VILNIUS UNIVERSITY

Aušra Sasnauskienė

ON THE PROCESSES OF CELL DEATH INDUCED BY PHOTODYNAMIC TREATMENT *IN VITRO*: IMPACT OF THE PHOTOSENSITISER LOCALISATION

Summary of doctoral thesis

Physical Sciences, Biochemistry (04 P)

Vilnius, 2011

This study was carried out at the Department of Biochemistry and Biophysics of Vilnius University during 2006 – 2011 years

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The thesis defense will take place at the open meeting held by Biochemistry Sciences doctoral thesis defense board at 3 pm on 21 October, 2011 in the auditorium No 214, Faculty of Natural Sciences, Vilnius University.
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The summary of doctoral thesis has been sent on the ___ of September, 2011

Thesis is deposited at the library of Vilnius University

VILNIAUS UNIVERSITETAS

Aušra Sasnauskienė

FOTODINAMINIO POVEIKIO SUKELTI LĄSTELIŲ ŽŪTIES VYKSMAI *IN VITRO*: FOTOSENSIBILIZATORIAUS LOKALIZACIJOS REIKŠMĖ

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Disertacijos santrauka išsiuntinėta 2011 m. rugsėjo ___ d.

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Introduction

Actuality of the problem

Photodynamic treatment (PDT) is used to treat cancer and other diseases caused by cellular overgrowth. Photodynamic effect is achieved by irradiating light sensitive compound, a photosensitiser, with visible light. This results in the formation of singlet oxygen and other reactive oxygen species. It is generally accepted that the main damaging agent in photodynamic treatment is the singlet oxygen, which can diffuse, according to different studies, 20 or 100 nm from the site of its generation [1; 2]. Therefore, the initial damage of the photodynamic treatment is restricted to the site of the photosensitiser accumulation. Clinically used photosensitisers (e.g., Photofrin, 5-aminolevulinic acid-induced endogenous porphyrins, mTHPC) show affinity to multiple cellular organelles: endoplasmic reticulum, Golgi complex, mitochondria, lysosomes. It has long been accepted that the most effective inducers of apoptosis are the photosensitisers that localise to mitochondria, whereas those that bind to the plasma membrane or lysosomes, but not to mitochondria, kill cells less efficiently and by a non-apoptotic mechanism [3].

Mitochondria play an active role in apoptosis, since permeabilisation of outer mitochondrial membrane results in the release of soluble apoptogenic factors from the intermembrane space [4].

Recently it has become evident that lysosomes also play an important role in the progression of apoptosis. Lysosomal membrane permeabilization occurs in response to a large variety of cell death stimuli causing release of cathepsins from the lysosomal lumen into the cytosol where they participate in apoptosis signaling [5].

We have studied cellular response to photodynamic treatment (apoptosis, autophagy, cell cycle disturbance, expression of some genes) induced by photosensitisers, which localise selectively to mitochondria (Rh123, Safr) or

lysosomes ($AlPcS_4$), and compared it to the response evoked by the clinically efficient photosensitiser (mTHPC), which diffusely localises to multiple cellular organelles – endoplasmic reticulum, Golgi complex, mitochondria, lysosomes [6-8].

The objective of the dissertation work –

Evaluation of the cellular response to photodynamic treatment *in vitro* induced by photosensitisers localised to different cellular compartments.

Towards this goal, **the following specific tasks have been formulated:**

- To determine the effect of light and 2-(6-amino-3-imino-3H-xanthen-9-yl)benzoic acid methyl ester (Rhodamine 123, Rh123), localised to mitochondria, on mouse hepatoma MH-22A cells and compare it with photodynamic effect of meso-tetra(3-hydroxyphenyl)chlorin (mTHPC), localised to multiple cellular organelles;
- To evaluate cell viability determining processes (apoptosis, autophagy, cell cycle disturbance) in human epidermoid carcinoma A-431 cells after photodynamic treatment, induced by:
  - meso-tetra(3-hydroxyphenyl)chlorin (mTHPC),

localised to various cellular organelles;

  - 3,7-diamino-2,8-dimethyl-5-phenylphenazinium chloride (Safr),

accumulated in mitochondria;

  - aluminium (III) phthalocyanine tetrasulfonate ($AlPcS_4$),

localised to lysosomes.

- To evaluate the expression of interleukin-1 alpha (IL-1α), vascular endothelial growth factor A (VEGF-A) and hypoxia inducible factor 1, alpha subunit (HIF-1α), at RNA level, after photodynamic treatment on A-431 cells induced by Safr, $AlPcS_4$ and mTHPC.

Scientific novelty

This work is a complex research on the processes (apoptosis, autophagy, cell cycle disturbance), induced by photodynamic treatment, mediated by photosensitisers localised selectively to mitochondria and lysosomes or various cellular organelles.

For the first time, cellular response to photodynamic effect induced by the delocalised cation Safr, accumulated in mitochondria, was investigated .

New insights into cell response to the treatment induced by photosensitisers Rh123 or Safr, localised to the mitochondrial inner space, and light [9; 10] were gained. We determined, that the photodynamic treatment targeted to mitochondrial inner space evokes the specific cellular response, different from that induced by the photosensitiser localised to various cellular organelles or lysosomes: the treatment at medium dose (CD50) induces the damage to mitochondrial matrix, but does not induce cell death. The cell viability decrease following the photodynamic treatment, induced by Safr, can be ascribed to the cell cycle arrest, not to the induction of cell death. At high doses (CD80), the Safr-mediated photodynamic treatment induces apoptosis.

Although it has been demonstrated, that amount of autophagosomes increases after photodynamic treatment, induced by a photosensitiser, localised to lysosomes [11], there are no data concerning autophagic flux in the treated cells. In this work, we demonstrated, that in response to the photodynamic treatment mediated by $AlPcS_4$, localised to lysosomes, the amount of autophagosomes was increased, but the autophagic flux proceeded only after a weak cytotoxic dose (CD20); the higher doses (CD50 and CD80) resulted in the increased amount of autophagosomes, but the late steps of the autophagy were blocked.

For the first time, we demonstrated that after sub-lethal photodynamic treatment, inducing the damage to the matrix of mitochondria (Safr) or lysosomes ($AlPcS_4$), the amount of RNA, coding the cytokines VEGF-A, IL-1α and transcription factor HIF-1 α subunit, was increased in A-431 cells.

Doctoral thesis contents. The doctoral thesis (in Lithuanian) contains the following parts: Introduction, Literature review, Material and Methods, Results and Discussion, Conclusions, List of references (288 citations), List of publications (3 positions), participation at conferences (5 positions), Figures (15), Tables (5). Total 129 pages.

Materials and Methods

Materials

DMEM, FCS and streptomycin were obtained from Biochrom AG, Germany. Culture flasks and Petri dishes were from TPP. mTHPC (a generous gift of R. Bonnett, Queen Mary, University of London, UK) was dissolved in ethanol as 1 mg/ml stock solution. The stock solutions of Safr and Rh123 (Sigma) were prepared in the same way. The stock solution of $AlPcS_4$ (Porphyrin Products) was prepared as 5 mg/ml in PBS. All experiments were performed using dilutions of the stock solutions with cell incubation media. Other chemicals were from Sigma, unless indicated otherwise.

Cell culture

MH-22A murine hepatoma cells (Inst. of Cytology, Saint-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.

A-431 human epidermoid carcinoma cells (DSMZ, Germany) were cultured in monolayer in 75 or 150 cm^2 flasks in DMEM supplemented with 5% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, and 5 mM HEPES, at 37°C in a humidified atmosphere with 5% CO_2. Cells were subcultured by dispersal with 0.025% trypsin in 0.02% EDTA and replated at at 1:5 dilution twice a week.

Photochemical treatment of cells

mTHPC-PDT. MH-22A were washed twice with PBS buffer, and FCS-free DMEM, containing 0.15 μg/ml mTHPC for 18 h. After incubation, the cells were washed twice with PBS, and DMEM, containing 10% of FCS was added. Cells loaded with mTHPC were exposed to light for 45 sec. The light source was LED array at $\lambda = 660 \pm 5$ nm, the fluence rate at the level of the cells was 16 W/m^2.

A-431 cells were washed twice with PBS buffer, and FCS-free DMEM, containing 0.1 μg/ml mTHPC, was added for 18 hours. After incubation, the cells were washed twice with PBS, and DMEM, containing 5% of FCS was added. The cells were illuminated for 0.3-3 min with the LED array at $\lambda = 660 \pm 5$ nm (16 W/m^2).

Rh123-PTT. MH-22A cells were washed twice with PBS and FCS-free DMEM, containing 5 μg/ml Rh123, was added. After incubation, the cells were washed twice with PBS, and DMEM, containing 10% of FCS was added. The cells were illuminated for 60 min with LED array at $\lambda = 509 \pm 5$ nm (29 W/m^2).

