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Cytotoxicity of $PP(Arg)_2$- and $Hp(Arg)_2$-mediated photodynamic therapy and early stage of apoptosis induction in prostate carcinoma *in vitro*

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Porphyrin photosensitizers tend to localize in mitochondria. The depolarization of mitochondrial membrane is one of the early stages of apoptosis and Laser Scanning Fluorescence Microscopy allows to determine changes in transmembrane mitochondrial potential under influence of PDT depending on the kind of photosensitizer ($PP(Arg)_2$, $Hp(Arg)_2$), the energy dose (5, 10, 30 and 50 J/cm²) and time periods (24 and 48 hours after irradiation) in the LNCaP (lymphonodal metastasis of prostate carcinoma, the androgen dependent cell line). Cyototoxicity induced by $PP(Arg)_2$- and $Hp(Arg)_2$-based PDT depending on energy dose and time after irradiation in prostate carcinoma is determined with MTT. Generally, it was shown that lower energy doses induce greater changes in transmembrane mitochondrial potential. $Hp(Arg)_2$-based PDT was more effective causing greater mitochondrial membrane depolarization and cell viability decrease in comparison to $PP(Arg)_2$-mediated PDT (in the case of maximal nontoxic photosensitizer doses used).

Keywords: PDT, confocal microscopy, prostate carcinoma

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INTRODUCTION

Photodynamic therapy (PDT) is a photochemical reaction requires simultaneous presence of the photosensibilizator, light energy source adequate to photosensibilizator and oxygen concentration as high as possible (Henderson & Dougherty, 1992; Schuitmaker *et al*., 1996; Graczyk, 1999; Kaymond, 1999; Kessel & Dougherty, 1999; Sharman *et al*., 1999; Kessel, 2004). PDT is a low invasive and selective method of the cancer diagnosis and therapy. It can be a modern diagnostic method and complementary method to chemotherapy, radiotherapy and especially to surgery applied before or after PDT (Pass, 1993; Ochsner, 1997; Oleinck, 1998; Sharman *et al*., 1999; Brown & Brown, 2004; Castano *et al*., 2005a; 2005b).

The most frequent chemical compounds applied in the PDT are porphyrins and their derivatives (Sternberg & Dolphin, 1998; Graczyk, 1999; You *et al*., 2006). Porphyrin photosensitizers are derivatives of red blood dye — heme, and as such, the endogenic dyes possesses low toxicity and their metabolism in organisms is well known (Mauzerall, 1998; Ryter & Tyrrell, 1999).

Strong absorption band within the range of 390–405 nm (called Soret band) and four weaker absorption bands within the range of 450–700 nm (called Q bands) are characteristic for porphyrins. Soret band due to its small penetration depth of 2.0 mm into a tissue can be applied for diagnosis and treatment of flat skin lesions. Moreover, the depth of Soret band penetration is sufficient for cell line studies. The Q band, on the other hand, is used for therapy because of the tissue penetration depth up to 8.0 mm, which depends on the wavelength of the aforementioned (Graczyk, 1999; Sternberg & Dolphin, 1998; Mauzerall, 1998; Ryter & Tyrrell, 1999; Gomer, 1990).

Pre-clinical and clinical trials reveal that many photosensitizers, especially porphyrins'-based ones used in PDT, are gathering in mitochondria (Kessel, 1997; Kessel & Luo *et al*., 1999; Desagher & Martinou, 2000; Hilf *et al*., 1987; 1987), implying cell death *via* intrinsic apoptosis pathway (Green & Reed, 1998; Skulachev, 2000; Pedersen, 1987).

A number of metabolic processes proceeding in mitochondria, include a basic reaction of the ATP synthesis undergoing in the mentioned mitochondria (of which the compound is crucial for cell energetics). Bioenergetic properties of violently growing cancer cell differ from the ones of a normal tissue, mainly due to a rising of glicolysis with access to air and a cellular respiration inhibition with the help of glucose (Crabtree effect). Glicolysis is more important source of ATP in cancer cells than in the healthy ones. The functional disturbances of mitochondria induce the decrease of ATP synthesis. Both inhibition of glicolysis and mitochondrial respiration are necessary to destroy the cells mentioned before (Pedersen, 1987; Graczyk, 1999). The disturbances in respiration chain due to the proton leakage by internal mitochndrial membrane lead to decrease of electrochemical membrane potential (Graczyk, 1999; Oleinck, 1998; Chen *et al*., 1988). Depolarisation of transmembrane mi-

