

EXPERIMENTAL INVESTIGATIONS

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mTHPC-mediated photodynamic treatment of Lewis lung carcinoma in vitro and in vivo

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Key words: photodynamic treatment; LLC1 cell line; Lewis lung carcinoma; photosensitizer; mTHPC.

Summary. Background and objective. The ongoing search for the enhancement of efficacy of photodynamic therapy stimulates the interest in molecular mechanisms of the response to the treatment. Looking for the cell line suitable for investigation of cellular response both in vivo and in vitro, we evaluated phototoxicity of *m*-tetrakis-(3-hydroxyphenyl)-chlorin (mTHPC) on viability of Lewis lung carcinoma (LLC1) cells in vitro, growth of murine transplantable tumor, and mice survival.

Material and methods. LLC1 cell culture and male C57BL/6 mice bearing Lewis lung carcinoma were used for the experiments. Photodynamic treatment was mediated by *m*-tetrakis-(3-hydroxyphenyl)-chlorin as a photosensitizer. Light emitting diode array was used for illumination. The effect of the photodynamic treatment was evaluated by comparison of viability of control and treated cells, growth of tumors, and survival of the control and treated mice.

Results. In vitro, a cytotoxic dose inducing a reduction in viability of LLC1 cells by 50% was achieved at 60 mJ/cm² and approximately 400 ng/mL of the photosensitizer, or 30 mJ/cm² and 600 ng/mL of mTHPC. Both the concentration of the photosensitizer and duration of light exposure were significant determinants of cytotoxic effect. In vivo, an injection of 0.25 mg/kg of mTHPC to mice bearing Lewis lung tumor and illumination at 120 J/cm² taking place after 24 h significantly inhibited tumor growth and prolonged mice survival. However, the tumors regained their growth potential after 9 days.

Conclusions. Photodynamic treatment mediated by *m*-tetrakis-(3-hydroxyphenyl)-chlorin had a significant effect on LLC1 cells in vitro and growth of Lewis lung carcinoma in vivo.

Introduction

Photodynamic treatment (PDT) is a mode of therapy for eradication of tumors and other formations produced by cell overgrowth. It is based on the excitation of a cell-localized photosensitizer with visible light and the following generation of reactive oxygen species, which induce oxidative damage to cellular components (1). Recent progress in PDT as well as the recorded limitations of the treatment has stimulated attempts to establish details of the molecular mechanism of cellular response to the treatment. It was found that PDT caused overexpression of vascular endothelial growth factor (VEGF) (2) as well as other cytokines (3). Some of them are considered essential for tumor growth and necessary for the spread of tumor cells (4). These findings stimulated the idea to increase the efficacy of PDT by combination with immunotherapy (5). The combination of antiangiogenic and PDT therapy gave the first promising experimental results (6).

For the successful application of immunotherapy, it is necessary to reveal details of the molecular mechanism of cellular response to the treatment. Our previous investigations of photodynamic effect on cells of murine hepatoma MH-22A (7) and human epidermoid carcinoma A431 (8) revealed the significant differences in the pattern of cytokine expression following PDT (unpublished results). However, MH-22A cell line demonstrated a limited repertoire of overexpressed cytokines, and A431 could be cultivated only in vitro. Looking for the cell line suitable for investigation of cellular response to PDT both in vivo and in vitro, we started a study of LLC1 cells. LLC1 is a cell line established from the lung of a C57BL mouse bearing a tumor resulting from an implantation of primary Lewis lung carcinoma 1 (9). This line is widely used as a model for metastasis, implying an overexpression of cytokines, and is useful for studying the mechanisms of cancer chemotherapeutic agents both in vitro and in vivo.

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In this study, starting the investigation of LLC1 cell response to PDT, we evaluated the efficacy of photodynamic treatment in LLC1 cells and Lewis lung tumors. Neutral lipophilic chlorin derivative m-tetrakis-(3-hydroxyphenyl)-chlorin (mTHPC, generic name Temoporfin, commercial name Foscan®) (10) was used for PDT. It is one of the most potent photosensitizers currently available for clinical use, and it has been successfully applied for treatment of head and neck cancers (11). LLC1 cells as well as the tumors have already been used for PDT studies (12–15); however, to our knowledge, this is the first report of mTHPC-mediated PDT of LLC1 cells in vitro and tumors in vivo.

