VILNIUS UNIVERSITY

Daiva Dabkevičienė

EXPRESSION OF CYTOKINES VEGFA AND IL-1α STIMULATED BY CYTOTOXIC TREATMENT *IN VITRO* AND *IN VIVO*

Summary of doctoral thesis

Physical Sciences, Biochemistry (04 P)

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The thesis defense will take place in the open meeting held by Biochemistry Sciences doctoral thesis defence board at 15 pm on 24 September, 2010 in the auditorium No 216 at the Faculty of Natural Sciences of Vilnius University.
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Thesis is deposited at the libraries of Vilnius University and Institute of Biochemistry

VILNIAUS UNIVERSITETAS

Daiva Dabkevičienė

CITOTOKSINIO POVEIKIO SKATINAMA CITOKINŲ VEGFA IR IL-1α RAIŠKA *IN VITRO* IR *IN VIVO*

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Introduction

Actuality of the problem. Photodynamic therapy (PDT) is a novel and promising treatment for cancer and some non-malignant diseases. PDT is based on the use of photosensitizers that localize quite selectively in neoplastic/hyperplastic tissues and become cytotoxic when exposed to light due to production of reactive oxygen species [1]. Since PDT is an oxygen-dependent process, the existing tumour hypoxia could lead to the decrease in PDT efficacy. The depth of light penetration is another limiting factor for PDT. Photosensitizers of second generation with improved properties and improved laser instrumentation were developed and are under the clinical trial or already available for the clinical use. Both, efficiency and limitations of PDT have stimulated attempts to combine it with conventional modes of cancer treatment such as chemotherapy [2], radiotherapy [3] or surgery [4] at the very dawn of PDT.

Here, we have carried out the study on combination of PDT and chemotherapy or immunotherapy. The photosensitizer, *meso*-tetrakis(3-hydroxyphenyl)-chlorin (mTHPC, trade name Foscan®), used in this study is one of the most potent photosensitizers currently available for clinical use. The cytostatic drugs used in this study are Doxorubicin (Dox) of anthracyclines group and Taxotere (Tax) of taxanes family. The rationale behind the combination of PDT and Dox or Tax is the different modes of cytotoxic action. Light-activated photosensitizer generates reactive oxygen species that oxidize various biomolecules in close proximity to the site of localization of the photosensitizer [5]. The preferential sites of mTHPC localization are cellular membranes [6]. Meanwhile, Dox localizes to the cell nucleus, mainly [7], and Tax binds specifically to microtubules and alters their dynamics [8].

Our recent results of cDNA macroarray assay of gene expression in A-431 cells following the photodynamic treatment (PDT) in combination with cytostatic drugs revealed that among more than 2000 genes investigated the genes of the

vascular endothelial growth factor A (VEGF) and interleukin 1α (IL-1α) were the only genes overexpressed in every set of the experiments.

VEGF is a cytokine, overexpression of which has already been recorded after PDT [9]. VEGF is an inflammatory mediator and a strong promoter of angiogenesis [10]. Angiogenesis is considered to be essential for tumour growth and necessary for the spread of tumour cells. Overexpression of IL-1α has not been recorded after PDT in cancer cells, yet. IL-1α is a pleiotropic intracellular cytokine with properties of both a cytokine and a transcription factor, known to contribute to cell growth and repair [11]. The data on its role in tumour progression are contradictory.

Having in mind the deleterious effect of these cytokines on the tumour growth inhibition, we aimed to follow the details of the cytokines expression *in vitro* and *in vivo*.

The objective of dissertation work was to investigate the expression of the cytokines VEGF and IL-1α following the combined treatment of PDT and cytostatic drugs in cancer cells *in vitro*, and to evaluate the efficiency of the combined treatment of PDT and anti-VEGF or anti-IL-1α immunotherapy *in vivo*.

Towards these goals, **the following specific tasks have been formulated:**

- investigation of cell death following mTHPC-mediated PDT as a single treatment modality or in combination with cytotoxic drugs (doxorubicin or taxotere) and statistical evaluation of the efficacy of the combined treaments in A-431, MH-22A and LLC1 cell lines;
- investigation of changes in the expression of VEGF and IL-1α on mRNA and protein levels in A-431, MH-22A and LLC1 cell lines;
- assessment of the IL-1α effect on the VEGF expression in A-431 cells;
- investigation of changes in the expression of VEGF and IL-1α in murine LLC1 tumours after PDT *in vivo*;
- observation and evaluation of changes in tumour growth following PDT and / or anti-VEGF-A or anti- IL-1α immunotherapy.

Scientific novelty. In this study, the cytotoxic effects of mTHPC-mediated PDT or its combination with Dox or Tax were investigated in three cell lines. We applied the additive composite curve, the modern statistical method, for evaluation of the character of the combined effects on cell viability. The results of the study revealed that the character of the cytotoxic effect (additivity, synergism or antagonism) depends on the total dose in the combination. The finding was supported by the assays of caspase-3 activity after the combined treatments.

The present study was aimed at the detailed investigation on the expression of the cytokines VEGF and IL-1α *in vitro* following suboptimal PDT as a single treatment modality or in combination with Dox or Tax. In consistency with other findings [12], our results showed that suboptimal PDT-induced the VEGF expression in the carcinoma cells. To our knowledge, this is the first study to show that PDT increases expression of IL-1α in the carcinoma cells.

Our data indicate that PDT-induced VEGF and IL-1α promote the tumour growth and that PDT in combination with immunotherapy might be the effective therapeutic strategy.

Doctoral thesis contents. The doctoral thesis (in Lithuanian) contains the following parts: Introduction, Literature review, Material and Methods, Results and Discussion, Conclusions, Supplements, List of references (273 citations), List of publications (4 positions), participation at conferences (5 positions), Figures (39), Tables (24). Total 151 pages.

Materials and Methods

Photosensitizer. *meso*-tetrakis(3-hydroxyphenyl)-chlorin (**mTHPC**, trade name Foscan®) was kindly provided by R. Bonnett (London University, UK).

Anticancerous drugs. Solutions of **doxorubicin** hydrochloride 2 mg/ml (Ebewe Arzneimittel, Austria); **taxotere®** 40 mg/ml (Rhone-Poulenc Rorer, France).

Cell cultures and growth conditions. A-431, human squamous carcinoma cells (ATCC: CRL-1555), were grown in DMEM containing 5% FCS (Gibco BRL, Australia), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine and 10 mM HEPES at 37°C in 5% CO_2. Cells were replated at 10^5 cells/ml density twice a week. **MH-22A,** murine hepatoma cells (Inst. of Cytology, Sankt-Petersburg, Russia), were cultured in DMEM containing 10% FCS, 100 U/ml of penicillin, 100 μg/ml of streptomycin, 2 mM glutamine at 37°C in 5% CO_2. The cells were replated at 1:3 dilution twice a week. **LLC1,** murine Lewis lung carcinoma 1 cells (ATCC: CRL-1642), were cultured in sub-confluent monolayer (60-70%) in DMEM with 10% FCS, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine at 37°C in 5% CO_2 atmosphere. The cells were 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 viability assessment. MTT assay; staining with crystal violet (CV).

Tumour inoculation and observation. Mice were injected subcutaneously with 0.2 ml of five times diluted Lewis lung tumour mass suspension in a right groin. Tumour volume was determined by measuring the tumour diameters. After tumour exposure to treatment, tumour growth was monitored three times per week for up to 8 days. The anticancerous activity was evaluated by the index of tumour growth inhibition (TGI) in treated vs. control mice.

The scheme for treament *in vitro* and *in vivo*. For experiments *in vitro*, cells were exposed to the chemicals and light according to the scheme in Fig 1, a. For experiments *in vivo*, mice, bearing 90 ± 10 mm^3 tumours at the 7th day after transplantation, were coded and randomized into groups (n=5-6 in each group) following treatment according to the experimental schedule shown in Fig. 1, b.