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Abbreviations: ANOVA, one way analysis of variance; FBS, foetal bovine serum; $Hp(Arg)_2$, diarginine hematoporphyrin; LNCaP, lymphonodal metastasis of prostate carcinoma responsive to androgen therapy; LSCM, laser scanning confocal microscope; MANOVA, multi way analysis of variance; MTT, 3-(4,5-dimetylthiazol-2-yl)2,5-diphenyl tetrazolium bromide; MitoLight™, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine chloride; PBS, phosphate-buffered saline; PDT, photodynamic therapy; $PP(Arg)_2$, diarginine protoporphyrin IX.

tochondrial potential is an early stage of apoptosis — a programmed cell death (Lu, 1996; Kessel & Luo, 1999; Otsuki, 2000; Shi, 2001; Oleinick *et al*., 2002; Plaetzer *et al*., 2003; Buytaert *et al*., 2007).

Confocal Laser Scanning Microscopy (CLSM) is applied in measurements of the transmembrane mitochondrial potential changes (Reers *et al*., 1991; Smiley *et al*., 1991; Masters, 1995).

Fluorescent microscopy has been known for about 100 years and the confocal microscope is one of the most modern modifications to the conventional optical microscope. It allows improvement of the microscope image quality and widens the range of fluorescent microscopy studies (Sheppard, 1993; Masters, 1995; Furrer & Gurny, 2010).

Prostate cancer is a pathological proliferation of prostate, a gland set at the base of the man urinary bladder. It is also the most often diagnosed cancer among men (except skin cancer), and is fourth most common reason of cancer death in the world. The occurrence of prostate cancers in the family, is one amongst of the primary factors that increases the probability of prostate cancer incidence taking place. About 10% of prostate cancer is inherited, but to date, little is known about it. Prostate cancer can metastasize to different organs and it has a tendency of metastasizing to nearest lymph nodes and bones. Backbone, pelvis, ribs, humeral and femoral bones are the main targets of metastases (Steinberg *et al*., 1990; Issacs *et al*., 1995; Ponder, 2001; Lee, 2001; Suresh, 2007).

At the early stage (tumor limited to prostate gland) of prostate cancer, a standard treatment like: radical prostatectomy (surgical removal of prostate gland), internal and external radiotherapy or hormonotherapy in the case of hormone-dependent cancers can be used. In the case of bone metastasis there are additional bone targeted therapies (such as: radiotherapy or chemotherapy with use of biophosphonate) used in treatment or prevention of problems during spread of prostate cancer to the skeletal system.

Early diagnosis and treatment are the most important factors in prevention of the cancer prostate metastasis to bone or other organs. Because of it, there is a need to invent a new and more effective method of early stage of prostate cancer treatment (Gittes, 1991; Boyle P & Ferlay, 2005).

The purpose of this work was to describe the influence of $Hp(Arg)_2$ and $PP(Arg)_2$-based PDT on induction of the early stage of mitochondrial apoptosis — change in the transmembrane mitochondrial potential. The $Hp(Arg)_2$ and $PP(Arg)_2$-mediated PDT cytotoxicity towards LNCaP prostate cancer was described by cell viability measurements (MTT). All performed experiments should help to optimize the conditions for prostate carcinoma destruction, mostly *via* the mitochondrial apoptosis mechanism.

MATERIALS AND METHODS

Chemicals. Diarginine protoporphtrin IX and hematoporphyrin were synthesized at the Biochemistry and Spectroscopy Laboratory, at the Institute of Optoelectronics, Military University of Technology (patent number PL 1652 49 B1, EP 0539 960 B1) (Fig. 1). Two arginines substituents in PPIX and Hp ensure solubility of these compounds in water which implies improvement of the photosensitizers solubility.

Eagle'a medium 1959 with L-Glutamine and phenol red came from Serum and Vaccine Company Biomed (Lublin, Poland). Penicillin and streptomycin were bought at Polfa Tarchomin (Warsaw, Poland). Foetal bovine serum was provided by Gibco Invitrogen (Carlsband, USA). PPIX, Hp, trypsin-EDTA and other chemicals were obtained from Sigma-Aldrich Norway (Oslo, Norway).