Materials and methods

Photosensitizer. m-Tetrakis-(3-hydroxyphenyl)-chlorin (mTHPC, kindly provided by R. Bonnett, Queen Mary's College, the University of London, UK) was dissolved in ethanol as 3-mg/mL stock solution and stored at -20°C in the dark. All experiments in vitro were performed using dilutions of the stock solutions with cell incubation media. For experiments with mice, the stock solutions were diluted with water for injection to a final concentration of 0.25 mg/kg in 0.2 mL for mTHPC.

Cell culture. Culture flasks and Petri dishes were from Techno Plastic Products AG. Fetal calf serum (FCS) was from Gibco BRL. Other tissue culture products were obtained from Sigma. The murine Lewis lung carcinoma 1 cells, LLC1, were obtained from the American Type Culture Collection (ATCC). The cells were cultured in subconfluent monolayer (60–70%) in 25-cm² flasks in the Dulbecco's minimal essential medium (DMEM) supplemented with

10% FCS, 100 IU/mL penicillin, 100 $\mu\text{g}/\text{mL}$ streptomycin, and 2 mM glutamine at 37°C in 5% CO_2 atmosphere. The cells were subcultured by dispersal with 0.025% trypsin in 0.02% EDTA and replated at 1:6 dilutions three times a week.

Mice. Male C57BL/6 mice (the facility of Immunology Institute, Lithuania) at 8–10 weeks of age and 22–25 g body weight were used throughout the study. The animals received care in accordance with the guidelines established by the Lithuanian Animal Care Committee, which approved the study.

Cell exposure to photodynamic treatment. Cell exposure to a photosensitizer and light was carried out in 9.2-cm² Petri dishes according to the experimental scheme shown in Fig. 1, part A. The cells were seeded out as a suspension in DMEM supplemented with 10% FCS at a density of 0.7×10^5 cells/mL in 1.85 mL per dish. After 30 h, mTHPC was added to the cell monolayer in log phase to a final concentration of 100, 200, 400, or 600 ng/mL. When handling the samples containing mTHPC, precautions were taken to avoid irradiating the samples with room light by reducing the sources of illumination to a minimum and by protecting the samples from light with aluminum sheets. After incubation for 18 h at 37°C in the dark, an extracellular photosensitizer was removed by rinsing the cell monolayer 2 times with room temperature DPBS, and DMEM containing 10% FCS was added. The cells were exposed to light from an LED array UNIMELA-1 ($\lambda = 660 \pm 20$ nm, VU Laser Research Center, Lithuania) for 0.5 and 1.0 min, the fluence rate at the level of the cells being 1 mW/cm², as measured using an IMO (Russia). After light exposure, the cells were incubated for 24 h until cell viability was estimated.

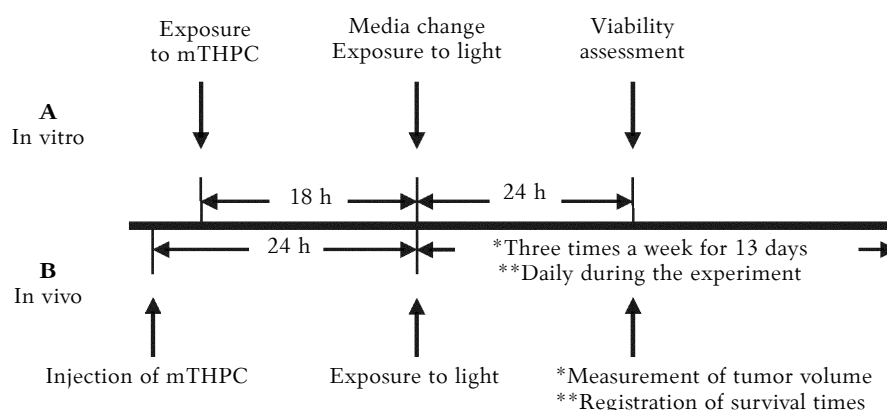


Fig. 1. Scheme for treatment of cells (A) and mice (B)