Caspase-3 activity. The activity of caspase-3 was monitored in the control and treated cells at various post-treatment times. Caspase-3 Cellular Activity Assay Kit PLUS (AK-703) (Biomol GmbH, Germany) was used. Assay is based on the cleavage of caspase-specific fluorogenic peptide substrate (Ac-DEVD-AFC). Fluorescence of AFC, which is proportional to the caspase-3 activity in the lysed cell samples, was recorded on fluoroscan Ascent FL (Labsystems, Finland) (λ_{ex}=390 nm, λ_{em}=510 nm).

Quantitative real time polymerase chain reaction (qPCR). The cells were treated according to the scheme (Fig.1, a) and harvested at 1-24 h post-exposure. Control (untreated) cells served as baseline samples for comparison. Total RNA was isolated from pellets of treated or untreated cells using the isolation kit Purescript® (Gentra Systems, MN, USA) following the manufacturer‘s protocol. The quality and concentration of RNA were checked by agarose gel electrophoresis and spectrophotometry, respectively, and samples were stored at -70°C until further processing. cDNA was synthesized from approximately 250 ng of total RNA using RevertAid™ H Minus First

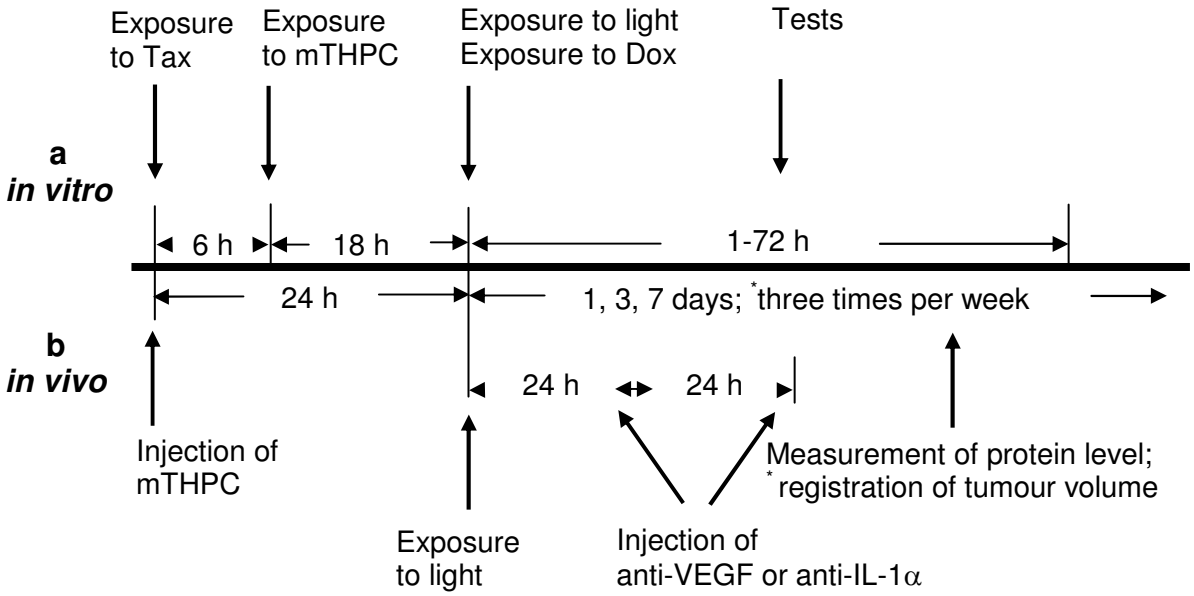

Fig 1. Shedule for treatment of cells (**a**) and mice (**b**).

(a) Tax was added to the medium 24 h prior to light exposure. mTHPC was added to cells and incubated in the dark for 18 h. Then the medium was replaced with the fresh one, and the cells were exposed to light at 660±20 nm, 1.6 mW/cm^2, and incubated in the dark, as follows: 1) for 24 h until cell viability assay; 2) for 1-72 h until caspase 3 activity assay; 3) for 1-24 h until mRNA assay, 4) for 6 or 24 h until protein level measurement. Dox was added to the medium immediately after light exposure.

(b) mTHPC was injected i.v. to mice bearing subcutaneous carcinoma LLC. After 24 h, tumours were exposed to light from the diode laser at 650±2 nm and 135 mW/cm^2 for 15 min, reaching a dose of 120 J/cm^2. sVEGFR1, anti-mouse VEGF or anti-mouse IL-1α antibodies were injected i.p. at 24 h and 48 h after light exposure. For the measurement of VEGF and IL-1α levels in tumours, PDT-treated and untreated mice were sacrificed at 1, 3 and 7 day post-exposure. The tumour volume was measured three times per week.

Strand cDNA Synthesis Kit (AB Fermentas, Lithuania) following manufacturer's protocol and samples were stored one day at -20°C. cDNA from each sample was divided into four aliquots (for analysis of two target genes and two 'housekeeping' genes) and amplified by qPCR together with a parallel blank reaction without cDNA as a negative control. The gene-specific primers (Metabion GmbH, Germany) were designed using the Primer3 program. qPCR reactions were performed on an Applied Biosystems Prism 7900HT system using one-step RNA amplification SYBR Green I kit and standard protocol (Roche Inc.). Mean C_t (threshold cycle, the crossing point, when amplification starts its exponential phase) for every gene was calculated from two to three qPCR reactions. To normalize for variations in input RNA amount and efficiency of reverse transcription, amplification of two 'house-keeping' genes (ACTB and GAPDH or *Actb* and *Hprt*) was performed as an endogenous control. Relative gene induction was calculated using $\Delta\Delta C_t$ method: induction ratio IR = $2^{-\Delta\Delta Ct}$, were $\Delta\Delta C_t$ is ($C_{t\ \text{Gene of interest}}$ -

$C_{t\ Housekeeping\ gene})_{treated}$ - ($C_{t\ Gene\ of\ interest}$ - $C_{t\ Housekeeping\ gene})_{control}$ (Applied Biosystems Inc.). Results are presented as mean ± SD of IR.

ELISA assay. For ELISA in vitro, cell culture supernatants and cell lysates were collected as follows: the cell culture media was centrifugated to pellet cell debris, the supernatants were collected and samples were stored at -80°C. The cells were washed, pelleted, counted and resuspended in the ice cold lysis buffer containing 1x peptidase inhibitor cocktail (Sigma) at 5×10^6 cells/ml. After 20 min, the lysate was centrifugated to pellet cell debris, the supernatants of cell lysates were collected and samples were stored at -80°C. For ELISA in vivo, mice were sacrificed, tumours were removed, homogenized and lysed in disruption buffer (PARIS™ Kit, Ambion) following manufacturer's protocol. The sandwich ELISA was carried out using anti-human or anti-mouse VEGF and IL-1α capture antibodies (R&D system), biotinylated antibodies as the detection antibodies (R&D system), recombinant human and mouse proteins (R&D system) as the standard, streptavidin-alkaline phosphatase (Amersham Biosciences, England) as the secondary reagent. ELISA procedure and dilutions of reagents were performed according recommendations of manufacturer. Duplicates were made of serial two-fold dilutions of the reference material in parallel with at least three consecutive dilutions of the samples. Colour development was performed by adding substrate buffer with 1 mg/ml of *p*-nitrophenylphosphate. The absorbance at 405 nm was detected with a microplate reader (Sunrise, Tecan). Results were corrected for the 10^6 cell *in vitro* and for the total sample protein concentration (estimated by BCA-Standard method).

Fluorescence microscopy. Cells were fixed in 4% para-formaldehyde and permeabilized with 0.1% Triton X-100 in PBS for 3 min. After blocking with 0.5% BSA in PBS, cells were treated with a 1:35 dilution of anti-human VEGF or IL-1α antibody (R&D systems) and stained with a 1:200 dilution of anti-mouse IgG-NL637 (R&D systems). For staining of nuclei, cells were incubated with 1 µg/ml DAPI (Molecular probes). Immunofluorescence observations were performed with Leica SP5 microscope equipped with 63x, HCX PLAPO lambda blue 1.4 oil immersion objective. Excitation/emission wavelenghts were 633/640-700 nm and 405/406-535 nm.