Safr-PDT. A-431 cells were washed twice with PBS and FCS-free DMEM, containing 0.7 μg/ml Safr, was added. Following incubation for 1 h, the cells were washed twice with PBS, and DMEM supplemented with 5% of FCS was added. The cells were illuminated for 1-10 min with the LED array at $\lambda = 509 \pm 5$ nm (29 W/m^2).

$AlPcS_4$-PDT. A-431 cells were washed twice with PBS buffer, and FCS-free DMEM, containing 20 μg/ml $AlPcS_4$, was added for 18 hours. After incubation, the cells were washed twice with PBS, and DMEM, containing 5% of FCS was added. The cells were illuminated for 4-12 min with the LED array at $\lambda = 660 \pm 5$ nm (16 W/m^2).

Imaging

For *colocalisation studies* of mTHPC and mitochondrial marker Rh123 in MH-22A cells, the cells were incubated with mTHPC (0.15 μg/ml for 18 h) and for the last 15 min of incubation, Rh123 (2 μg/ml) has been added to the incubation medium. Fluorescence has been visualized with an Olympus AX70 fluorescence microscope equipped with a 60×, NA 1.25 oil immersion objective and filter cube (400-410 nm for excitation and beyond 590 nm for emission) for mTHPC fluorescence; WIBA filter cube (460-490 nm for excitation and 515-550 nm for emission) for Rh123 fluorescence. The images were captured with a CCD camera Orca (Hamamatsu).

For colocalisation studies of photosensitisers in A-431 cells, the cells cultured on coverslips were incubated with Safr (0.7 μg/ml for 1 h), mTHPC (0.1 μg/ml for 18 h) or $AlPcS_4$ (20 μg/ml for 18 h). MitoTracker Deep Red 633 (25 nM) (Molecular Probes) was added to incubation medium together with Safr. MitoTracker Green (20 nM) (Molecular Probes) was added to incubation medium for the last 45 min of incubation with mTHPC. LysoTracker Yellow-HCK-123 (75 nm) (Molecular Probes) was added to incubation medium for 1 h after incubation with $AlPcS_4$. Excitation/emission wavelengths for each of the fluorescent substances were as follows: Safr, 514/560-630 nm; mTHPC, 633/640-670 nm; $AlPcS_4$, 633/640-690 nm; MitoTracker Deep Red 633, 633/650-750 nm; MitoTracker Green, 488/500-540; LysoTracker Yellow-HCK-123, 488/494-605 nm. The images were captured with Leica SP5 confocal microscope equipped with 63×, NA 1.2 water immersion objective.

For *studies of morphology* changes, fluorescence and Nomarski microscopy (DIC) of cells following PDT was performed with epi-fluorescence microscope Olympus AX70 equipped with a 60×, NA 1.25 oil immersion objective. For staining of chromatin, cells were incubated with 5 μg/ml of Hoechst 33342 (Molecular Probes) for 30 min at 37°C. WU filter cube (excitation/emission wavelengths, 330-385/400 nm) was used for fluorescence detection. The images were captured with a CCD camera Orca (Hamamatsu) and processed with MicroImage 4.0 (Media Cybernetics) software.

For *immunofluorescent detection of LC3*, cells cultured on coverslips were fixed with 4% formaldehyde and permeabilized with PBS, containing 0.1% Triton X-100, at 24 h after light exposure. After blocking with 0.5% BSA in PBS, cells were incubated with anti-LC3 antibody (PM036, MBL) and labeled with anti-rabbit IgG-NL637 (NL005, R&D Systems). LC3 immunofluorescence analysis was performed with Leica SP5 confocal microscope equipped with 63×, NA 1.4 oil immersion objective. Excitation/emission wavelengths were 633/640-680 nm.

Cell viability assay

MTT assay or staining with crystal violet (CV).

Citrate synthase activity assay

Cells were detached from substratum by 0.025% trypsin, washed with PBS and lysed in 50 mM Tris-HCl (pH 7.5), containing 0.3% Triton X-100 at the density of 10^7 cells/ml. Then, citrate synthase activity was determined by a two-step procedure. At first, acetyl-CoA hydrolase activity was determined: 30 μl of cell lysate was added to a mixture of 100 μl of DTNB solution (0.4 mg/ml in 1 M Tris-HCl, pH 8.1), 30 μl of acetyl-CoA solution (10 mg/ml in water, acidified to pH 5) and 790 μl of water, and absorbance at

412 nm was recorded for 2 min with Perkin Elmer spectrometer Lambda 20. On the second step, 50 μl of 1.32 mg/ml of oxaloacetic acid was added and the absorbance recorded for additional 2-3 min in order to determine citrate synthase activity. The change in absorbance during the last minute of the first step, was subtracted from the linear change in absorbance during the first minute of the second step. Citrate synthase activity was then expressed as percentage of that, measured in the control cells.

β-N-acetylglucosaminidase activity

After washing of cells monolayer with PBS, the cells were lysed in lysis buffer (solution of 0.1% Triton X-100 in 100 mM of sodium acetate, pH 4.6). 100 μl of lysate was mixed with 400 μl of substrate solution (2 mM p-nitrophenyl N-acetyl-β-D-glucosaminide solution in 100 mM of sodium acetate, pH 4.6). After incubation for 2 h at 37°C, reaction was stopped by adding 500 μl of ice-cold 0.5 M Na_2CO_3. Absorbtion at 410 nm was registered by spectrophotometer Perkin Elmer λ20. β-N-acetylglucosaminidase activity was then expressed as percentage of that, measured in the control cells.

Caspase-3 activity

Caspase-3 Cellular Activity Assay Kit PLUS (AK-703) (Biomol, Germany) was used. The assay is based on the cleavage of the specific fluorogenic peptide substrate acetyl-aspartyl-glutamyl-valyl-aspartyl-7-amino-4-trifluoromethylcoumarin (Ac-DEVD-AFC) for caspase-3-like activity. Sample cells were harvested at various times post-exposure and treated according to manufacturer's instruction. Fluorescence of AFC, which was proportional to the DEVD-ase activity in the cell lysates, was recorded on fluoroscan Ascent FL (Labsystems, Finland) at an excitation/emission wavelengths of 390/510 nm, respectively.

Flow cytometric analysis

Cell cycle was investigated by biparametric flow cytometric analysis, performing BrdU incorporation and DNA staining with propidium iodide. During DNA synthesis, BrdU replaces thymidine, so BrdU pulse labeling detects S-phase cells engaged in DNA synthesis at that time. We analysed cell cycle by two experimental approaches: i) BrdU was added immediately after PDT and the fate of cells, which were in S-phase at the time of labeling, was analysed after fixation at 6 h following BrdU pulse; ii) cells were incubated with BrdU at 24 h after PDT, allowing to distinguish S-phase cells from G0/G1 and G_2/M cells, then fixed and analysed.

BrdU was added to culture medium to a final concentration of 10 μM. The cells were incubated with BrdU in CO_2 incubator at 37°C for 30 min. Then the cells were detached from growth dish, washed in PBS and fixed by 70% ethanol. Fixed cells were resuspended in 2N HCl with 0.5% Triton X-100 for 30 min to denature the DNA. After washing with 0.1 M $Na_2B_4O_7$, pH 8.5, to neutralise the acid, the cells were resuspended in PBS, containing 0.5% Tween 20 and 1% BSA for 15 min. Anti-BrdU FITC antibody (347583, Becton Dickinson) was added for 30 min at room temperature. After incubation with the antibody, the cells were washed with PBS and resuspended in PBS, containing 5 μg/ml of propidium iodide. The cellular DNA content and the amount of incorporated BrdU were simultaneously measured using a FACSort flow cytometer (Becton Dickinson) and acquired data were analysed by WinMDI 2.8 software.

Quantitative PCR

The cells were detached from substratum by 0.025% trypsin and washed once with TE buffer (10 mM Tris, 1 mM EDTA, pH 7.4 in DEPC treated water). Total RNA was isolated from 2×10^6 cells using total RNA isolation kit Purescript® (Gentra Systems, USA). cDNA for RT-PCR was synthesized using RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas), according to manufacturer's protocol. RT-PCR reactions were performed on ABI Prism® 7000 Sequence Detection System (Applied Biosystems), using SYBR® Green PCR Master Mix (Applied Biosystems). The thermal profile was as follows: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s, 60°C for 1 min. Expression of VEGFA, IL1A, HIF1A was normalised to ACTB. Results were analysed by the Relative Standard Curve Method.

Western blotting

For detection of cytochrome c, 4×10^6 cells were lysed for 30 min on ice in 60 µl of lysis buffer: PBS, containing 10 µg/ml saponine, 1 mM PMSF, 1 µM leupeptin and 0.01 µM aprotinin. Cell lysates were centrifuged at 1300×g for 5 min to remove cell debris. The supernatant was centrifuged at 17 000×g for 15 min to pellet mitochondria.