A, 100–600 ng/mL of mTHPC was added to LLC1 cells and incubated in the dark for 18 h. Then the incubation medium was replaced with the fresh one, and the cells were exposed to light at $\lambda = 660 \pm 20$ nm, 1 mW/cm², and the cells were incubated for 24 h postexposure; B, mTHPC, 0.25 mg/kg, was injected intravenously to mice bearing subcutaneous Lewis lung carcinoma. After 24 h, the mice were exposed to laser illumination at 650 ± 2 nm, 120 J/cm². During following 13 days, tumor volume was measured every two or three days. The survival of mice was observed every day until demise.

Cell viability assessment. For the assessment of cell viability, tetrazolium dye 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was used. Briefly, the cells were incubated with MTT for 1 h at 37°C. Then, the MTT solution was discarded, and the blue dye was extracted with 1 mL of 2-propanol. The optical density (OD) was then recorded at 570 nm using an ASYS UVM 340 microplate reader. The mean OD₅₇₀ of the control cells exposed to test compound-free culture medium was set to represent 100% of viability, and the results were expressed as a percentage of these controls (16).

Tumor inoculation and observation. Mice were injected subcutaneously with 0.2 mL of 5-time diluted suspension of Lewis lung tumor mass in the right groin. Tumor volume (TV) was determined by measuring the tumor diameter with vernier calipers and calculating according to the formulae:

$$TV=L \times W \times H \times \pi/6,$$

where L is length, W is width, and H is height of the tumor. After tumor exposure to treatment, tumor growth was monitored three times per week for up to 13 days, and survival was recorded daily throughout the experiment. The antitumor activity was evaluated by the index of tumor growth inhibition (TGI) in treated (T) vs. control (C) mice, which was calculated according to the formulae $TGI=100-(TV_T/TV_C \times 100)$.

Tumor exposure to treatment. For evaluation of antitumor activity, mice were treated according to the experimental schedule shown in Fig. 1, part B. mTHPC was administered 10 days after transplantation when tumor volume was $94 \pm 31 \text{ mm}^3$. Mice were coded and randomized into two groups (n=6 in each group): group 1, control mice and group 2, PDT-treated mice. In the group 1, mice were not treated. In the group 2, PDT was performed as follows: 0.2 mL of mTHPC was injected intravenously at a dose of 0.25 mg/kg, and after 24 h, tumors were exposed to light from the diode laser (Institute of Oncology, Vilnius University, Lithuania) at $650 \pm 2 \text{ nm}$ wavelength and fluence rate of 135 mW/cm^2 for 15 min, reaching a dose of 120 J/cm^2 .

Data analysis. Results were expressed as the mean \pm standard error. The data of experiments in vitro were analyzed using two-way ANOVA. Significance of the volume differences between two groups of tumors was assessed by *t* test. The Kaplan-Meier method was used for the survival analysis. The level of significance of the differences between the survival curves was assessed by the Gehan-Breslow test. Statistical significance was set at $P < 0.05$. SigmaPlot 10 software was used for the statistical analysis.

Results

The effect of PDT in vitro was characterized by the dose-response relationship of cytotoxicity

measured by reduction of cell viability at 24 h post-exposure. The cells were preincubated in the medium containing 100–600 ng/mL of mTHPC and exposed to light for 0.5 or 1 min at a fluence rate of 30 and 60 mJ/cm², respectively. The residual viability of the cells was assessed by the MTT assay, which is a reliable cytotoxicity test for photodynamically treated cells (17). Neither cytotoxicity of the photosensitizer at the selected concentration without light exposure nor that of light without mTHPC photosensitization was registered. CD50, a cytotoxic dose inducing a reduction in cell viability by 50%, was achieved at 60 mJ/cm² and approximately 400 ng/mL of mTHPC, or 30 mJ/cm² and 600 ng/mL of mTHPC (Fig. 2). The contribution of the treatment factors to the overall result was evaluated by the two-way analysis of variance. The analysis revealed that both concentration of mTHPC and duration of light exposure in the presence of mTHPC were significant determinants of the cytotoxic effect of PDT on LLC1 cells in vitro (Table 1).