Flow cytometry. Detached and adherent cells were collected, fixed in 2% paraformaldehyde (Thermo Scientific) and permeabilized with 0.1% (v/v) saponine in PBS with 1% BSA. After incubation on ice and centrifugation, cells were resuspended in 100 µl of buffer with anti-mouse IL-1α-PE (0.25 µg antibody per 10^6 cells) (BD Biosciences) and incubated for 30 min at 4°C in the dark. After staining, the cells were washed and resuspended in 1 ml of Cell Wash (BD Biosciences) buffer and samples were analysed with BD FACS Calibur flow cytometer, „Cell quest" software (BD Biosciences, Germany). Excitation/emission wavelenghts were 488/575 nm. Generally, 10,000 cells were analyzed for each measurement.

Data analysis using the additive composite curve method. The effect character of PDT in combination with Dox or Tax was evaluated using the additive composite curve method.

Statistical data analysis. The data of two groups were analyzed using t-test. One-way ANOVA and the *post hoc* criterion were used for multiple comparisons. If data showed normal distribution, parametric statistics were used, if not, nonparametric statistics were used. SigmaStat 3.0.1 and SigmaPlot 7.101 softwares were used for the statistical analysis. The data are presented from at least two independent assays, each one at least in duplicate (unless overwise indicated).

Results and Discussion

Statistical analysis of the character of combined effects of PDT and antitumour drugs on cell viability

The first task of this study was to evaluate the effect of mTHPC-mediated PDT in combination with cytostatic drugs of different mode of action on cell viability.

When the combined effect produced by the action of two agents is equal to the sum of their separate effects, the character of the combination is additive. In contrast, the combination may either exaggerate the effect (synergism) or attenuate it (antagonism). From the clinical point of view, synergism is especialy important, for it allows the use of smaller amounts of the constituent drugs. The statistical methods based on the additivity of effects or the additivity of doses are the key techniques to evaluate the character of the cytotoxic effect.

The statistical methods based on the additivity of effects (ANOVA, regression analysis) use a set of unequally effective dose combinations. They are adaptable to diverse dose-response relations. However, limitation of the methods is that statistical conclusion about the effect of the combination regards to the overall process, though the sets of unequally effective doses are used.

A good example of the evaluation of the character of PDT combination with the cytostatic drugs Dox or Tax is the treatment of LLC1 cells. This cell line has been introduced in our laboratory during this study as a line appropriate for experiments both *in vitro* and *in vivo*. Following the selection of optimal cytotoxic doses of single cytostatic drugs or single PDT (Fig. 2), the factorial design was made for the experiments studying the effect of the combined treatment. Different concentrations of Dox or Tax and durations of light exposure were applied. The concentration of mTHPC was fixed at 0.2 ng/ml. The significance of the contribution of each treatment factor to the combined effect on cell viability was evaluated by two-way ANOVA. In case of Dox+PDT, the analysis revealed that concentration of Dox and duration of light exposure in the presence of mTHPC

were significant determinants (p=0.04 and p=0.01, respectively) of the cytotoxic effect, whereas the effect determined by the interaction of both factors in combination was not significant (p=0.26). In case of Tax+PDT, the cytotoxic effect of the combination was significantly determined by the concentration of Tax and duration of light exposure in the presence of mTHPC (p=0.024 and p=0.007, respectively), and the impact of interaction of Tax and PDT to the final result was not significant (p=0.875). The absence of the interaction determinants corresponding to the combination of PDT with Dox or Tax suggests that the overall loss of cell viability induced by PDT+Dox or Tax+PDT results from the summing up the cytotoxicity of Dox or Tax and that of PDT. Thus, the results show the additivity of either combinations.

The statistical evaluation of data showed that the character of the combined effect of Dox and PDT on viability of A-431 and MH-22A cells was additive when Dox was added just after light exposure. When Tax was added before PDT, the combination also had an additive character.

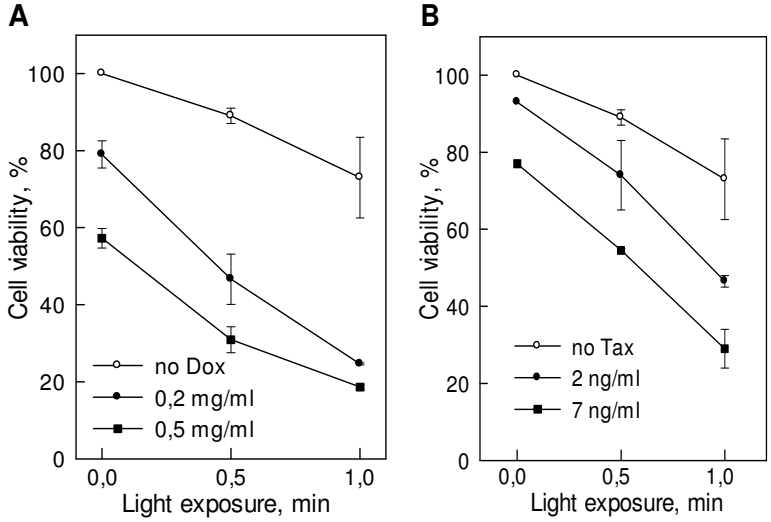

Fig. 2. Viability of LLC cells treated with PDT and cytostatic drugs**.**

A, the combined PDT+Dox treatment. Cells were incubated with 0.2 μg/ml mTHPC for 18 h following light-exposure (660 ± 20 nm, 1.6 mW/cm^2) at 48 mJ/cm^2 and 96 mJ/cm^2; Dox was added just after irradiation.

B, the combined Tax+PDT treatment. Tax was added to the incubation media at 24 h before irradiation (6 h in DMEM with serum and 18 h together with 0.2 μg/ml mTHPC in serum-free DMEM); the cells were exposed to light (660 ± 20 nm, 1.6 mW/cm^2) at 48 mJ/cm^2 and 96 mJ/cm^2. *Bars*, ± SD.

However, the character of the combined effect depends not only on the combination schedule, but also on the total dose in the combination. The modern statistical method, the additive composite curve, examines drug combination over the range of the effects and provides more detailed information that is especially useful in screening for the most effective doses of compounds in combination [13]. In this method the individual dose-response data (curves) are used to construct the theoretic additive curve for a fixed-ratio combination in which the total dose over the range of effects comprises constant proportions of the drugs. An experiment with this proportion produces an actual total dose-effect relation that may then be statistically compared with the composite additive curve. In order to evaluate the data using this method, the special fixed-ratio experimental design has to be applied. If the potency of one drug is much more effective than the other one, this method might be inapplicable.

A-431 cell line was selected as the optimal one for this method. The fixed-ratio design was applied for the experiments on cell viability. Application of additive composite curve for evaluation of experimental data revealed the additive character of PDT and Dox effects in combination (Fig. 3, a). In case of PDT combined with Tax, the overall deviation of the experimental composite curve from additivity was not significant, however the results were more complex. The effect of combination depended on the treatment doses: the effect of low doses (up to CD20) was less than additive implying the presence of antagonistic character at some extent, while the effect of high doses (from CD80) was greater than additive implying the contribution of synergism at some extent (Fig. 3, b).

The effect of PDT and cytostatic drugs on caspase-3 activity

Apoptotic cell death is the significant indicator of cellular response to cytotoxic treatment. The cell viability relies on the activity of the apoptotic enzymes. Consequently, the previous finding suggested the new question: do the effects of both combinations (Tax+PDT and PDT+Dox) on cell viability and caspase-3 activity have the same character?