For detection of LC3, a marker of autophagy, cells were lysed in RIPA buffer: 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 1 mM PMSF, 1 µM leupeptin and 0.01 µM aprotinin. 2×10^5 cells (10^7 cells/ml) were lysed for 30 min on ice and centrifuged for 5 min at 14 000×g and 4°C.

Cytosolic fraction (supernatant) was mixed at 1:5 with 6×sample buffer (30% glycerol, 10 % SDS, 0.012 % bromophenol blue, 0.6 M DTT and 0.35 M Tris-HCl, pH 6.8). Samples were subjected to 15% SDS-PAGE for cytochrome c and 12% SDS-PAGE for LC3 detection at 120V. Proteins were transferred to nitrocellulose membrane (BioRad) by semi-dry blotting. Blots were probed with anti-cytochrome c antibody (SA-226, Biomol), or anti-LC3 antibody (PM036, MBL). In addition, the blots were probed with anti-actin antibody (sc-8432, Santa Cruz Biotechnology) for detection of β-actin as a loading control. Membrane-bound primary antibodies of actin and cytochrome c were detected by horseradish-peroxidase-conjugated secondary anti-mouse antibody (sc-2031, Santa Cruz Biotechnology). The antibody against LC3 was detected by horseradish-peroxidase-conjugated secondary anti-rabbit antibody (sc-2030, Santa Cruz Biotechnology). The immunoreactive bands were developed using enhanced chemiluminescence reagents (Amersham Biosciences). *Inhibition of LC3 degradation.* The stock solutions of lysosomal protease inhibitors E64d and pepstatin A were prepared in DMSO at concentration of 10 mg/ml each. Inhibitors (10 µg/ml, each) were added to cell media immediately after light exposure and remained in the growth media for 24 hours.

Data analysis

The data are presented as means ± SD. Student's unpaired t-test was used for assessing significant difference. Sigma Stat version 3.5 software was used for the assessment.

Results and Discussion

Cell death induced by photosensitisers localised to different organelles

Effect of Rh123-phototoxic and mTHPC-photodynamic treatment on MH-22A cells

The first task of the study was to determine the effect of Rh123, localised to mitochondria, and light to mouse hepatoma MH-22A cells and compare it with cell death response to light and mTHPC, localised to multiple organelles. First, we have determined the localisation of mTHPC in MH-22A cells. It was partly colocalised with mitochondrial marker Rh123 (Fig. 1, A), so part of mTHPC in MH-22A cells localise to mitochondria. Since the data of other authors, concerning the importance of oxygen for Rh123-induced phototoxic effect are contradictory [12; 13], we entitle the treatment of cells with Rh123 and light not photodynamic (involving oxygen), but phototoxic (PTT). The greatest loss of cell viability achieved by Rh123-mediated PTT was approx. 40% (cytotoxic dose, CD40) and it took 60 min of light exposure (104.4 kJ/m^2). Meanwhile, the same loss by mTHPC-PDT was achieved at 0.72 kJ/m^2 (45 sec of light exposure). In order to evaluate the toxic effect of Rh123 and light, we assessed the activity of citrate synthase, the specific enzyme of the mitochondrial matrix (the putative site of Rh123 accumulation) following the treatment. The cell treatment with Rh123 and light significantly decreased the citrate synthase activity ($P<0.001$), indicating the damage to the inner space of mitochondria (Fig. 1, B). After mTHPC-PDT, which reaches the multiple organelles, the citrate synthase activity did not change. Probably, mTHPC due to its lipophilic nature resides in the membranes of mitochondria. It is interesting to note, that after light exposure of Rh123 pre-treated cells, the morphology of mitochondria has changed (Fig. 1, C).

In order to know, how the cell would respond to the damage targeted to mitochondrial interior, we focused our study on the changes in cell morphology and some biochemical parameters following Rh123-PTT and mTHPC-PDT.

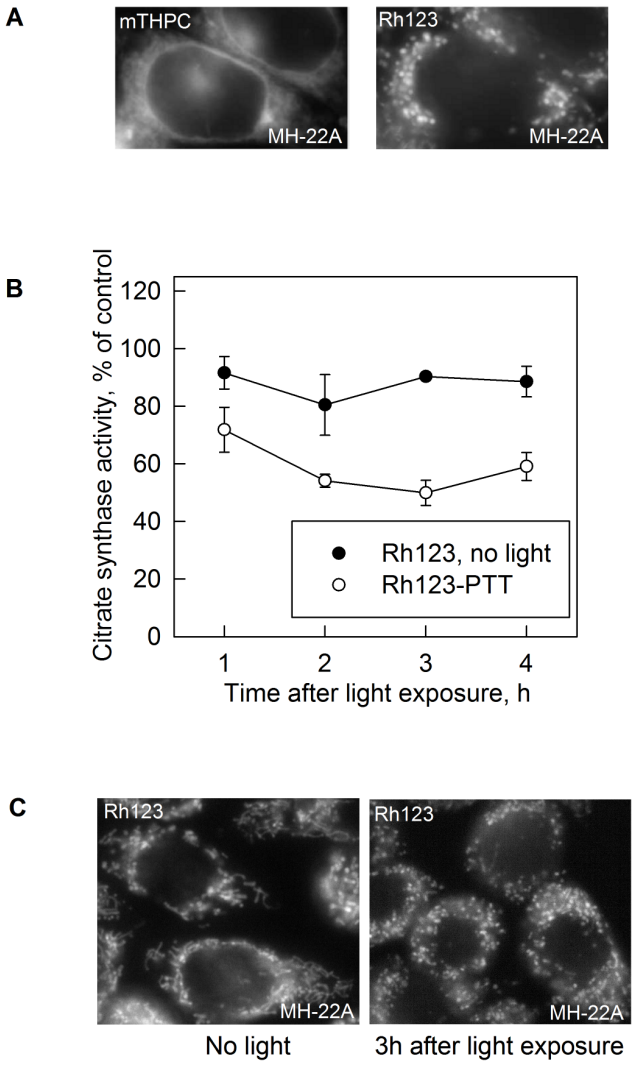

Fig. 1. Cellular localisation of mTHPC, citrate synthase activity and Rh123 fluorescence pattern after Rh123-PTT in MH-22A cells.

A - Cellular localisation of mTHPC in MH-22A cells. The cells were coincubated with mTHPC and mitochondrial marker Rh123.

B - Citrate synthase activity following light exposure expressed as % of untreated control. The cells were incubated for 1 h with 5 μg/ml of Rh123 (Rh123, no light) or exposed to Rh123-PTT.

C – Rh123 fluorescence after Rh123-PTT. Rh123 fluorescence was registered after incubation with 5 μg/ml Rh123 for 1 h (no light) and at 3 h (3 h after light exposure) following Rh123-PTT.

Rh123-PTT: the cells for 1 h were incubated with 5 μg/ml of Rh123 and for 60 min. exposed to light of λ=509±5 nm, 29 W/m^2.

Bars, ± SD.

After mTHPC-PDT, apoptotic features were observed, as follows: chromatin condensation (Fig. 2, A), cytochrome c release to cytosol (Fig. 2, B), and caspase-3 activation (DEVDase activity) (Fig. 2, C). After Rh123-PTT, only a couple of dead cells, exhibiting apoptotic or necrotic morphology, were observed, in the same extent as in control cells; no release of cytochrome c was detected in cytosolic fraction of the treated cells (Fig. 2, B), and no increase of caspase-3 activity in comparison with control cells was recorded (Fig. 2, C).

