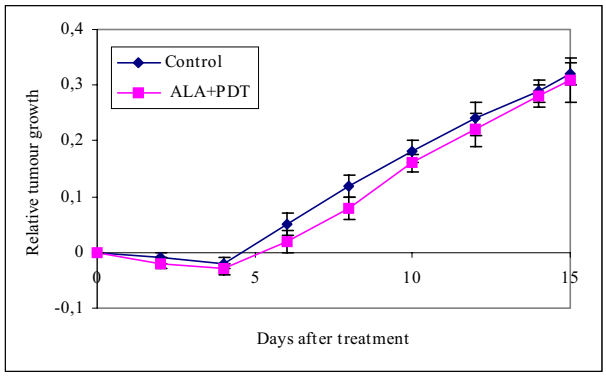

Fig. 3. EAT growth inhibition after 800 mg/kg ALA-based photodynamic treatment

Type of tumor damage after ALA-mediated PDT treatment. In order to estimate tumor damage after ALA-mediated PDT treatment, histological evaluation of tumor slices after treatment was performed. Macroscopically, immediately after PDT treatment no hemorrhage was observed on the surface of tumor, but following 24 hours histological pictures showed almost a full degradation of tumor structure – necrosis of the central part, necrobiotic cells under the capsuli, some small hemorrhagies between them. Small blood vesels in the capsule were unchanged (Fig. 7).

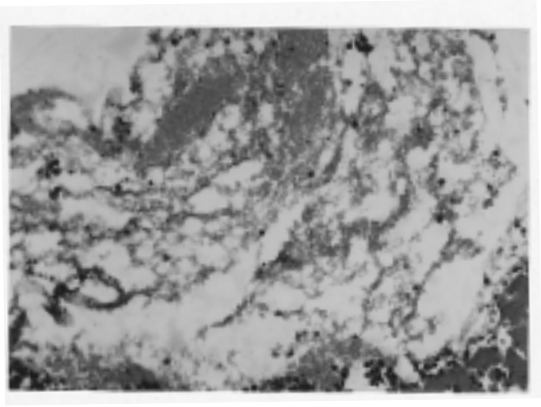

Fig. 4. A22 hepatoma tumor damage 24 h after ALA-mediated PDT treatment

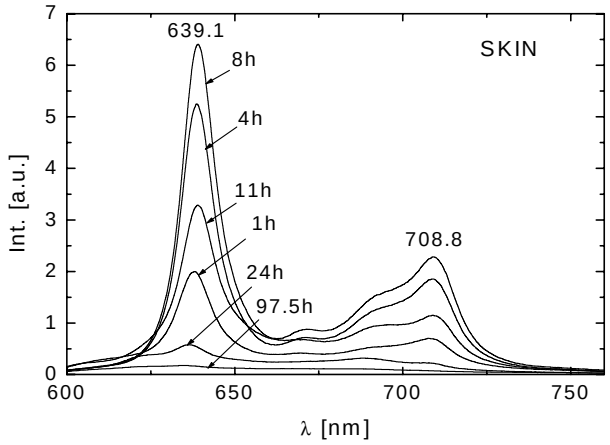

Fig. 5. Fluoresence spectra of intracellular ppIX at different time intervals after injection of 400 mg/kg

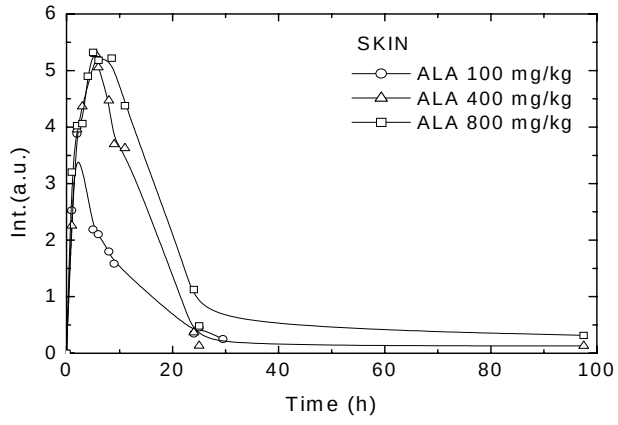

Fig. 6. PpIX accumulation and clearance from mice skin when different ALA concentrations were used

Investigation of skin photosensitivity in mice during ALA-mediated PDT. Using a spectrofluorimetric system, PpIX fluorescence spectra were obtained from the surface of the mouse skin. Spectra of intracellularly accumulated PpIX in the skin, performed at different time intervals after ALA injection are presented in Fig. 5. PpIX production 1–97.5 h after ALA injection was evaluated. According to the data obtained, the PpIX intracellular concentration in the skin is highest 4–8 hours after ALA injection. A subsequent decrease of PpIX content in the cells was observed (11–24 h), and absolute clearance was detected 97.5 hours after ALA injection (Fig. 6). It is important to note that the increase of ALA concentration (from 100 mg/kg to 800 mg/kg) increased the "production" time of PpIX in skin cells, as well as clearance from them.