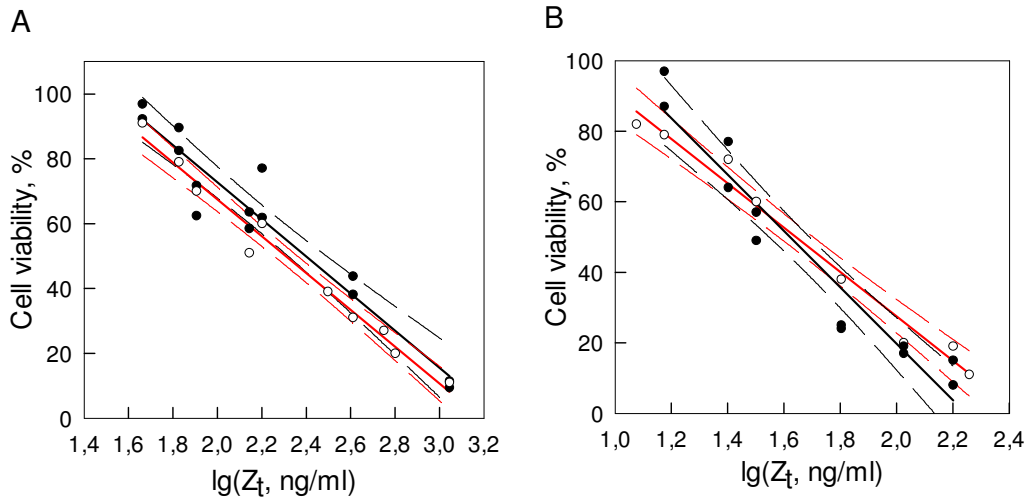

Fig. 3. The theoretic additive curve (black) and the experimental curve (red) for a fixed-ratio combination of PDT and cytostatic drug.

A, PDT in combination with Dox. **B,** PDT in combination with Tax. The cells were exposed to light (660 ± 20 nm, 1.6 mW/cm^2) for 60 s. After light exposure, cells were incubated for 24 h until cell viability was estimated by CV assay.
lg(Z_t), logarithm of the total dose.

The apoptotic cell death following the combined PDT+Dox and Tax+PDT treatment was followed by registration of the cleavage rate of peptide DEVD, that was a specific substrate of caspase-3. The experimental data were statisticaly evaluated using nonpara-metric one-way ANOVA.

PDT, as a single treatment modality and in combination with Dox or Tax, induced a significant caspase-3 activation in A-431 (Fig. 4) and MH (not shown) cells.

The effects of PDT and Dox in combination had the additive character on caspase-3 activity, however the effect of Dox in both cell lines was delayed and reached the peak just at 54 h post-exposure, so at the first hours post-exposure the cytotoxic effect of PDT+Dox was determined only by PDT.

Having in mind the results of analysis using additive composite curve, the investigation of Tax+PDT-induced changes in caspase-3 activity was of special interest. In case of Tax+PDT, caspase-3 activity was increased even at 1 h post-exposure, in comparison with negligible effects of PDT and Tax as single

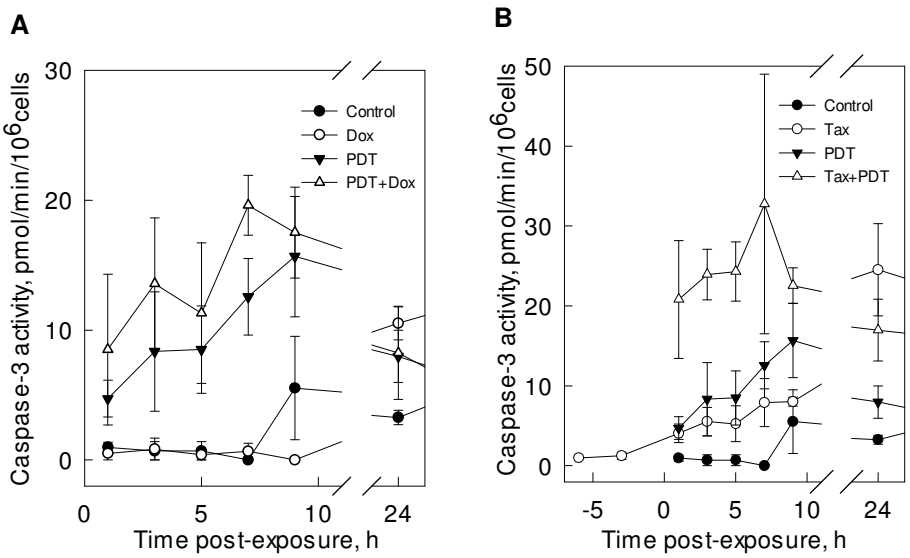

Fig. 4. Time-dependent caspase-3 activity in A-431 cells.

A, PDT in combination with Dox. Cells were incubated in serum-free DMEM with 0.1 μg/ml mTHPC for 18 h. The medium was replaced with DMEM containing serum, and cells were exposed to light (660 ± 20 nm, 1.6 mW/cm^2) at 60 mJ/cm^2. Dox was added to DMEM with serum just after irradiation to a final concentration of 50 μg/ml.

B, PDT in combination with Tax. Tax was added to the incubation media to a final concentration of 2 ng/ml 24 h before irradiation (6 h in DMEM with serum and 18 h together with 0.1 μg/ml mTHPC in serum-free DMEM); the medium was replaced with DMEM containing serum, and cells were exposed to light (660 ± 20 nm, 1.6 mW/cm^2) at 60 mJ/cm^2. *Bars,* ±SE, n=3

treatment modalities, and the sum of single factors-induced caspase-3 activity values was less than the value of caspase-3 activity after Tax+PDT, suggesting the synergetic character of combination. Data analysis using additive composite curve suggested that Tax+PDT dose, close to that used in caspase-3 activity assay, had the synergetic character as well.

Summing up, the results of additive composite curve analysis were supported by the results of caspase-3 activity assay, and it all goes to show that the character of the combined effect of PDT and Tax depends on the treatment dose and might deviate from additivity.

The effect of PDT and cytostatic drugs on expression of cytokines VEGF and IL-1 α in vitro

Though the response of the cancer cells to PDT in combination with cytotoxic drugs was encouraging, cDNA macro array technique revealed significant overexpression of vascular endothelial growth factor A (*VEGF*) mRNA and especially interleukin 1α (*IL1A*) mRNA following PDT and combined treatments [14]. Some cytokines play an important part in cancer progression [15,16] and could resist the inhibition of tumour growth. In current investigation, the detailed analysis of cytotoxic effect-induced *VEGF* and *IL1A* expression was carried out in three cell lines: the time course was followed in A-431 cells and the dose effect analysis was carried out in A-431, MH and LLC cells.

Neither Tax, nor Dox did alter the amount of mRNA of *IL1A* and *VEGF*. The significant increase of *IL1A* and *VEGF* expression was detected only when cells were treated with PDT, single or in combination with Tax or Dox. Therefore, we suggest that PDT has been the leading inducer of the cytokine expression. The character of the cytokine expression after the treatment of both A-431 and LLC cells was similar. Here, we represent the results of the cytokine expression after PDT in A-431 cells.

The response of MH cells to the treatment was a special case: i) mRNA of VEGF but not IL-1α was present in MH, and ii) neither PDT nor cytostatics, single or in combination, had any significant effect on VEGF expression.