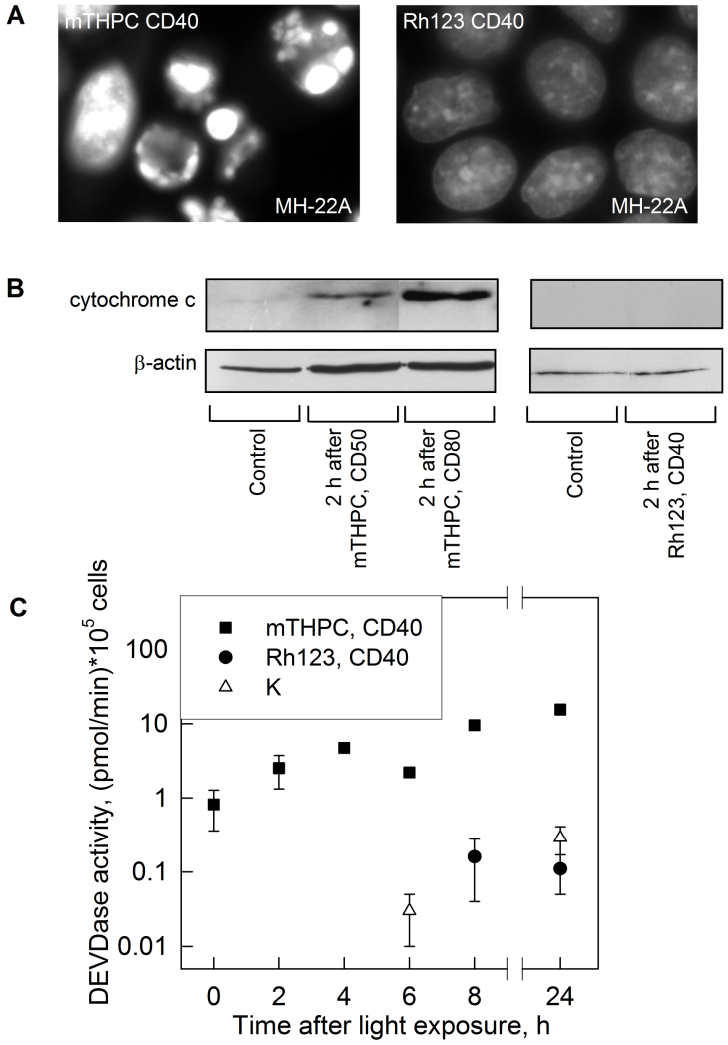

Fig. 2. Hallmarks of apoptosis in MH-22A cells.
A - Fluorescence of Hoechst 33342 in cells after treatment. Duration of post-exposure incubation: mTHPC-PDT, 1.5 h; Rh123-PTT, 4 h.
B – Western blots of cytochrome c in cytosolic fraction after mTHPC-PDT and Rh123-PTT at 2 h after light exposure.
C - DEVDase activity following mTHPC-PDT and Rh123-PTT.
mTHPC-PDT: the cells were incubated for 18 h with 0.15 μg/ml of mTHPC and for 45 sec. exposed to light of 660 ±5 nm, 16 W/m^2.
Regimen of Rh123-PTT see in the legend of Fig.1. Bars, ± SD.

These results demonstrate that cell response to the damage to the mitochondrial interior by Rh123-PTT at CD40 does not induce cell death. The loss of cell viability indicates that growth arrest or other pathways of cell death such as autophagy should be involved.

Cell death after Safr- or mTHPC-mediated photodynamic treatment of A-431 cells

Since Rh123 was inefficient as a photosensitiser, for further studies we have chosen another delocalised cation Safr, which has a high singlet oxygen quantum yield (0.24) [14]. Human epidermoid carcinoma A-431 cell line was chosen for further studies. We checked the subcellular localisation of mTHPC and Safr in A-431 cells. Fluorescence of mTHPC was distributed all over the cell in accordance with its lipophilic character and presumed localisation to the cell membranes. Mitochondria were among the cell compartments accumulating mTHPC, since the fluorescence pattern of the mitochondrial probe Mitotracker Green partially coincided with that of mTHPC, indicating that under the incubation protocol applied in this study some part of mTHPC was localised to mitochondrial area (Fig. 3, A). The fluorescence pattern of Safr coincided with that of the mitochondrial probe Mitotracker Deep Red (Fig. 3, B).

By light exposure, both photosensitisers efficiently mediated the light-exposure dependent reduction of cellular viability. Most of the further experiments were carried out at light exposures reducing amount of viable cells at 24 h post-treatment to either 50% (CD50) or 80% (CD80).

In order to evaluate the damage to mitochondrial matrix, we assayed the cell lysates for the activity of citrate synthase, residing in the mitochondrial matrix (Fig. 3, C). Immediately after light exposure, no decrease in citrate synthase activity was detected in lysates of cells pre-incubated with mTHPC, while in the lysates of cells pre-treated with Safr, the enzyme activity was decreased to 40% at CD80 and the decrease was statistically significant ($P=0.049$).

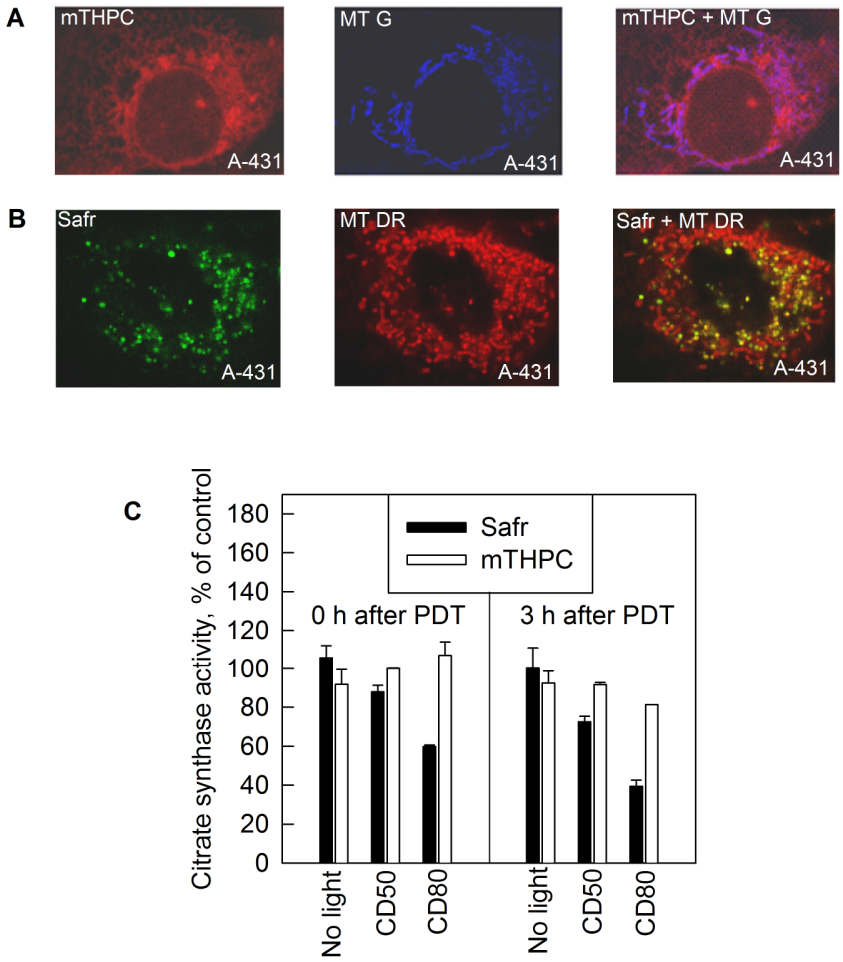

Fig. 3. Cellular localisation of mTHPC, Safr and citrate synthase activity after Safr-PDT and mTHPC-PDT in A-431 cells.

A - Fluorescence pattern of and MitoTracker Deep Red 633 (MT DR). The cells for 1 h were simultaneously incubated with 0.7 μg/ml Safr and 25 nM MT DR.

B - Fluorescence pattern of mTHPC and MitoTracker Green (MT G). The cells were incubated for 18 h with 0.1 μg/ml mTHPC and for the last 45 min of incubation with mTHPC was added MT G (20 nM).

C - Citrate synthase activity immediately (0 h) and 3 h after light exposure, expressed as % of untreated control.

Safr-PDT: The cells were incubated for 1 h with 0.7 μg/ml Safr, not exposed (No light) or exposed for 3 min (CD50) or 10 min (CD80) or other (if indicated otherwise), to light of 509±5 nm, 29 W/m^2.

mTHPC-PDT: The cells were incubated for 18 h with 0.1 μg/ml mTHPC, not exposed (No light) or exposed for 1 min (CD50) or 3 min (CD80), to light of 660±5 nm, 16 W/m^2.
Bars, ± SD.

No significant decrease in citrate synthase activity was detected in cells pre-incubated with mTHPC following 3 h post-light exposure at CD50, yet, and a significant reduction for 22% was registered in Safr-treated cells (P=0.012). At CD80, a significant decrease for 20% was observed in mTHPC-treated cells (P<0.001), which was much exceeded by the enzyme activity reduction for 61% in Safr-treated cells (P=0.003). It should be noted, that the immediate response of citrate synthase activity to Safr-PDT could be a proof that cationic Safr accumulates in mitochondrial interior and mediates damage to mitochondrial matrix.

We examined hallmarks of cell death following PDT. After mTHPC-PDT at CD50, apoptotic features were prominent, as follows: cell rounding, shrinkage and condensation of chromatin (Fig. 4, A and B), cytochrome c release to cytosol (Fig. 4, C) and caspase-3 activation (DEVDase activity) (Fig. 4, D). A share of necrotic cells was not significant, less than 2%, at the same extent as in control cells. After Safr-PDT at CD50, only few dead cells, exhibiting apoptotic or necrotic morphology, were observed, at the same extent as in control cells; cytochrome c was not detected in cytosolic fraction of the treated cells (Fig. 4, C); and insignificant caspase-3 activation was recorded (Fig. 4, D). At the doses lower than CD50, we observed no significant hallmarks of apoptosis or necrosis even at 72 h post-exposure. However, high doses of Safr-PDT, beyond CD70, induced pronounced apoptosis: characteristic changes of cellular and chromatin morphology (Fig. 4, A and B), substantial release of cytochrome c to the cytosol (Fig. 4, C), and caspase-3 activation (Fig. 4, E). After Safr-PDT inducing extensive apoptosis, a small share of necrosis was detected, at the same extent as in control cells (approx. 2%).