The stock solutions of $PP(Arg)_2$ and $Hp(Arg)_2$ in distilled water at a concentration of 0.25 mg/ml were sterilized with filter 0.45 μm CORNING® (Wiesbaden, Germany) and stored at 4°C in darkness in sterile Becton Dickinson (Franklin Lakes, USA) probes, in order to be used for further experimentation. Immediately prior to experiment, the stock solutios of $PP(Arg)_2$ and $Hp(Arg)_2$ were diluted in a sterile phosphate buffer (pH 7.4) to the appropriate concentrations.

Cell culture. LNCaP (Lymphonodal metastasis of prostate carcinoma responsive to androgen therapy) was received from the Hirschfeld Institute (Wroclaw, Poland). Cancer cells were cultured in completed medium (100 ml medium supplemented with 10% foetal bovine serum (FBS) and 1 ml of antibiotics solution containing penicillin (10000 U/ml) and streptomycin (4 mg/ml)). Cells were maintained in humidified air containing 5% CO_2 at 37°C, in sterile cell culture dishes Nunc (Kamstrupvej, Denmark).

Completed medium was changed thrice a week and washed with PBS (cells were trypsinized and re-seeded into fresh medium twice a week). Every time, sterile, serological pipettes purchased from Becton Dickinson (Franklin Lakes, USA) were used.

Cell visualization. Experimental cell images were performed with the Scanning Confocal Microscope OLYMPUS IX70. FV500.

MTT assay. MTT assay determines a metabolic acitivity of cells. Soluble in water, yellow solution of MTT (3-(4,5-dimetylthiazol-2-yl)2,5-diphenyl tetrazolium bromide] is absorbed by cells, and reduced to the insoluble in water purple formazan. The reduction of MTT to formazan is performed by mitochondrial dehydrogenase which exhibits activity only in viable cells. On the basis of cells abilities to MTT reduction, cell viabillity is determined. Formazan crystals were dissolved in isopropyl alcohol and the concentration of reaction product was determined spectrophotometrically at 570 nm wavelength.

Cancer cells were seeded into 96-well plates and experiments were carried out according to the procedure described below.

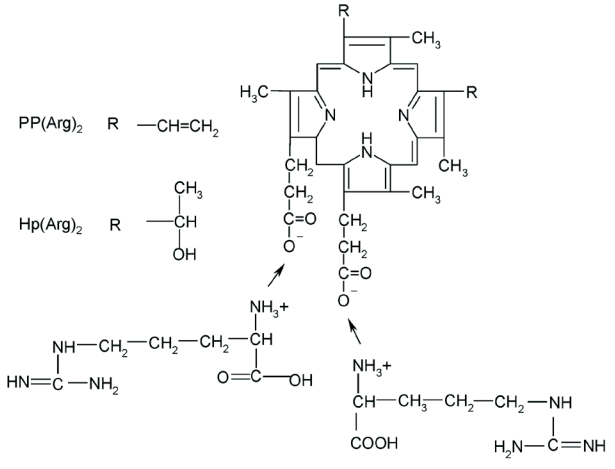

Figure 1. Structure of diarginine protoporphyrin IX ($PP(Arg)_2$) and hematoporphyrin ($HP(Arg)_2$)

Plates with cells were centrifuged at 3000 rpm, medium was removed and the cells were washed twice with PBS (150 μl/well) and centrifuged. Next, 50 μl of MTT solution in PBS (5 mg/ml, diluted 1:20) was added to each well and incubated for 3 h at 37°C in humidified atmosphere containing 5% CO_2. Afterwards, formazan crystals were dissolved in isopropyl alcohol (200 μl/well). Absorbance of solution product was measured with microplate spectrophotometer Power Wave XS (Bio Tek, USA). The ratio of formazan absorbance in the sample (cells under influence of different factors: photosesitizer alone, light alone and PDT) to the control one (cells without photosensitizer, light and PDT) was given in percentage.

Evaluation of the LNCaP cell viability under influence of PP(Arg)$_2$ and Hp(Arg)$_2$ in darkness. LNCaP human prostate cancer cells were seeded into sterile 96-well plate Nunc (Kamstrupvej, Denmark) at the densities of 1.5×10^4 cells/ml and incubated, in order for the cells to settle down. Six hours after seeding, an appropriate volume of the photosensitizer (PP(Arg)$_2$, Hp(Arg)$_2$) stock was added to the final concentrations: 10; 20; 30; 40; 50; 60; 70; 80; 90; 100; 110; 130; 150; 200; 400; 800 and 1600 μg/ml. Cancer cells with photosensitizer were incubated for 24 h in darkness. Then, the cultured medium was changed to get rid off the photosensitizer. After time periods: 24 h + 24 h; 24 h + 48 h and 24 h + 120 h, cancer cell viability was described by MTT (24 h is the time when cancer cells were being irridiated during PDT experiments, but in analogous experiments without irradiation the cells were stored in the incubator). To simplify, in further text the time intervals of 24, 48, 120 h will correlate with 24, 48, 120 h after irradiation in experiments below respectively.