VEGF expression in A-431 cells. Investigating the time course of the PDT-induced overexpression of *VEGF,* we registered the highest increase of mRNA amount, up to 10-fold, at 8 h post-irradiation (Fig. 5, a). For the studies of the impact of treatment intensity on the expression of *VEGF*, we chose 4 h post-irradiation, as an analysis time point for gene expression profiling, since at that time induction of the target gene was already pronounced. Cytotoxic doses of PDT

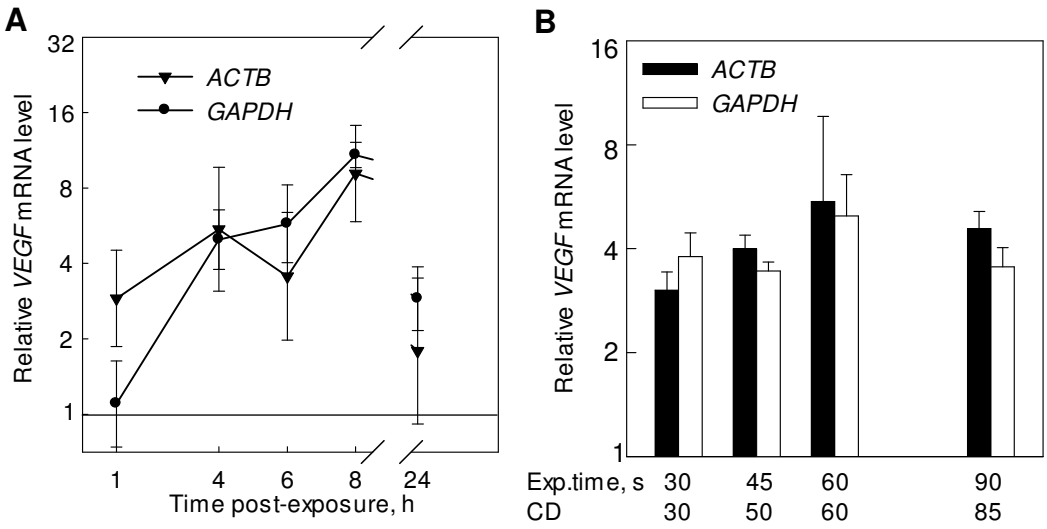

Fig. 5. Changes in mRNA expression of *VEGF* in PDT-treated A-431 cells.

The amount of cytokines mRNA was normalized to the amount of *ACTB* and *GAPDH*. **A, time-dependent** changes in mRNA expression of *VEGF*. The cells were incubated with 0.1 μg/ml mTHPC and exposed to light (660 ± 20 nm, 1.6 mW/cm^2) at 96 mJ/cm^2, harvested at various times post-irradiation, as indicated on the axis, and qPCR was carried out. PDT-treated cells received CD40 as determined at 24 h post-exposure.

B, dose-dependent changes in mRNA expression of *VEGF*. The cells were incubated with 0.1 μg/ml mTHPC and exposed to light (660 ± 20 nm, 1.6 mW/cm^2) at various doses, as indicated on the axis, harvested at 4 h post-irradiation and qPCR was carried out. Cytotoxic dose (CD) of PDT (indicated below x-axis) was determined at 24 h post-exposure. Data represent the mean ± SD.

varied between 30 (48 mJ/cm^2) and 85 (144 mJ/cm^2) and induced a significant overexpression of *VEGF* (Fig. 5, b). Statistical analysis using one-way ANOVA has shown that the induction of *VEGF* expression was not dose-dependent ($p=0.183$, for *ACTB*, and $p=0.107$, for *GAPDH*).

To confirm the PDT-induced overproduction of VEGF, protein analysis in A-431 (Fig. 6) and LLC1 (not shown) in cells was performed. ELISA assay revealed the significant increase of VEGF in the A-431-cell cultivation medium at 24 h after PDT ($p<0.01$) (Fig. 6, a). On the other hand, the increase of VEGF concentration in culture medium of cells that were treated at CD70, was significantly less than in the case of CD40 ($p=0.02$). VEGF secretion depends on the classical ER/Golgi secretory pathway. It might be possible that, due to accummulation of the photosensitizer in ER and Golgi, higher PDT doses caused

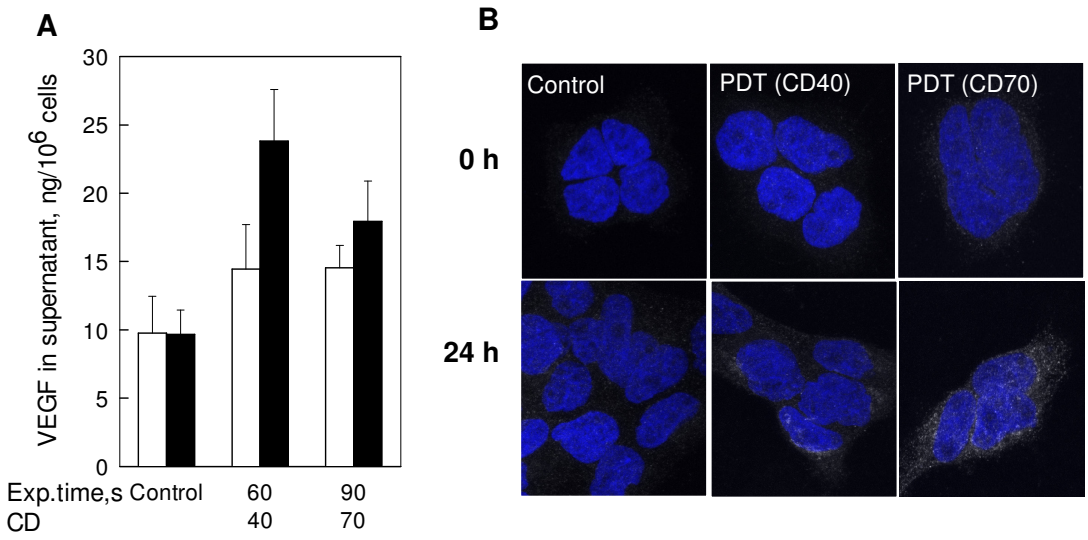

Fig. 6. Dose-dependent VEGF expression in A-431 cells after PDT.

The cells were incubated with 0.1 μg/ml mTHPC and, according to Fig. 1, exposed to light (660 ± 20 nm, 1.6 mW/cm^2) at 96 mJ/cm^2 and 144 mJ/cm^2. Cytotoxic dose (CD) of PDT was determined at 24 h post-exposure.

A, the changes of VEGF expression in cell culture supernatants were studed at 6 h (white bars) and 24 h (black bars) post-irradiation using ELISA; data represent the mean ± SD.

B, The changes of VEGF expression in cells was studied at 0 and 24 h post-exposure (indicated above the picture) using Leica SP5 microscope.

Blue, nuclei stained with DAPI ($\lambda_{ex}/\lambda_{em}$=405/406-535 nm), white, anti-mouse IgG-NL637 secondary antibody ($\lambda_{ex}/\lambda_{em}$=633/640-700 nm), conjugated with VEGF molecules via anti-human VEGF monoclonal antibody; the merged images are shown.

more damage to the elements of the secretion pathway of VEGF.

The increased amount of VEGF in the PDT-treated A-431 cells was shown using fluorescence microscopy (Fig. 6, b) and in in the PDT-treated LLC1 cells - using ELISA method (not shown).

Summing up, results of the studies of gene expression on a protein level have supported the results of mRNA assays: VEGF expression was significantly induced by PDT in A-431 and LLC1 cells. These results imply that PDT could be potentiated by anti-VEGF therapy, since VEGF-mediated angiogenesis promotes tumour growth and renewal [17].