Having in mind the absence of hallmarks of apoptosis or necrosis following Safr-PDT at CD50, we hypothesized that the loss of viability of Safr-PDT cells up to 50% should be ascribed to the reduced proliferation of the treated cells, i. e.

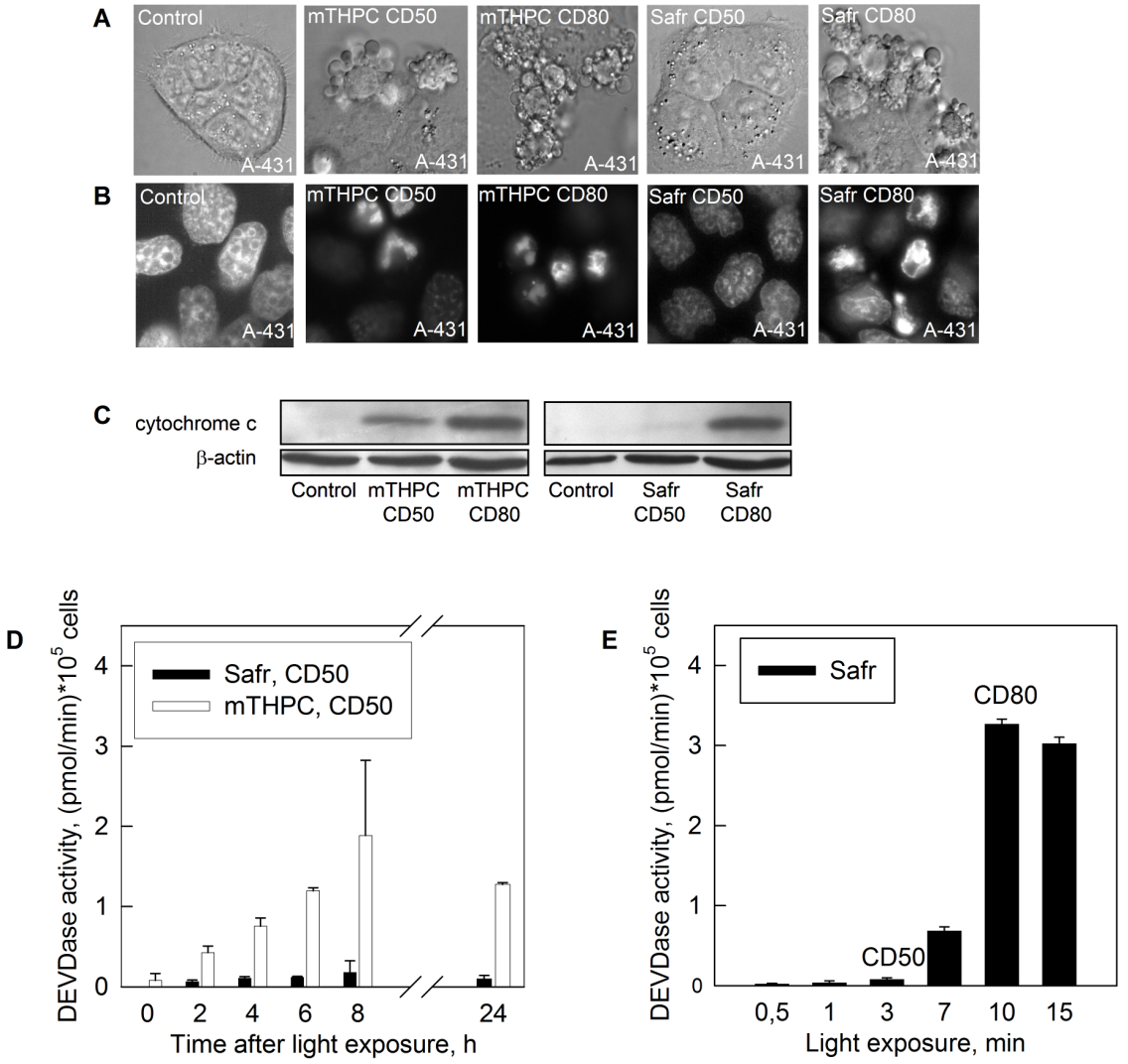

Fig. 4. Hallmarks of mTHPC-PDT and Safr-PDT induced apoptosis in A-431 cells.

A - Nomarski microscopy (DIC) images of cells following PDT. Duration of incubation after light exposure: mTHPC-PDT at 1.5 h; Safr-PDT at 4 h.

B - Fluorescence of Hoechst 33342 in cells after treatment. Duration of post-exposure incubation: mTHPC-PDT, 1.5 h; Safr-PDT, 4 h.

C – Western blots of cytochrome c in cytosolic fraction after mTHPC-PDT and Safr-PDT at 3 h after light exposure.

D - DEVDase activity following Safr-PDT and mTHPC-PDT.

E - DEVDase activity after Safr-PDT at 3 h after light exposure.

Regimen of PDT see in the legend of Fig. 3.

Bars, ± SD.

growth inhibition. Cell cycle effects of PDT were investigated in a series of experiments based on BrdU incorporation using two different protocols. Fig. 5, A

shows dot plots of the biparametric flow cytometric analysis of DNA-BrdU at 6 h after light exposure, with BrdU labeling in the first 30 min after light was switched off. BrdU-positive control cells, which were in S-phase at the time of the labeling, reached the late S-phase, G_2/M or subsequent G_0/G_1 in 6 h. BrdU-negative control cells, which were in G_0/G_1 or G_2/M at the time of the labeling, were caught being in G_0/G_1 and early S at 6 h. An S-phase delay was observed after PDT, since a significant amount of the treated cells in S-phase at the time of the labeling were still in the S-phase at 6 h and few ones had divided.

To follow the cell cycle related distribution of the surviving cells, we used the different design of the experiment, giving BrdU for 30 min at 24 h after PDT and harvesting the cells immediately after labeling (Fig. 5, B). In average, after Safr-PDT at CD50, amount of S-phase cells decreased by 10 ± 2% and G_0/G_1 cells increased by 11.5 ± 2%, as compared to control.

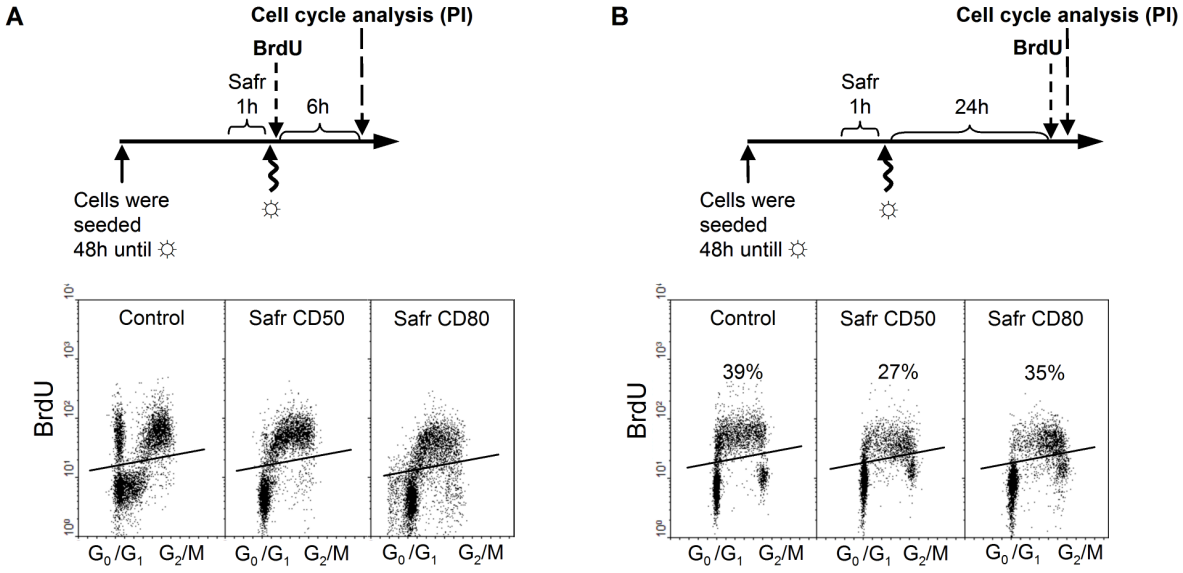

Fig. 5. Cell cycle analysis following Safr-PDT in A-431 cells.

A - Pulse-chase DNA-BrdU analysis at 6 h after light exposure, with BrdU given in the first 30 min immediately after light exposure.