Evaluation of the LNCaP cell viability under influence of the energy dose. Cells were seeded according to the procedure described above. 48 hours after seeding cells were irradiated ($\lambda = 410$ nm) with energy doses: 5, 10, 30, 50, 80 and 120 J/cm^2 using lamp manufactured in MUT (Warsaw, Poland) as the light source and the light intensity was set to 140 mW. After 24, 48 and 120 h cancer cell viability was described by MTT. Applied wavelenghts were within the range of Soret band, and the depth of the Soret band penetration was sufficient for cell line studies (Graczyk, 1999).

Evaluation of the LNCaP cell viability under the influence of PDT effect. Cells were seeded according to the procedure described above. The maximal non-toxic photosensitizer doses were applied to LNCaP cell line 6 hours after seeding (for PP(Arg)$_2$ and Hp(Arg)$_2$ with the dose of: 60 and 110 μg/ml respectively). 24 hours later photosensitizer was flushed out. After 48 hours the seeded cells were irradiated with energy doses of ($\lambda = 410$ nm): 5, 10, 30; 50; 80 and 120 J/cm^2. 24, 48 and 120 h after irradiation, further actions were performed in accordance with the procedure described above.

Statistical analysis. MTT assay experiments were carried out in 6 parallel attempts and repeated twice, and the data was normalized for comparison. Multi Way Analysis of Variance (MANOVA) was performed in order to estimate the significance of main effects (energy dose, type of photosensitizer and time after irradiation) and their interactions with viability of investigated cancer cells. To estimate the significant differences among parameters, the post-hoc test of Tukey was performed, in which the significance level was set at $P < 0.05$. In the case of photosensitizers concentration studies, the one-way analysis of ANOVA, followed by post-hoc test of Dunnett was performed. The significance level was set at $^*P < 0.05$ as statistically significant difference in comparison to the control sample. The calculations were made with Statistica 9.0 StatSoft, Inc. USA.

Measurements of the transmembrane mitochondrial potential changes by LSCM (Laser Scanning Confocal Microscope). Synthesis of ATP proceeds thanks to the transmembrane mitochondrial potential $\Delta\Psi$. Potential disturbance is a one of the early change in mitochondrial apoptosis (Reers *et al*., 1991; Smiley *et al*., 1991; Masters, 1995; Misiewicz *et al*., 2003; Misiewicz *et al*., 2004; Misiewicz-Krzemińska *et al*., 2009).

MitoLight™ (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine chloride) is a mitochondrial indicator, which interacts in a different way with the mitochondria of living and apoptotic cell. Healthy cells have polarized mitochondria. In such cells dye accumulate and form J-aggregates giving red fluorescence. Apoptotic cells have depolarized mitochondria and monomeric dye give green fluorescence (dye stays in the cytoplasm). Cells of investigated line were seeded to 8-well microplates in the density of 1.5×10^4 cell/ml. PDT experiments were carried out with cancer cells where: concentrations of PP(Arg)$_2$ and Hp(Arg)$_2$ determined in MTT assay were 60 and 110 μg/ml for PP(Arg)$_2$ and Hp(Arg)$_2$ respectively; energy doses of 5, 10, 30 and 50 J/cm^2 were used. The change of mitochondrial transmembrane potential after irradiation time of 24 and 48 h was studied. For this purpose the medium was removed, cells were washed twice with PBS, and 200 μl of freshly made MitoLoght solution (1 μl/ml dye) was added to each well. Incubation time was 15 min at 37°C in humidified atmosphere containing 5% CO_2. Next dye solution was removed and 200 μl of PBS was added. The changes of mitochondrial transmembrane potential of studied cancer cell line under influence of PDT was investigated with Laser Scanning Confocal Microscope Olympus IX70 FV 500, while the fluorescence intensity analysis was performed with Fluo View 3.4c. To excite fluorescence of dye monomers and aggregates, blue dye laser was used (Argon laser 488 nm). To observe monomeric fluorescence (green fluorescence) 505–525 nm filter was used. Meanwhile, red fluorescence from aggregates was collected through BP560 filter.