IL-1α expression *in vitro*. Investigation of the time-dependent and dose-dependent mRNA accumulation in A-431 cells (Fig.7) and LLC1 cells (not shown) revealed significant overexpression of *IL1A* after PDT, and it was more

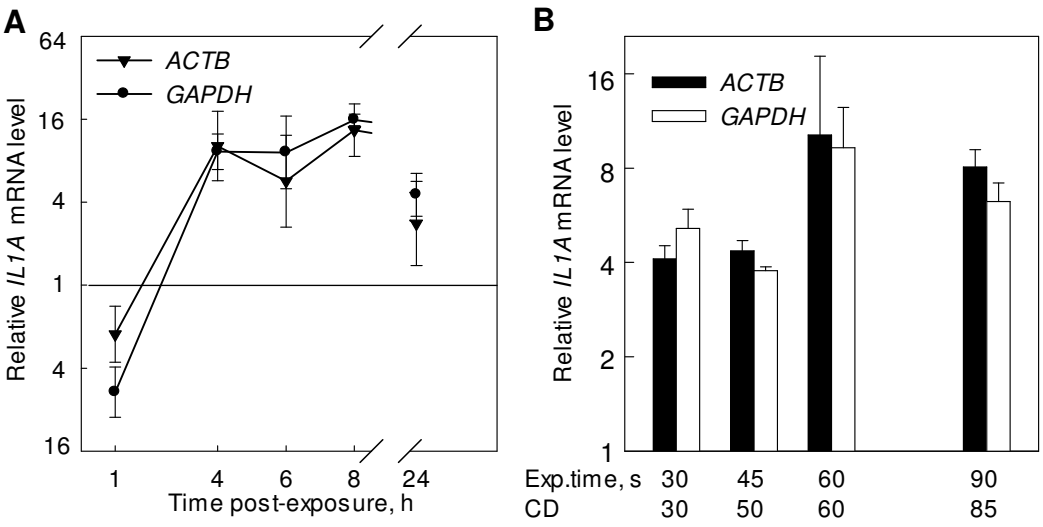

Fig. 7. Changes in mRNA expression of *IL1A* in PDT-treated A-431 cells. The amount of cytokines mRNA was normalized to the amount of *ACTB* and *GAPDH*.

A, time-dependent changes in mRNA expression of *IL1A*: the cells were incubated with 0.1 μg/ml mTHPC and exposed to light (660 ± 20 nm, 1.6 mW/cm^2) at 96 mJ/cm^2, harvested at various times post-irradiation, as indicated on the axis, and qPCR was carried out; viability of the PDT-treated cells was 60% as determined at 24 h post-exposure.

B, dose-dependent changes in mRNA expression of *IL1A*: the cells were incubated with 0.1 μg/ml mTHPC and exposed to light (660 ± 20 nm; 1.6 mW/cm^2) at various doses, as indicated on the axis, harvested at 4 h post-irradiation and qPCR was carried out; cytotoxic dose (CD) of PDT (indicated below x-axis) determined at 24 h post-exposure. Data represent the mean ± SD.

substantial than the *VEGF* expression. The highest amount of *IL1A* mRNA, up to 15-fold increase, was registered at 8 h post-irradiation. Moreover, the cytotoxic effect of PDT, inhibiting cell growth by more than 50%, resulted in the increase of *IL1A* expression from 4- to 8-fold in comparison with the less cytotoxic effect.

To confirm the PDT-stimulated production of IL-1α in cells, protein analysis using ELISA (A-431, Fig. 8) and flow cytometry (LLC1, not shown) was performed. In consistency with the results of *VEGF* overexpression, a study of *IL1A* expression on a protein level has supported the results of mRNA assays. PDT significantly induced the the increase of IL-1α in the A-431 culture medium (Fig. 8, a) and in the cell lysates (Fig.8, b). In contrast to VEGF, the increase of IL-1α concentration in the medium was dose-dependent, and at 6 h post-treatment

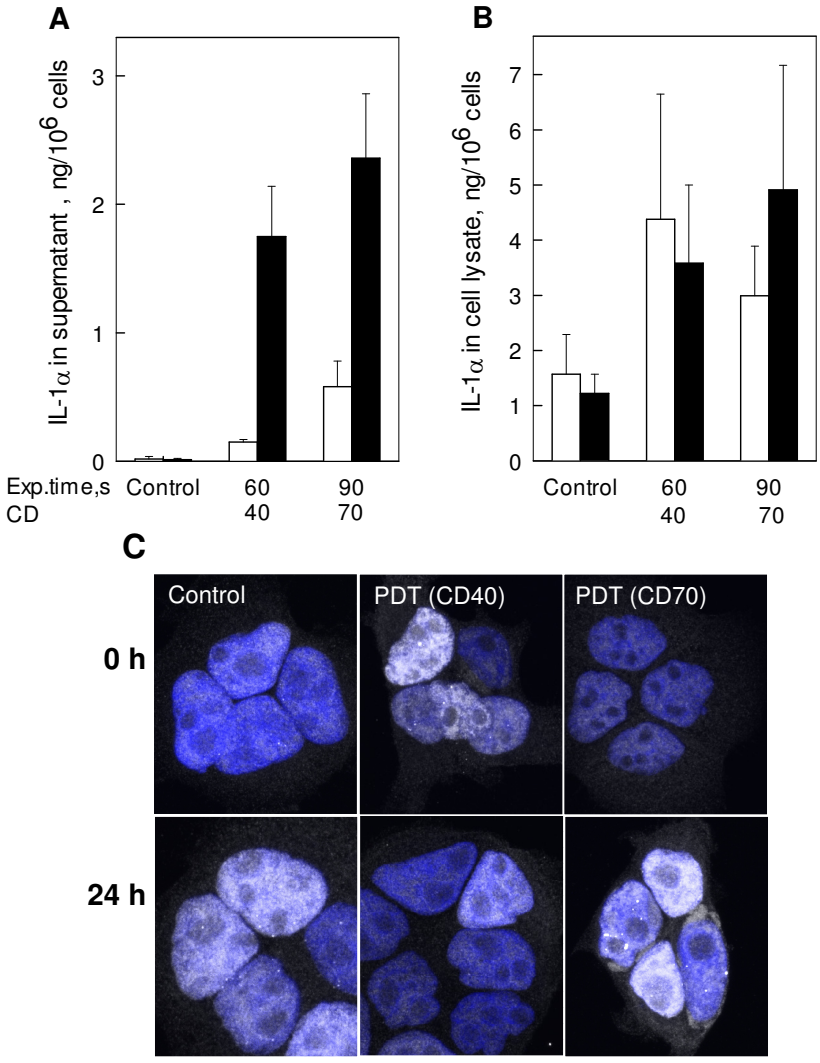

Fig. 8. Dose-dependent IL-1α expression in A-431 cells after PDT.

The cells were incubated with 0.1 μg/ml mTHPC and exposed to light (660 ± 20 nm, 1.6 mW/cm^2) at 96 mJ/cm^2 and 144 mJ/cm^2. Cytotoxic dose (CD) of PDT was determined at 24 h post-exposure. The changes of VEGF expression in cell culture supernatants **(A)** and in cell lysates **(B)** were studied at 6 h (white bars) and 24 h (black bars) post-irradiation using ELISA; data represent the mean ± SD. The changes of VEGF expression in cells **(C)** was studied at 0 and 24 h post-exposure (indicated above the picture) using Leica SP5 microscope.

Blue, nuclei stained with DAPI ($\lambda_{ex}/\lambda_{em}$=405/406-535 nm), white, anti-mouse IgG-NL637 secondary antibody ($\lambda_{ex}/\lambda_{em}$=633/640-700 nm), conjugated with IL-1α molecules via anti-human IL-1α monoclonal antibody; the merged images are shown.

the difference between CD40- and CD70-treated cells was statisticaly significant (p=0,001).

Secretory pathway of IL-1α differs from that of VEGF. IL-1α peptide lacks a hydrophobic leader sequence and is secreted via an alternative pathway [18].

Our results suggest that biosynthesis of IL-1α is constitutive, and the secretion of this cytokine is an inducible process in A-431 cells. Secretion of IL-1α is a rare occurrence, and from cells it might be released only if plasma membrane is damaged by acute inflammation [19]. It should be noted, that an oxidative stress-induced release of IL-1α might be associated with cancerogenesis [20].

The results of fluorescence microscopy have shown that IL-1α is localized not only in the cytosol but also in the nucleus of A-431 cell (Fig 8, c). The IL-1α peptide has nuclear localization sequence, and nuclear IL-1α is associated with potentiation of cell proliferation [21].

The impact of IL-1α to VEGF secretion *in vitro*. The fact that PDT had no effect on VEGF expression in MH cells that do not produce IL-1α suggest some link between IL-1α and VEGF.