B - DNA-BrdU analysis at 24 h after light exposure, with BrdU given for 30 min just before fixation of the cells. Cells were labelled, fixed and analysed for DNA and BrdU content. Cells were considered BrdU positive, when detected above the solid line. Values above the line, amount of BrdU positive cells as % of total amount of cells

Regimen of PDT see in the legend of Fig. 3.

Cell death after $AlPcS_4$-mediated photodynamic treatment of A-431 cells

In A431 cells, $AlPcS_4$ colocalised with the lysosomal marker LysoTracker Yellow-HCK-123 (Fig. 6, A).

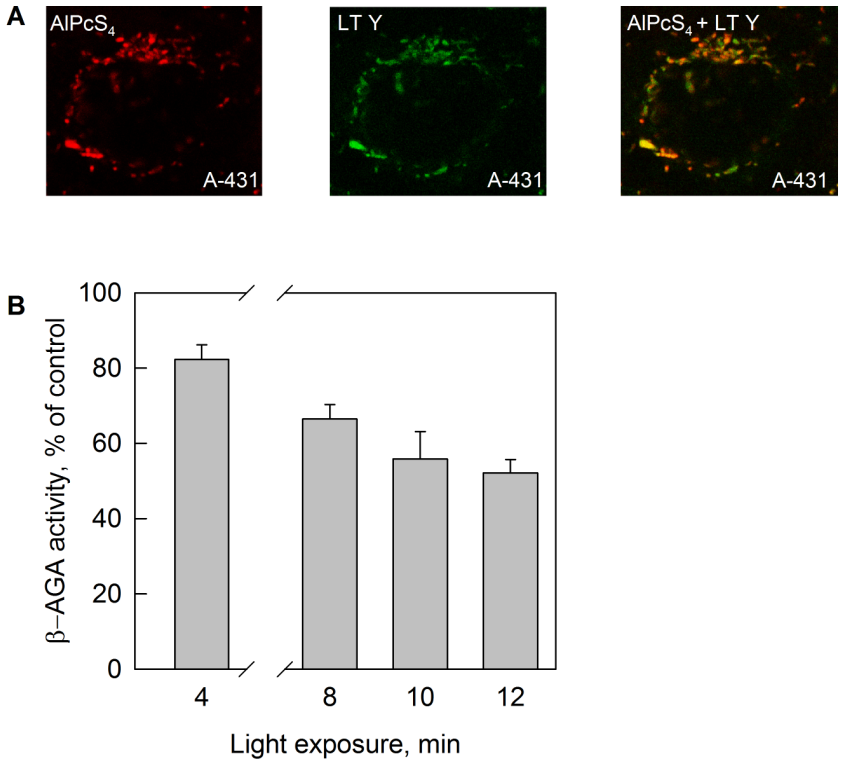

Fig. 6. Cellular localisation of $AlPcS_4$ and β-N-acetylglucosaminidase activity after $AlPcS_4$-PDT in A-431 cells.

A - Fluorescence pattern of $AlPcS_4$ and LysoTracker Yellow-HCK-123 (LT Y). The cells were incubated for 18 h with 20 μg/ml $AlPcS_4$ and for 1 h with 75 nm LT Y.

B - β-N-acetylglucosaminidase (β-AGA) activity after $AlPcS_4$-PDT immediately after light exposure.

$AlPcS_4$-PDT: The cells were incubated for 18 h with 20 μg/ml $AlPcS_4$ and exposed for indicated time (5 min corresponds to CD20, 9 min – CD50, 12 min – CD80) to light of 660±5 nm, 16 W/m^2.

Bars, ± SD.

$AlPcS_4$-mediated PDT reduced the activity of the lysosomal enzyme β-N-acetylglucosaminidase (Fig. 6, B), and this change was significant ($P \leq 0.003$). $AlPcS_4$-PDT induced apoptosis, as determined by changes in cellular morphology:

cell rounding, chromatin condensation and fragmentation (Fig. 7, A and B), cytochrome c release to cytosol (Fig. 7, C) and caspase-3 activation (DEVDase activity) (Fig. 7, D).

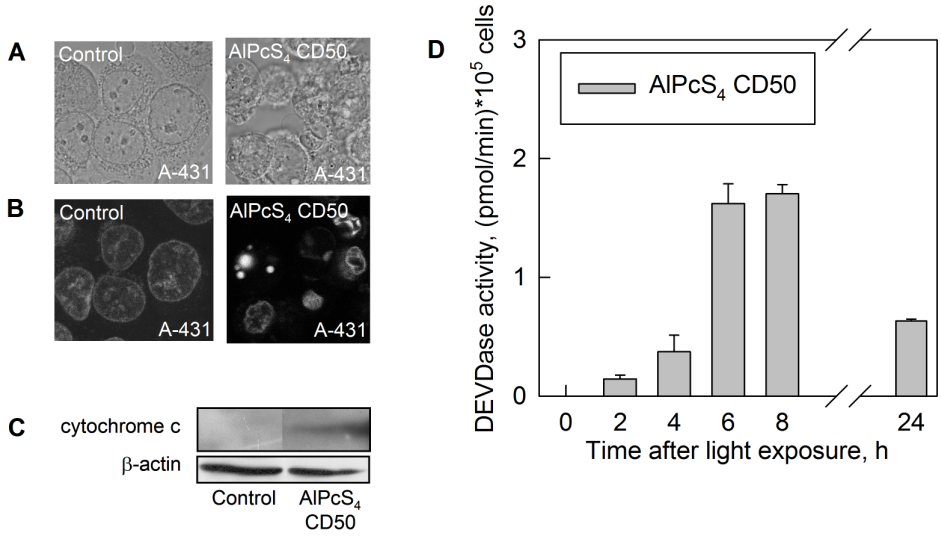

Fig. 7. Hallmarks of $AlPcS_4$-PDT induced apoptosis in A-431 cells.

A - Nomarski microscopy (DIC) images of cells following $AlPcS_4$-PDT (CD50) at 3 h after light exposure.

B - Fluorescence of Hoechst 33342 in cells after $AlPcS_4$-PDT (CD50) at 3 h after light exposure.

C – Western blots of cytochrome c in cytosolic fraction after $AlPcS_4$-PDT (CD50) at 3 h after light exposure.

D - DEVDase activity following $AlPcS_4$-PDT (CD50).

Regimen of PDT see in the legend of Fig. 6.

Bars, ± SD.

Autophagy induced by the photosensitisers, localised to different cellular organelles in A-431 cells

Autophagy can be both: cytoprotective response activated by dying cells in the attempt to cope with stress and cell death mode [15]. First, we determined autophagy by immunofluorescence microscopy, detecting microtubule-associated protein 1 light chain 3 (LC3), its lipidated form is known to be associated with autophagosomes. In control cells, a comparatively week fluorescence of LC3 was more or less diffusely distributed over the cytoplasm (Fig. 8). However, in the

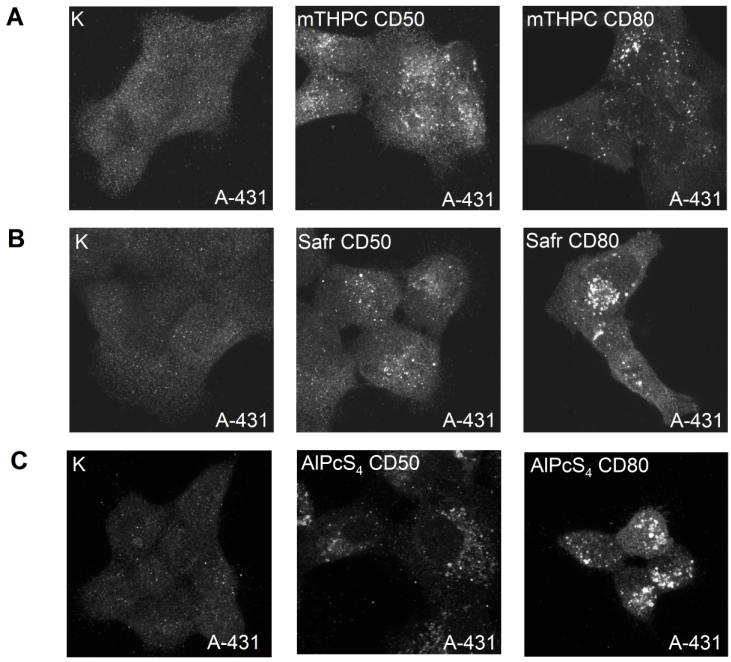

Fig. 8. Hallmarks of PDT-induced autophagy in A-431 cells. Immunofluorescence microscopy of LC3 at 24 h after light exposure following:

A - mTHPC-PDT;

B - Safr-PDT;

C - $AlPcS_4$-PDT.

mTHPC-PDT: The cells were incubated for 18 h with 0.1 μg/ml mTHPC, exposed for 1 min (CD50) or 3 min (CD80) to light of 660±5 nm, 16 W/m^2.