RESULTS

The effect of type and concentration of the photosensitizer on cell viability, depending on time in darkness, was studied first (Table 1 and 2). Statistical analysis shows that concentration, type of photosensitizer, time and interactions: concentration–time, concentration–type of photosensitizer and time–type of photosensitizer, are statistically significant factors. For each investigated time and photosensitizer, it is possible to determine a bordering value of concentration after crossing the cell viability is decreasing. The highest nontoxic doses of PP(Arg)$_2$ and Hp(Arg)$_2$ were determined: 200 for 24 h (this time correlates with 24 h after irradiation in further experiments), 130 for 48 h (this time correlates with 48 h after irradiation in further experiments), 60 μg/ml for 120 h (this time correlates with 120 h after irradiation in further experiments) and 400 for 24 h, 200 for 48 h, 110 μg/ml for 120 h respectively. The highest nontoxic doses of PP(Arg)$_2$ and Hp(Arg)$_2$ are significantly different to the control. For both PP(Arg)$_2$, and Hp(Arg)$_2$ differences among results for 24 h, 48 h and 120 h are

Table 1. The effect of PP(Arg)2 concentration on cell viability rate dependinf from time: 24 h, 48 h and 120 h.
Cells were incubated with photosensitizer for 24 h in darkness (Materials and Methods). Values are means ± S.D. (standard deviation).

LNCaP			
Concentration of $PP(Arg)_2$ (mg/ml]	Cell viability rate (%)		
	24 h	48 h	120 h
10	106±8	103±7	101±11
20	10513	102±12	101±8
30	103±8	102±11	101±6
40	103±11	101±11	100±10
50	102±7	101±7	100±11
60	102±6	101±5	100±5
70	102±7	100±7	95±8*
80	100±6	100±7	95±9*
90	100±7	100±7	93±6*
100	100±8	100±10	87±10*
110	100±7	100±7	81±7*
130	100±11	96±6	79±9*
150	97±5	92±10*	76±7*
200	94±10	88±11*	71±9*
400	90±9*	86±7*	63±7*
800	86±7*	73±10*	56±7*
1600	81±7*	60±6*	44±4*

*$P < 0.05$ in comparison to control.

Table 2. The effect of $Hp(Arg)_2$ concentration on cell viability rate: 24 h, 48 h and 120 h.
Cells were incubated with photosensitizer for 24 h in darkness (Materials and methods). Values are means ±S.D. (standard deviation).

LNCaP			
Concentration of $Hp(Arg)_2$ (mg/ml]	Cell viability rate (%)		
	24 h	48 h	120 h
10	106±8*	109±7*	113±13*
20	105±10	103±9	112±13*
30	104±8	102±6	106±9*
40	103±7	102±9	105±8*
50	101±7	102±8	101±8
60	103±8	102±8	101±6
70	103±8	102±8	101±6
80	103±10	100±9	100±8
90	101±12	101±10	100±10
100	101±13	100±8	100±10
110	101±12	100±8	100±6
130	100±11	100±10	93±8*
150	100±11	100±10	88±9*
200	100±12	96±12	80±7*
400	97±8	91±8*	70±11*
800	88±8*	80±9*	61±11*
1600	83±8*	71±9*	48±8*

*$P < 0.05$ in comparison to control.

also significant. The highest nontoxic concentrations of $Hp(Arg)_2$ are higher for each investigated time in comparison to $PP(Arg)_2$. The differences between $PP(Arg)_2$ and $Hp(Arg)_2$ results for 48 h and 120 h are statistically significant as well (for 24 h no significant differences were noticed). So generally, cells of LNCaP are more susceptible to $PP(Arg)_2$ than to $Hp(Arg)_2$ in darkness (significant differences). Results showed that cell viability of studied cells decreases with the passing of time in the case of both investigated photosensitizers (significant differences).

It was shown that photosensitizers interact with LNCaP cells in darkness, without irradiation. It was also noticed that low doses of photosensitizers stimulate cancer cell growth, in comparison to the control probes.