The impact of IL-1α on the amount of VEGF protein was studied in A-431 cells. Incubation of untreated and PDT-treated cells with *IL-1A*-siRNA decreased the amount of IL-1α protein in cells approximately up to 80% and it resulted in the decreased secretion of VEGF (Fig. 9, a) ($p=0.01$ and $p=0.125$ and for untreated and PDT-treated cells, respectively). Incubation of untreated and PDT-treated cells with exogenous recombinant IL-1α significantly increased the amount of the secreted VEGF (Fig.9, b) ($p=0.006$ and $p<0.001$, respectively). Both silencing of *IL1A* and addition of IL-1α revealed the contribution of IL-1α to the control of VEGF expression.

Summing up, the results of IL-1α expression study show that IL-1α could contribute to the tumour regrowth by stimulation of angiogenesis.

The role of cytokines VEGF and IL-1α in tumour growth

The findings *in vitro* stimulated the *in vivo* studies of the impact of anti-VEGF and anti-IL-1α therapies on the tumour growth. First of all we examined

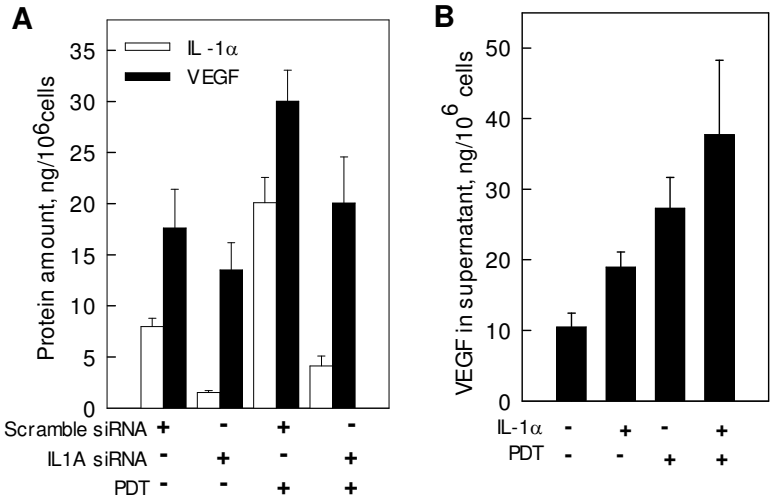

Fig. 9. Effects of *IL1A*-siRNA **(A)** and recombinant cytokine IL-1α **(B)** on VEGFA secretion in PDT-treated and PDT-untreated A-431 cells.

A, cells were incubated with *IL1A*-siRNA for 4-6 h, then treated with 0.1 μg/ml of mTHPC and exposed to light (660 ± 20 nm, 1.6 mW/cm^2) at 96 mJ/cm^2. Culture supernatants and cell lysates were collected at 24 h post-irradiation and analyzed by ELISA.

B, cells were incubated with 0.1 μg/ml of mTHPC for 18 h and exposed to light (660 ± 20 nm, 1.6 mW/cm^2) at 96 mJ/cm^2. 5 ng/ml of recombinant IL-1α was added to the medium just after irradiation. Culture supernatants were collected at 6 h post-irradiation and analyzed for VEGFA production by ELISA.
PDT-treated cells received CD40 as determined at 24 h post-exposure. Data represent the mean ± SD.

the production of VEGF and IL-1α in LLC tumours *in vivo* after exposure to mTHPC-mediated PDT. LLC tumours bearing mice were treated according to the scheme represented in Fig. 1, b. VEGF level in PDT-treated tumour extracts were significantly increased for 3 days, and on the 7th day the protein levels in treated and untreated tumours were similar, indicating that PDT induces transient augmentation of VEGF (Fig. 10, a).

IL-1α level in untreated tumour extracts remained relatively constant (Fig 10, b). By contrast, IL-1α level in the PDT-treated tumours was increased significantly for 3 days compared with that in the untreated tumours.

Summing up, results of studies on VEGF and IL-1α expression *in vivo* have supported the results of *in vitro* assays: there was a significant induction of both cytokines expression by PDT.

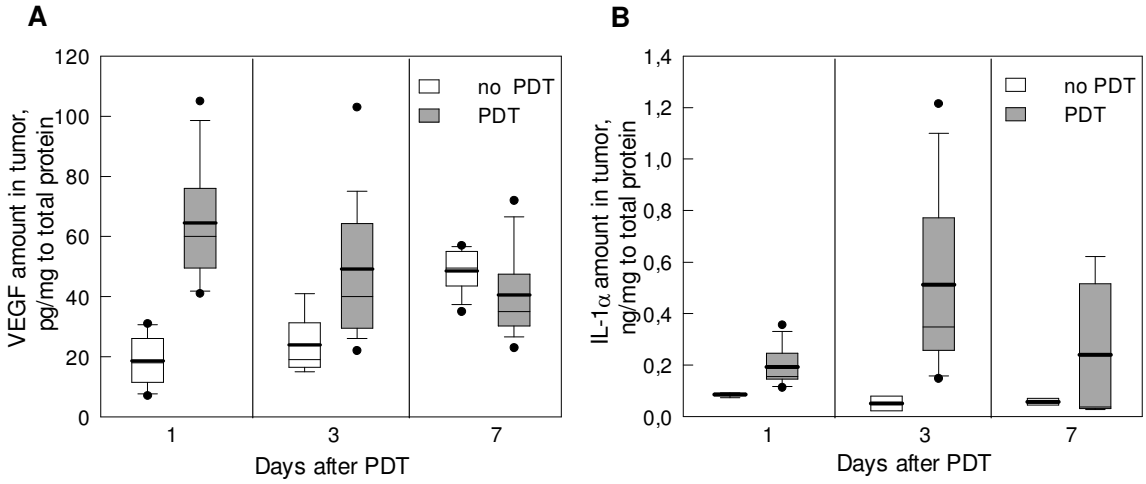

Fig 10. VEGF **(A)** and IL-1α **(B)** expression in control (white boxes) and treated (grey boxes) LLC1 tumours after PDT.

0.2 ml of mTHPC was injected i. v. at a dose of 0.25 mg/kg, and after 24 h, tumours were exposed to light at 650±2 nm, 135 mW/cm^2 for 15 min, reaching a dose of 120 J/cm^2. The protein amount was detected using the ELISA method.

The boundary of the box closest to zero indicates the 25th percentile, the thin and bold lines within the box mark the median and the mean, the boundary of the box farthest from zero indicates the 75th percentile, the error bars above and below the box indicate the 90th and 10th percentiles, the black symbols indicate the data points that lie outside the 10th and 90th percentiles.

To determine whether the increased secretion of VEGF or IL-1α in the PDT-treated tumours affects the anticancerous response, we applied the anti-VEGF or anti-IL-1α immunotherapy to LLC1 tumours-bearing mice.

Anti-murine VEGF antibody neutralizing VEGF-120 and -164 isoforms and a soluble VEGFR1 (sVEGFR1) were used for VEGF blockade. The antibody neutralize VEGF-120 and -164 isoforms and mainly suppresses the activity of VEGFR2, as the receptor binds to the isoforms of VEGF of the lower molecular mass (110-165 amino acid residues). VEGFR2 is the predominant mediator of VEGF-stimulated angiogenesis. sVEGFR1 binds to VEGF and PIGF with high affinity, and suppresses the activity of both VEGFR2 and VEGFR1. Application of these selective peptides enabled us to evaluate the impact of VEGFR1- and/or VEGFR2 binding proteins to tumour growth.

Anti-murine IL-1α antibody was used for IL-1α blockade.