In mice bearing Lewis lung tumor, we investigated the effects of PDT in vivo following the injection of 0.25 mg/kg of mTHPC and illumination at 120 J/cm^2 taking place after 24 h. The experimental conditions were established during a series of pilot

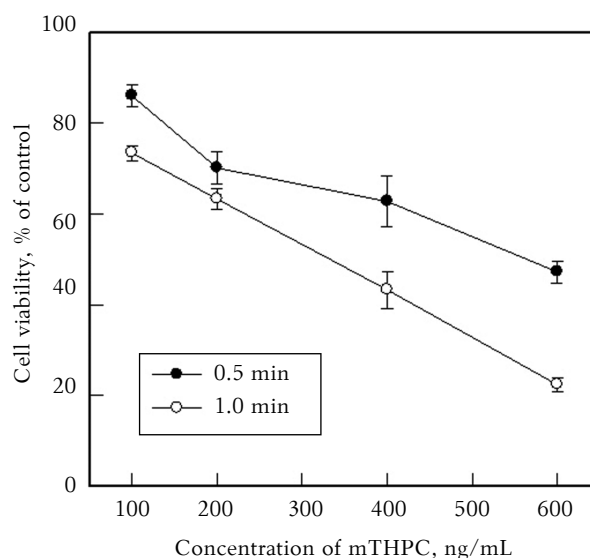


Fig. 2. Viability of LLC1 cells after mTHPC-mediated photodynamic treatment

The cells were incubated with mTHPC as shown in Fig. 1 and exposed to light at $660 \pm 20 \text{ nm}$ and 1 mW/cm^2 . Cell viability was evaluated by the MTT assay following incubation for 24 h in the dark. Error bars represent SE.