DISCUSSION

Effective PpIX production in tumor cells is one of the most important determinants in ALA-mediated PDT treatment (9). According to our data, PpIX production strongly depends on the type of tumor, its histogenesis and cell membrane histoarchitecture. It seems obvious that the histological type of tumor has its individual characteristics of PpIX production at the same experimental protocol. Measurements of tumor growth inhibition after PDT treatment confirmed a clear correlation between intracellular PpIX concentrations and therapeutic effect: the growth of EAT was not inhibited, because this type of tumor does not produce sufficient amounts of the endogeneous photosensitizer PpIX. On the contrary, A22 hepatoma tumor growth was inhibited for more than 21 days, probably due to a rather effective PpIX production in hepatoma cells.

What are the mechanisms of tumor damage? It is well known that after PDT tumor can be damaged in two ways: by destroying tumor cells directly or through destroying tumor vasculature. Hydrophilic photosensitizers ($TPPS_4$, Hpde) are mostly accumulating in the endothelium of the vasculature, but hydrophobic photosensitizers (PpIX) are localized in the tumor cells. According to histological evaluations, immediately after PDT treatment an extensive damage of vasculature was observed in A22 hepatoma after $TPPS_4$-mediated PDT, but not after ALA-mediated PDT. Following 24 hours, total tumor necrosis and some not extensive damage of vessels was observed in this case.

Prolonged skin photosensitivity is one of the most serious disadvantages of conventional photofrin-mediated PDT. All patients undergoing PII-PDT will remain photosensitive for up to 6 weeks after PDT treatment. No well controled, prospective clinical studies of skin photosensitivity have yet been carried out. One of the most important benefits of ALA-mediated PDT is reduced skin photosensitivity. Pharmacokinetic studies of PpIX levels in mice skin have shown that retention of the endogeneous photosensitizer lasted in the skin no more than 24–97.5 hours after ALA injection.

In conclusion, response to PDT is a very complicated function and depends on a lot of factors. Our results confimed the idea that the intracellular concentration of the photosensitizer is one of the factors determining PDT efficiency. Determination of the intracellular concentration of photosensitizer proves to be an important step in predicting the clinical outcome as well as the response of tumor to treatment. It also may assist in modulating current therapy concepts by devising individual treatment protocols according to the degree of sensitizer intracellular accumulation.

ACKNOWLEDGEMENTS

This study was financially supported by the Lithuanian Science and Studies Foundation.

The authors wish to thank Prof. G. Maksimov and Dr. S. Jurkonienė (Lithuanian Institute of Botany) for help in the interpretation of fluorescence measurements, D. Kaskelytė and L. Rutkovskienė for technical assistance.

Received 15 April 2002
Accepted 30 May 2002

References

1. Dougherty TJ, Gomer CJ, Henderson BW, Jori G, Kessel D, Korbelik M, Moan J, Peng Q. Photodynamic Therapy. J Natl Cancer Inst 1998; 90: 889–905.
2. Peng Q, Berg K, Moan J, Kongshaug M, Nesland JM. 5-aminolevulinic acid-based photodynamic therapy: Principles and experimental research. Photochem Photobiol 1997; 65: 235–51.
3. Peng Q, Warloe T, Berg K, Man J, Kongshaug M, Giercksky KE. Photodynamic therapy with 5-aminolevulinic acid-induced porphyrins and DMSO/EDTA for basal cell carcinoma. SPIE 1995; 2371: 226–35.
4. Luksiene Z. Experimental study of simultaneous photodynamic and hyperthermic treatment on EAC and A22 hepatoma. SPIE 1997; 3191: 338–42
5. Amano T, Prout Gr, Lin Chi-Wei. Intratumoral injection as a more effective means of porphyrin administration for photodynamic therapy. J Urology 1988; 139: 391–5.
6. Lukšienė Ž, Rutkovskienė L, Griciūtė L, Plešnienė L, Čepulis V, Vaičaitis V, Sirutkaitis V. New noncoherent light source for photodynamic treatment of cancer. Acta medica Lituanica 2002; 9(1): 58–61.
7. Luksiene Z, Kalvelyte A, Supino R. On the combination of photodynamic therapy with ionizing radiation. Photochem Photobiol 1999; 52: 35–42.
8. Berg K, Luksiene Z, Moan J, Ma Li Wei. Combined treatment of ionizing radiation and photosensitization of 5-ALA-induced protoporphyrin IX. Radiation Research 1995; 142: 340–6.
9. Lukçienė Ž, Rutkovskienė L, Griciūtė L, Jurkonienė S, Maksimov G. Evaluation of $TPPS_4$ photobiological efficiency in two different types of mice tumours. Acta medica Lithuania 2002; 9(1): 62–6.

Ž. Lukšienė, L. Rutkovskienė, L. Griciūtė, V. D. Kaskelytė, R. Gadonas, V. Vaičaitis, V. Sirutkaitis

ALA MEDIJUOTOS FOTODINAMINĖS VĖŽIO TERAPIJOS IKIKLINIKINIAI TYRIMAI

Santrauka

Aminolevulininės rūgšties (ALA) medijuota fotodinaminė terapija (EDT) yra naujas ir daug žadantis vėžio gydymo būdas. Endogeninio fotosensibilizatoriaus protoporfirino (PpIX) gamybos skirtingų navikų tipuose, navikų pažeidimo ir odos fotojautrumo mechanizmų ikiklinikiniai tyrimai parodė, kad skirtingos histogenezės navikai produkuoja skirtingą PpIX kiekį ir tai tiesiogiai koreliuoja su terapiniu efektyvumu.