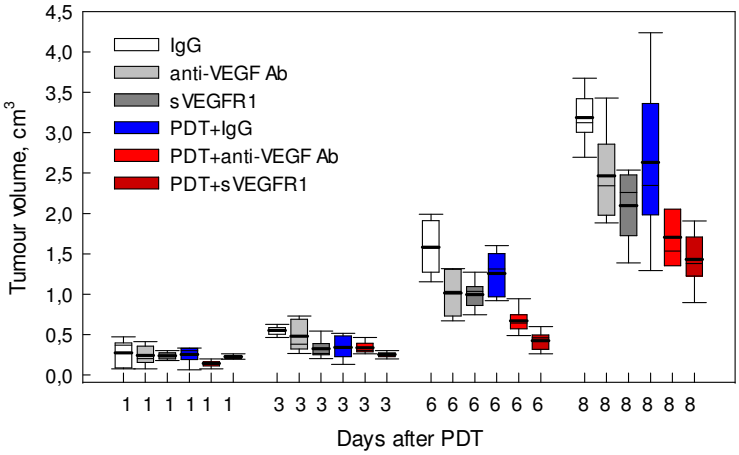

Fig. 11. Effect of VEGF blockade in mice bearing LLC1 tumours.

For PDT, 0.2 ml of mTHPC was injected i. v. at a dose of 0.25 mg/kg, and after 24 h, tumours were exposed to light at 650±2 nm, 135 mW/cm^2 for 15 min, reaching a dose of 120 J/cm^2. For immunotherapy, anti-mouse VEGF antibody (anti-VEGF Ab) or soluble VEGFR1 (sVEGFR1) was injected i.p. at a dose of 10 μg at 24 h and 48 h post-exposure.

The boundary of the box closest to zero indicates the 25th percentile, the thin and bold lines within the box mark the median and the mean, the boundary of the box farthest from zero indicates the 75th percentile, the error bars above and below the box indicate the 90th and 10th percentiles.

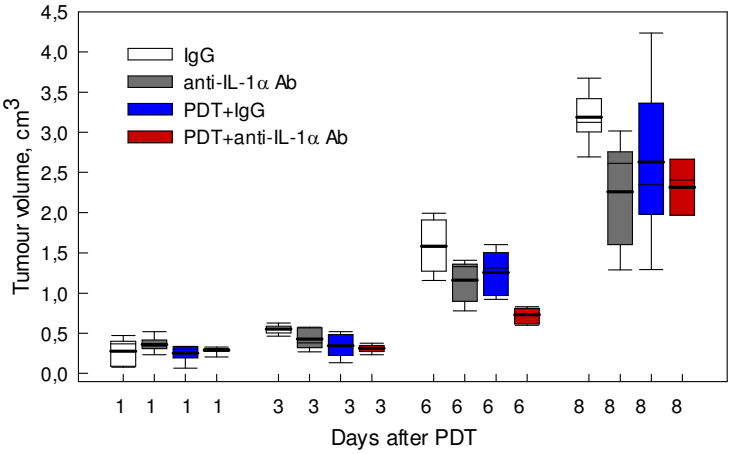

Fig. 12. Effect of IL-1α blockade in mice bearing LLC1 tumours.

For PDT, 0.2 ml of mTHPC was injected i. v. at a dose of 0.25 mg/kg, and after 24 h, tumours were exposed to light at 650±2 nm, 135 mW/cm^2 for 15 min, reaching a dose of 120 J/cm^2. For immunotherapy, anti-mouse IL-1α antibody (anti-IL-1α Ab) was injected i.p. at a dose of 10 μg at 24 h and 48 h post-exposure.

The boundary of the box closest to zero indicates the 25th percentile, the thin and bold lines within the box mark the median and the mean, the boundary of the box farthest from zero indicates the 75th percentile, the error bars above and below the box indicate the 90th and 10th percentiles.

The experiments revealed the significant inhibition of tumour growth by anti-VEGF (Fig. 11) or anti-IL-1α therapy (Fig. 12), as a single treatment. In the case of PDT, a suboptimal dose that inhibited the tumour growth by 30%, was used. Neutralization of PDT-induced VEGF and IL-1α increased the anticancerous effect of PDT, i.e., the combined treament in all cases produced greater tumour growth delay than either single treatment.

The effect of sVEGFR1 on tumour growth was approximately 10% greater than the effect of anti-VEGF antibody, indicating the leading impact of VEGFR2-binding VEGF isoforms on LLC tumour growth.

The suppresssion of tumour growth was more efficient after immunotherapy with sVEGFR1 than after anti-IL-1α therapy. The effect of anti-VEGF antibody on tumour growth was similar to that of anti-IL-1α antibody. However, the impact of anti-IL-1α therapy to tumour growth was the weakest in comparison with sVEGFR1- or anti-VEGF antibody-treatment. Expression of pro-angiogenic and pro-inflammatory cytokines is a therapeutic concern as it may lead to the enhanced angiogenesis and tumour recurrrence. Combination of PDT with immunotherapy seems promising in cancer treatment, however the application of this combination for cancer treatment is still on the experimental level.

Our data indicate that PDT-induced VEGF and IL-1α promotes the tumour growth and that PDT in combination with immunotherapy might be the effective therapeutic strategy.

Conclusions

1. The effect of the mTHPC-mediated photodynamic treatment (PDT) in combination with doxorubicin (Dox) on viability of human epidermoid carcinoma A-431 and murine lung carcinoma LLC1 had an additive character.

2. The effect of the mTHPC-mediated photodynamic treatment in combination with taxotere (Tax) on viability of A-431 and LLC1 cells had additive character; however the low doses (up to CD20) produced less than additve effect on A-431 cells, while the high doses (from CD80) produced greater than additve effect.

3. mTHPC-mediated PDT, single and in combination with Dox or Tax, *in vitro* stimulates the expression of vascular endothelial growth factor (VEGF) and interleukin 1α (IL-1α) in A-431 and LLC1 cells, but not in murine hepatoma MH-22A cells.

4. Cytokine IL-1α contributes to the control of VEGF expression in A-431 cells *in vitro*.

5. mTHPC-mediated PDT induces the expression of VEGF and IL-1α in LLC1 tumour.

6. The blockade of cytokines VEGF or IL-1α inhibits the growth of both untreated and mTHPC-PDT-treated LLC1 tumours.

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Santrauka

Sergančiųjų vėžio ligomis gydymui veiksminga taikyti vadinamąją kombinuotą terapiją. Gydymo rezultatai ypatingai pagerėja, kai derinami priešvėžiniai veiksniai, kurių veikimo mechanizmai yra skirtingi. Šiuo požiūriu priešvėžinio vaisto derinimas su fotodinamine terapija yra perspektyvus: derinant šiuos du metodus, galima tikėtis veiksmingesnio gydymo. Gydymui patraukliausias yra sinergistiniu poveikiu pasižymintis vaistų derinys, nes mažinant veiksmingai derinamų vaistų dozes, sumažinami nepageidaujami šalutiniai poveikiai organizmui.

Šio darbo tikslas buvo ištirti citokinų VEGF ir IL-1α raišką po fotodinaminio poveikio ir jo derinio su citostatikais vėžinėse ląstelėse *in vitro* bei įvertinti PDT derinimo su imunine terapija, nukreipta prieš šiuos citokinus, perspektyvumą *in vivo*.

Nustatyta, kad 1) mTHPC sukelto fotodinaminio poveikio ir doksorubicino derinys, kai doksorubicino tirpalas pilamas iškart po švitinimo, A-431 ląstelių gyvybingumą *in vitro* mažina adityviai; mTHPC sukelto fotodinaminio poveikio ir taksotero derinio, kai ląstelės su takosoteru inkubuojamos prieš švitinimą, poveikio pobūdis A-431 ląstelių gyvybingumui *in vitro* priklauso nuo poveikio dozės; **2)** mTHPC sukeltas fotodinaminis poveikis, vienas arba kartu su doksorubicinu ar taksoteru, skatina citokinų VEGF ir IL-1α raišką A-431 ir LLC1 ląstelėse *in vitro*; **3)** citokinas IL-1α skatina VEGF raišką A-431 ląstelėse *in vitro*; **4)** mTHPC sukeltas fotodinaminis poveikis skatina citokinų VEGF ir IL-1α raišką LLC1 navikuose; **5)** citokinų VEGF ir IL-1α raiškos slopinimas lėtina LLC1 navikų augimą.

Curriculum vitae

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