The influence of energy dose ($\lambda = 410$ nm) on cell viability LNCaP depending on the time after irradiation (24, 48 and 120 h), was shown in Fig. 2. Statistical analysis showed that neither energy dose nor time are statistically significant factors. No significant energy dose effect on studied cell line was observed for all investigated energy doses (5, 10, 30, 50, 80 and 120 J/cm^2). However, small but insignificant stimulation of LNCaP cells growth due to low energy doses was observed.

$Hp(Arg)_2$- and $PP(Arg)_2$-based PDT on cell viability LNCaP depending on time was shown in Figs. 3 and 4. Statistical analysis shows that energy dose, type of photosensitizer, time after irradiation and interactions among them are the most significant factors. For both photosensitizers $PP(Arg)_2$ and $Hp(Arg)_2$ differences among PDT results of 24 h, 48 h and 120 h after irradiation were found to be significant. Differences between $PP(Arg)_2$- and $Hp(Arg)_2$-mediated PDT results for 24 h, 48 h and 120 h are also statistically significant. Moreover, differences among interactions results of $PP(Arg)_2$-based PDT for 24 h, 48 h and 120 h and $Hp(Arg)_2$-mediated PDT for 24 h, 48 h and 120 h (except interaction of $Hp(Arg)_2$-mediated PDT for 48 h and $PP(Arg)_2$-based PDT for 120 h are not significant). Data show that $Hp(Arg)_2$ more effectively decreases cancer cell viability

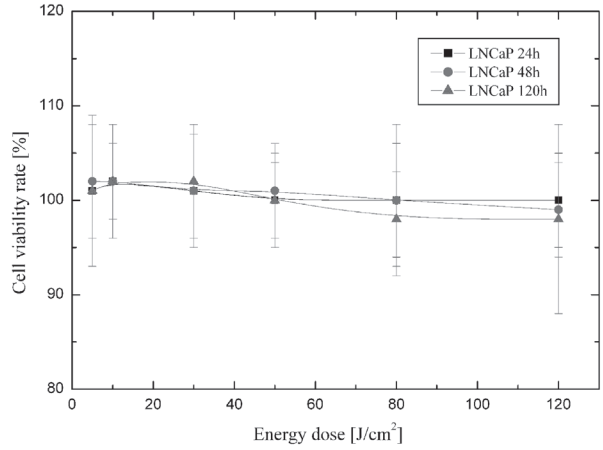

Figure 2. The influence of energy dose on cancer cell viability rate: (■) 24 h, (●) 48 h and (▲) 120 h after cell irradiation.
Values are means ± S.D.

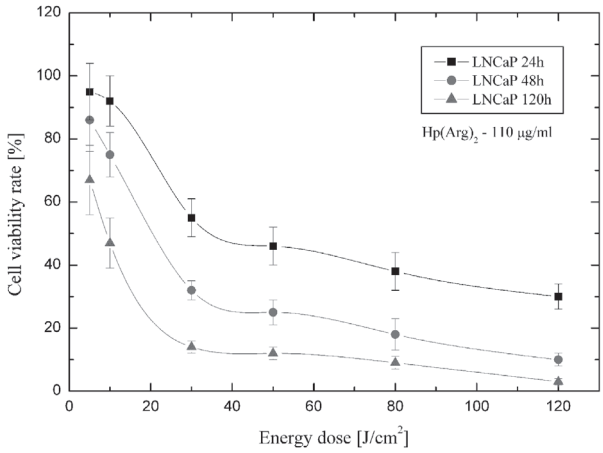

Figure 3. Hp(Arg)$_2$ mediated PDT effect on cell viability rate: (■) 24 h, (●) 48 h and (▲) 120 h after irradiation.
The maximal non-toxic doses were applied. Cells were incubated with photosensitizer for 24 h in darkness (Materials and methods). The Hp(Arg)$_2$ control probes were shown in Table 2. The energy dose control probes were shown in Fig. 3. Values are means ± S.D.

in comparison to PP(Arg)$_2$-mediated PDT. Results of the PP(Arg)$_2$- and Hp(Arg)$_2$-mediated PDT effect on cell viability show slow cells viability decrease with time at the lower energy dose range.

Accumulation of the investigated photosensitizers in LNCaP cell line was presented in Fig. 5. It was shown that photosensitizers accumulate in cells in different quantity, and as such the photodynamic effect cannot be equal to all of the cells presented in the experiments. Taking into consideration this aspect of therapy, it is better to induce apoptosis, mainly because of cell death due to of apoptotic cascade. In the non-damaged cells, apoptosis can be induced through contact with other apoptotic cells.