Table 1. Significance of the cytotoxic effect of treatment factors

Treatment factor	F ratio	P
Light exposure	50	<0.001
mTHPC	76	<0.001

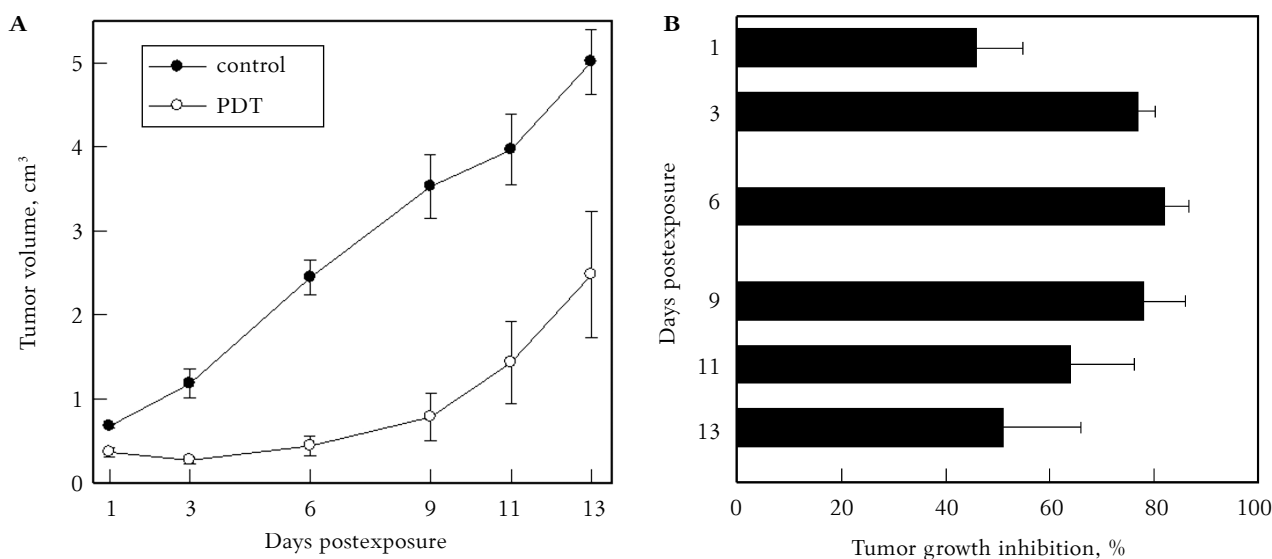


Fig. 3. Growth of subcutaneously transplanted Lewis lung carcinoma in photodynamically treated versus untreated mice. PDT, 0.25 mg/kg of mTHPC was administered, and after 24 h, tumors were exposed to light at 650 ± 2 nm and 120 J/cm². A, tumor growth in untreated mice (control) or photodynamically treated mice (PDT). B, tumor growth inhibition in photodynamically treated mice compared to untreated ones. Error bars represent SE.

Table 2. Significance of the difference between tumor volumes of control vs treated mice

Days posttreatment	<i>t</i>	<i>P</i>
1	4.9	<0.001
3	5.2	<0.001
6	8.4	<0.001
9	5.8	<0.001
11	3.9	0.004
13	2.8	0.02

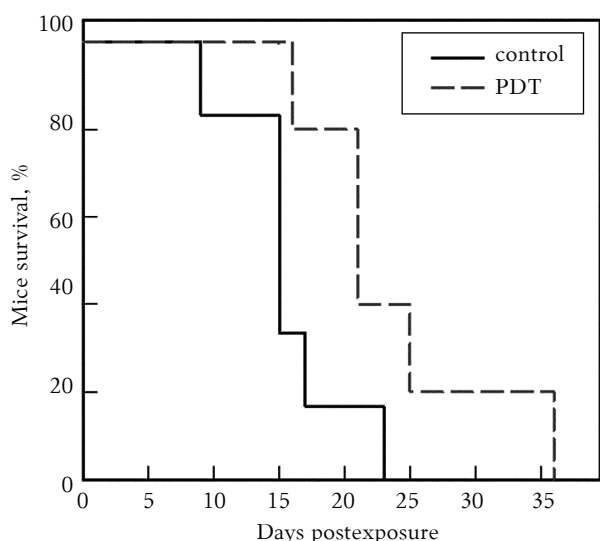


Fig. 4. Survival curves of photodynamically treated (PDT) versus untreated (control) mice. PDT, 0.25 mg/kg of mTHPC was administered, and after 24 h, tumors were exposed to light at 650 ± 2 nm and 120 J/cm².

experiments (data not shown). In addition, the pilot experiments revealed the absence of antitumor activity of light without mTHPC or mTHPC without light. Tumor volumes of the treated mice were smaller than those in the control group, as shown in Fig. 3A, and the difference was significant (Table 2). The most pronounced inhibition of tumor growth, exceeding 80%, was registered on days 3 to 9 (Fig. 3B).

The survival of the treated mice corresponded with tumor growth inhibition: the treated mice survived for a longer time than those in the control group (Fig. 4), and the difference was significant ($P=0.025$).

Discussion

Mouse tumor models have been extensively employed in preclinical studies investigating various aspects of host-tumor interaction following anticancer treatment. One of them is Lewis lung carcinoma, which has been widely used as a model for studying the mechanisms of cancer chemotherapeutic agents. In preclinical PDT studies, Lewis lung carcinoma has been used for investigations *in vitro* and *in vivo* involving various photosensitizers, such as porphyrin (12, 13), ALA (14), and phthalocyanines (15). Singlet oxygen is the main reactive oxygen species (ROS), produced by PDT. It induces spatially resolved responses in a cell (18). Thus, cell response depends on the site of ROS production, *i.e.* subcellular localization of the photosensitizer. Since the pattern of photosensitizer accumulation by the cells is unique to a photosensitizer, in our study on mTHPC-mediated PDT, we could not rely on the

published LLC1 cell response to PDT, mediated by other photosensitizers.