Safr-PDT: The cells were incubated for 1 h with 0.7 μg/ml Safr, exposed for 3 min (CD50) or 10 min (CD80) to light of 509±5 nm, 29 W/m^2.

$AlPcS_4$-PDT: The cells were incubated for 18 h with 20 μg/ml $AlPcS_4$ and exposed for 9 min (CD50) or 12 min (CD80) to light of 660±5 nm, 16 W/m^2.

cells after PDT mediated by mTHPC, Safr or $AlPcS_4$, quite a few intense punctate LC3 structures were visualised supposing the presence of autophagosomes.

Accumulation of autophagosomes does not provide the evidence of functional autophagy that includes delivery to, and degradation within lysosomes. So, additional experiments were carried out to determine the flux through the autophagy pathway. To validate membrane-associated form of LC3 in photodynamically treated cells, lipidation of LC3 was detected by immunoblot analysis.

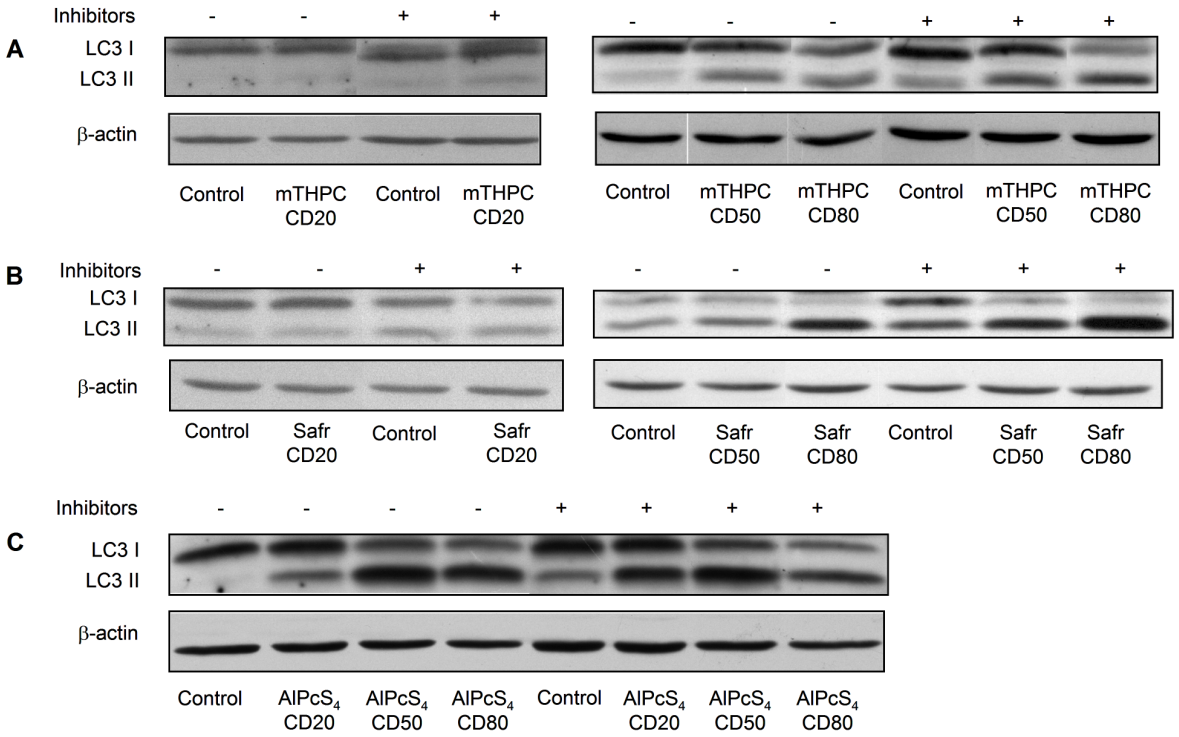

Fig. 9. Hallmarks of PDT-induced autophagy in A-431 cells. Western blots of LC3 at 24 h after light exposure following:

A - mTHPC-PDT;

B - Safr-PDT;

C - $AlPcS_4$-PDT.

mTHPC-PDT: The cells were incubated for 18 h with 0.1 μg/ml mTHPC, exposed for 20 sec (CD20), 1 min (CD50) or 3 min (CD80) to light of 660±5 nm, 16 W/m^2.

Safr-PDT: The cells were incubated for 1 h with 0.7 μg/ml Safr, exposed for 1 min (CD20), 3 min (CD50) or 10 min (CD80) to light of 509±5 nm, 29 W/m^2.

$AlPcS_4$-PDT: The cells were incubated for 18 h with 20 μg/ml $AlPcS_4$ and exposed for 4 min (CD20), 9 min (CD50) or 12 min (CD80) to light of 660±5 nm, 16 W/m^2.

The so-called LC3-II is produced when phosphatidylethanolamine attaches to LC3-I, which is unlipidated form of LC3. This conversion correlates with the number of autophagosomes [16]. Since LC3-II can be itself degraded by autophagy, lysosomal protease inhibitors, such as E64d and pepstatin A, were used to partially inhibit degradation of LC3-II [17]. All the examined photodynamic treatments: mTHPC-PDT, Safr-PDT, $AlPcS_4$-PDT, induced an increase in LC3-II amount at 24 h after light exposure (Fig. 9). After Safr-PDT at CD50, LC3-II increase was not intense, however, incubation with lysosomal

protease inhibitors revealed a prominent increase of autophagic flux. Although it has been shown that PDT, mediated by photosensitisers localised to lysosomes, increases the number of autophagosomes [11], the autophagic flux has not been determined. In this work, we have demonstrated that, after lysosome damage inducing $AlPcS_4$-PDT, the number of autophagosomes increases, but late steps of autophagy occur only after PDT at small dose (CD20). $AlPcS_4$-PDT at medium (CD50) and high (CD80) doses increase the number of autophagosomes, but the flux through autophagy pathway is blocked.

Expression of cytokines VEGF-A, IL-1α and transcription factor HIF-1 α subunit after photodynamic treatment in A-431 cells

The increase of the expression of cytokines: vascular endothelial growth factor A (VEGF-A) and interleukin 1, alpha (IL-1α), following mTHPC-PDT was determined in our laboratory and it was demonstrated that IL-1α overexpression stimulates the increase of VEGF-A [18]. In this study, we investigated the impact of the photodynamic treatment induced by photosensitisers localised selectively to mitochondria (Safr) and lysosomes ($AlPcS_4$) on the expression of VEGF-A and

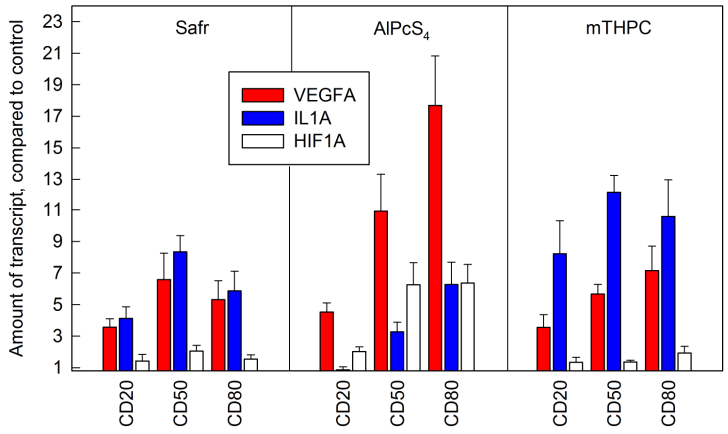

Fig. 10. Changes in mRNA expression of VEGFA, IL1A and HIF1A in Safr-PDT, mTHPC-PDT and $AlPcS_4$-PDT treated A-431 cells. Gene expression has been determined at 3.5 h after light exposure.

Regimen of PDT see in the legend of Fig. 8.

IL-1α. Since the transcription factor HIF1 is a known regulator of VEGF-A expression [19], we have studied the expression of HIF-1α after photodynamic treatment, as well.

The amount of VEGF-A and IL-1α transcripts was increased after Safr-PDT and $AlPcS_4$-PDT (Fig. 10). The highest increase of VEGF-A RNA was detected after $AlPcS_4$-PDT. It coincided with the high expression level of HIF-1α. Therefore, PDT mediated by photosensitisers selectively localised to lysosomes ($AlPcS_4$) or mitochondria (Safr) increases the expression of VEGF-A and IL-1α, alike PDT mediated by photosensitiser localised to multiple organelles (mTHPC).

Conclusions

1. Photocytotoxic treatment, targeted to different organeles, induces the complex cellular response, which depends on the treatment dose and the damage sites.

2. The photodynamic treatment of mouse hepatoma cells MH-22A mediated by mitochondria-localised rhodamine 123 at medium cytotoxic dose (CD40) decreases the activity of citrate synthase residing in mitochondrial matrix, but does not increase the permeability of the mitochondrial outer membrane, and apoptosis is not initiated. The same cytotoxic dose of photodynamic treatment induced by meso-tetra(3-hydroxyphenyl)chlorin, localised to multiple organelles, evokes apoptosis.