Results show that higher energy doses (80 and 120 J/cm^2) used in Hp(Arg)$_2$- and PP(Arg)$_2$-based PDT cause significant cell viability decrease just 24 hours after irradiation (Figs. 3 and 4). For this purpose, the measurement of the early stage of apoptosis was investigated for lower energy doses (5, 10, 30 and 50 J/cm^2) applied in

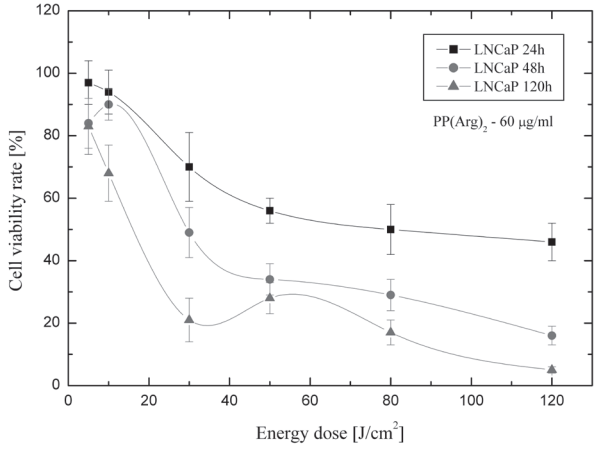

Figure 4. PP(Arg)$_2$ mediated PDT effect on cell viability rate: (■) 24 h, (●) 48 h and (▲) 120 h after irradiation.
The maximal non-toxic doses were applied. Cells were incubated with photosensitizer for 24 h in darkness (Materials and Methods). The PP(Arg)$_2$ control probes were shown in Table 1. The energy dose control probes were shown in Fig. 3. Values are means ± S.D.

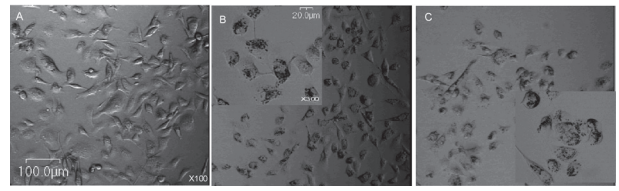

Figure 5. Accumulation of PP(Arg)$_2$ and HP(Arg)$_2$ in LNCaP cell line.
Cells were incubated with photosensitizers by 24h: a — LNCaP cells, b — cells with PP(Arg)$_2$, c — cells with Hp(Arg)$_2$. Cell images (magnification x 100 and x 300) were performed with the Scanning Confocal Microscope OLYMPUS IX70. FV500.

PP(Arg)$_2$- and Hp(Arg)$_2$-mediated PDT (Figs. 6 and 7). The changes in transmembrane mitochondrial potential (Ψ_m) under influence of PP(Arg)$_2$- and Hp(Arg)$_2$-based PDT depending on energy dose of: 5, 10, 30 and 50 J/cm^2 and time after irradiation of 24 h and 48 h were studied by confocal microscope (Figs. 6 and 7). In these figures, the images of the changes in transmembrane mitochondrial potential (Ψ_m) in LNCaP cell line evaluated with confocal microscope are shown (on the left side). Next to the images, there are attached plots of red and green fluorescence intensity of representative cells (on the right side). When mitochondrial membrane is depolarized, dye is in a monomeric form and gives-off green fluorescence (Figs. 6b-i and 7b–i). In the control sample, there is a dominance of red fluorescence, while tracks of green fluorescence (dye aggregates and mitochondrial membrane is polarized) are also present (Figs. 6a and 7a).

Hp(Arg)$_2$-based PDT induces green fluorescence level according to the series: approximately $5 < 10 > 30 > 50$ J/cm^2 24 h after irradiation, but differences between results of 10 and 30 J/cm^2 are not distinct (on the base of plots in Fig. 6b–e). The mitochondrial depolarization level in the case of 10 and 30 J/cm^2 Hp(Arg)$_2$-based PDT is greater than for PP(Arg)$_2$-mediated PDT (plots in Fig. 6cd and gh). 48 hours after irradiation the green fluorescence level is going to decrease for all the investigated energy doses, on the basis of plots in Fig. 7b–e. PP(Arg)$_2$-mediated PDT induces green fluorescence level according to the series: approximately $5 < 10 = 30 > 50$ J/cm^2 for 24 hours after irradiation (on the basis of plots in Fig. 6f–i) while 48 h after irradiation mitochondrial depolarization level change according to the series: $5 < 10 > 30 > 50$ J/cm^2 (on the base of plots in Fig. 7f–i).