The results of this study support our suggestion that Lewis lung carcinoma model is relevant for investigation of cellular response to mTHPC-mediated PDT, since both the cells *in vitro* and tumors *in vivo* were damaged by the treatment. LLC1 cells lost their viability *in vitro* following mTHPC-mediated photodynamic treatment at a significantly lower dose of the photosensitizer and significantly lower light exposure than in the case of ALA-mediated PDT *in vitro* (14). The main problem with the LLC1 cells is their weak attachment to the growth substrate and the need to be careful in dealing with the cultivation dishes.

The effect of mTHPC-mediated PDT on Lewis lung carcinoma *in vivo* was prominent and exceeded that of Photofrin-mediated PDT *in vivo* (13). The pattern of tumor growth inhibition by mTHPC-mediated PDT revealed the dual character of Lewis lung carcinoma response. During the first 9 days post-exposure, the tumor growth was inhibited at a great extent. However, afterward the tumors regained

their growth potential, and the growth seemed to be even accelerated in comparison with the tumors in untreated mice. The recurrence of tumor growth could be the consequence of the overexpression of cell/tumor proliferation-promoting cytokines, secreted by insulted tumor cells. This suggestion is the scope of our future investigation.

Conclusions

The photodynamic treatment mediated by m-tetrakis-(3-hydroxyphenyl)-chlorin had a significant effect on Lewis lung carcinoma both *in vivo* and cells *in vitro*. The tumors, which growth was inhibited by photodynamic treatment, regained their growth potential in approximately a week.

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mTHPC sukeltas fotodinaminis poveikis Luiso (Lewis) plaučių karcinomai *in vitro* ir *in vivo*

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Raktažodžiai: fotodinaminis poveikis, LLC1 ląstelių linija, Luiso (Lewis) plaučių karcinoma, fotosensibilizatorius, mTHPC.

Santrauka. *Pagrindimas ir tikslas.* Kuriant būdus, kaip padidinti fotodinaminės terapijos veiksmingumą, vis labiau domimasi atsako į šią terapiją molekuliniais mechanizmais. Ieškodami ląstelių linijos, tinkamos ląstelių atsakui tirti *in vivo* ir *in vitro*, įvertinome m-tetrakis-(3-hidroksifenil)-chlorino fototoksinį poveikį Luiso plaučių karcinomos linijos (LLC1) ląstelių gyvybingumui *in vitro* bei pelių Luiso karcinomos augimui ir jų išgyvenamumui *in vivo*.

Tyrimo medžiaga ir metodai. Eksperimentai atlikti panaudojus LLC1 linijos ląsteles ir C57BL/6 linijos pelių patinus, kuriems buvo įskiepyta Luiso plaučių karcinoma. Tyrimams naudotas fotosensibilizatorius mTHPC, o švitinimui – šviestukų gardelė. Fotodinaminis poveikis įvertintas pagal ląstelių gyvybingumą, gydytų navikų augimą ir pelių gyvenimo trukmę.

Rezultatai. *In vitro*, citotoksinė dozė, sumažinusi LLC1 ląstelių gyvybingumą 50 proc., buvo 60 mJ/cm² šviesos energijos dozė ir apie 400 ng/ml fotosensibilizatoriaus arba 30 mJ/cm² šviesos energijos dozė ir 600 ng/ml fotosensibilizatoriaus. Citotoksinis poveikis reikšmingai priklausė ir nuo fotosensibilizatoriaus koncentracijos, ir nuo šviesos ekspozicijos trukmės. *In vivo*, suleidus 0,25 mg/kg fotosensibilizatoriaus pelėms, kurioms buvo įskiepytas navikas, ir po 24 val. eksponavus navikus šviesai, sukauptam 120 J/cm² dozė, švitintų pelių navikai augo reikšmingai lėčiau, pelės gyveno reikšmingai ilgiau. Navikai pradėjo greičiau augti po devynių dienų.

Išvados. m-tetrakis-(3-hidroksifenil)-chlorinu indukuota fotosensibilizacija sukėlė reikšmingą poveikį LLC1 ląstelėms *in vitro* bei navikų augimui *in vivo*.

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