3. The photodynamic treatment of human epidermoid carcinoma cells A-431 mediated by mitochondria-localised safranin O at medium cytotoxic dose (CD50) does not induce the cell death, and the cell viability decrease could be attributed to the cell cycle arrest. The high cytotoxic dose (CD80) induces apoptosis. At medium (CD50) and high (CD80) cytotoxic doses, the photodynamic treatment mediated by meso-tetra(3-hydroxyphenyl)chlorin, localised to multiple organelles, evokes apoptosis.

4. The photodynamic treatment of A-431 cells mediated by lysosomes-localised aluminium (III) phthalocyanine tetrasulfonate induces apoptosis.

5. The photodynamic treatment mediated by safranin O or meso-tetra(3-hydroxyphenyl)chlorin at medium and high doses increases the autophagy in A-431 cells. Although the amount of autophagosomes increases after small (CD20), medium (CD50) and high (CD80) dose of photodynamic treatment mediated by aluminium (III) phthalocyanine tetrasulfonate, but

the flux through autophagy pathway proceeds only after small dose (CD20) of the treatment.

6. The Photodynamic treatment of A-431 cells mediated by safranin O, aluminium (III) phthalocyanine tetrasulfonate or meso-tetra(3-hydroxyphenyl)chlorin increases the expression of cytokines VEGF-A, IL-1α and transcription factor HIF-1 α subunit on the RNA level.

References

1. Moan, J. and Berg, K. Photochem Photobiol 1991; 53:549-553.
2. Hatz, S., Poulsen, L. and Ogilby, P.R. Photochem Photobiol 2008; 84:1284-1290.
3. Oleinick, N.L., Morris, R.L. and Belichenko, I. Photochem Photobiol Sci 2002; 1:1-21.
4. Tait, S.W. and Green, D.R. Nat Rev Mol Cell Biol 2010; 11:621-632.
5. Johansson, A.C., Appelqvist, H., Nilsson, C., Kagedal, K., Roberg, K. and Ollinger, K. Apoptosis 2010; 15:527-540.
6. Marchal, S., Francois, A., Dumas, D., Guillemin, F. and Bezdetnaya, L. Br J Cancer 2007; 96:944-951.
7. Triesscheijn, M., Ruevekamp, M., Aalders, M., Baas, P. and Stewart, F.A. Photochem Photobiol 2004; 80:236-241.
8. Leung, W.N., Sun, X., Mak, N.K. and Yow, C.M. Photochem Photobiol 2002; 75:406-411.
9. Sasnauskiene, A., Kadziauskas, J., Labeikyte, D. and Kirveliene, V. Biologija 2009; 55:51-56.
10. Sasnauskiene, A., Kadziauskas, J., Vezelyte, N., Jonusiene, V. and Kirveliene, V. Apoptosis 2009; 14:276-286.
11. Reiners, J.J., Jr., Agostinis, P., Berg, K., Oleinick, N.L. and Kessel, D. Autophagy 2010; 6:7-18.
12. Atlante, A., Passarella, S., Moreno, G. and Salet, C. Photochem Photobiol 1992; 56:471-478.
13. Morliere, P., Santus, R., Bazin, M., Kohen, E., Carillet, V., Bon, F., Rainasse, J. and Dubertret, L. Photochem Photobiol 1990; 52:703-710.
14. Bunting, J.R. Photochem Photobiol 1992; 55:81-87.
15. Galluzzi, L., Vitale, I., Abrams, J.M., Alnemri, E.S., Baehrecke, E.H., Blagosklonny, M.V., Dawson, T.M., Dawson, V.L., El-Deiry, W.S., Fulda, S., et al. Cell Death Differ 2011.
16. Kabeya, Y., Mizushima, N., Ueno, T., Yamamoto, A., Kirisako, T., Noda, T., Kominami, E., Ohsumi, Y. and Yoshimori, T. Embo J 2000; 19:5720-5728.
17. Mizushima, N. and Yoshimori, T. Autophagy 2007; 3:542-545.
18. Dabkevičienė, D. Daktaro disertacija. Vilniaus universitetas, Vilnius 2010.
19. Zelzer, E., Mamluk, R., Ferrara, N., Johnson, R.S., Schipani, E. and Olsen, B.R. Development 2004; 131:2161-2171.

List of publications

1. **A. Sasnauskiene**, J. Kadziauskas, N. Vezelyte, V. Jonusiene, V. Kirveliene. Apoptosis, autophagy and cell cycle arrest following photodamage to mitochondrial interior // Apoptosis. 2009, 14: 276-286.
2. **A. Sasnauskiene**, J. Kadziauskas**,** D. Labeikyte, V. Kirveliene. Rhodamine 123-mediated photodamage to mitochondrial interior does not develop hallmarks of apoptosis in MH22 cells // Biologija. 2009, 55: 51-56.
3. **A. Sasnauskiene**, J. Kadziauskas, N. Vezelyte, V. Jonusiene, V. Kirveliene. Damage targeted to the mitochondrial interior induces autophagy, cell cycle arrest and, only at high doses, apoptosis // Autophagy. 2009, 5: 743-744.

Conference reports

1. N. Vėželytė, **A. Sasnauskienė**, V. Kirvelienė (2009). Cell death induced by photodynamic treatment targeted to lysosomes // 17th ECDO Euroconference, Paris, France, September 23-26, Abstract book, P-237.
2. **A. Sasnauskienė**, V. Kirvelienė (2008). Programmed cell death induced by mitochondria-targeted photodynamic treatment // 16th ECDO Euroconference on apoptosis, Bern, Switzerland, September 6-9, Programme and book of Abstracts, P-140.
3. **A. Sasnauskienė**, N. Vėželytė, J. Kadziauskas, V. Kirvelienė (2008). Photodynamic damage to mitochondrial interior induces cell growth inhibition followed by extensive apoptosis // Apoptosis World 2008. From mechanisms to applications, Luxembourg, January 23-26, Proceedings and Program, p. 488.
4. **A. Sadauskaitė**, N. Vėželytė, U. Sūdžiuvienė, V. Jurgelevičius, V. Kirvelienė (2007). Cytokine expression following oxidative damage to different subcellular compartments // ELSO 2007 Conference, Dresden, Germany, September 1-4, Proceedings, p. 66.
5. **A. Sadauskaite**, J. Kadziauskas, V. Kirveliene (2006). On cellular effects of photosensitized damage by cationic dyes // 14th European Bioenergetics Conference, Moscow, Russian Federation, July 22-27, Biochim. Biophys. Acta: Astract Book, V. 14, p. 511.

Santrauka

Fotodinaminė terapija yra naudojama gydyti vėžį ir kitas ligas, kurias sukelia suintensyvėjusi ląstelių proliferacija. Fotosensibilizatoriaus (FS) lokalizacija ląstelėje lemia pažaidų vietą. Daugelis klinikoje naudojamų FS kaupiami įvairiose ląstelės organelėse. Šio darbo metu *in vitro* buvo tiriamas ląstelių atsakas į fotodinaminį poveikį (FDP), kurį sukėlė FS, atrankiai sukaupti skirtingose organelėse. Buvo tiriami apoptozės ir autofagijos vyksmai, ląstelės ciklo eiga, kai kurių genų raiška. Naudoti FS, kurie kaupiami 1) mitochondrijose - 2-(6-amino-3-imino-3H-ksanten-9-il)benzoinės rūgšties metilo esteris (rodaminas 123, arba Rh123) ir 3,7-diamino-2,8-dimetilo-5-fenilfenazino chloridas (Safr); 2) lizosomose - aliuminio (III) ftalocianino tetrasulfonatas ($AlPcS_4$); 3) įvairiose organelėse - mezo-tetra(3-hidroksifenil)chlorinas (mTHPC).

Buvo nustatyta, kad ir Safr, ir $AlPcS_4$ ir mTHPC atveju stiprus FDP, mažinantis ląstelių gyvybingumą 80% (CD80), sukelia apoptozę. Vidutinio stiprumo FDP dozė, sukelta naudojant Safr ir Rh123, neindukuoja ląstelių žūties, tačiau po Safr-FDP vyksta ląstelės ciklo areštas. Vidutinis (CD50) ir stiprus (CD80) FDP, kuriam sukelti naudojamas Safr arba mTHPC, indukuoja autofagiją. Nors autofagosomų padaugėjo po silpnos, vidutinės ir didelės dozės $AlPcS_4$ sukelto FDP, tačiau galutinės autofagijos stadijos vyko tiktai po silpno (CD20) poveikio. Po FDP, sukelto naudojant Safr, $AlPcS_4$ ar mTHPC, padidėjo citokinų VEGF-A, IL-1α ir transkripcijos veiksnio HIF-1 α subvieneto raiška RNR lygiu.

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