Generally, it was noticed that 48 hours after irradiation red fluorescence level decreases in comparison to the 24 one in the case of Hp(Arg)$_2$- and PP(Arg)$_2$-based PDT.

Changes in transmembrane mitochondrial potential were determined for different time after irradiation due to cascade apoptosis (Graczyk, 1999; Castano *et al.*, 2005a; 2005b; Oleinick *et al.*, 2002).

PP(Arg)$_2$- and Hp(Arg)$_2$-mediated PDT induce higher mitochondrial membrane depolarization for 24 h after irradiation than 48 h one, for all the energy doses. Moreover, lower (but not the lowest) energy doses induced higher depolarization level of mitochondrial membrane in general. MTT assay show that Hp(Arg)$_2$-based PDT cause better results (lower cell viability rate especially noticed for 120 h after irradiation). Finally, the fluorescence analysis shows that Hp(Arg)$_2$-mediated PDT cause mostly greater green fluorescence — better membrane depolarization.

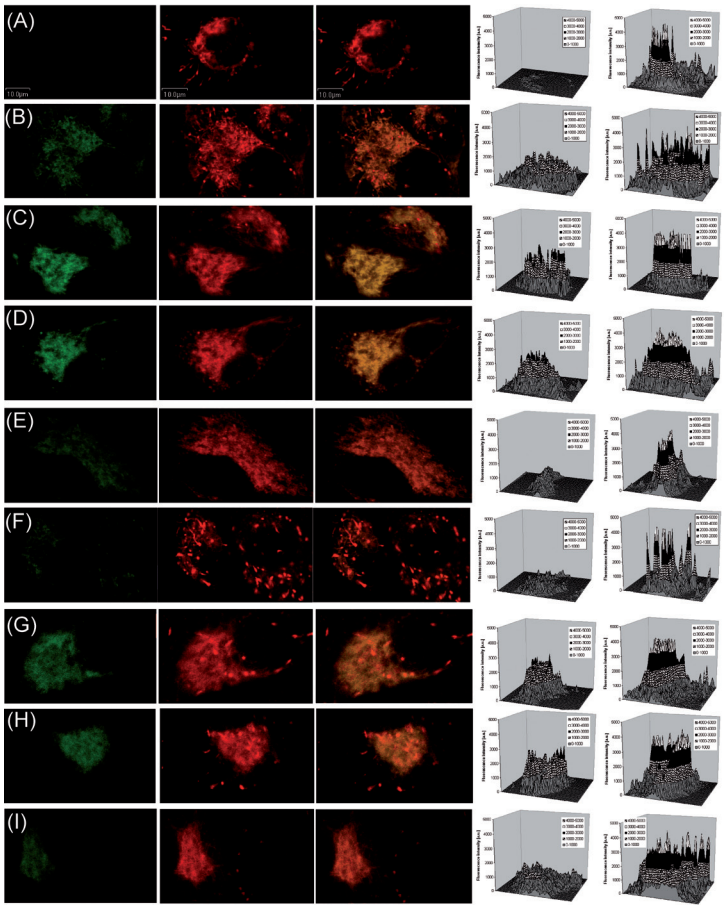

Figure 6. The changes of transmembrane mitochondrial potential in LNCaP cell line under influence of $Hp(Arg)_2$ and $PP(Arg)_2$-mediated PDT 24 hours after irradiation.
Cells were stained with apoptosis detection kit MitoLight™. On the left side there are images (magnification x 1200) of the following: green, red fluorescence and fluorescence of both signals put together. Green fluorescence represents dye monomers in apoptotic cells with depolarized mitochondrial membrane, while red one — dye aggregates bound to polarized mitochondrial membrane in healthy cells. On the right side there are plots of green and red fluorescence intensity of the representative cell.
A — control probe which represent also green and red fluorescence level of other control probes (cells without photosensitizer and not irradiated, cells under influence of $Hp(Arg)_2$ and $PP(Arg)_2$, cells only irradiated with: 5, 10, 30 and 50 J/cm^2).
B — $Hp(Arg)_2$ (110 μg/ml)-mediated PDT 24 hours after irradiation of 5 J/cm^2.
C — $Hp(Arg)_2$ (110 μg/ml)-mediated PDT 24 hours after irradiation of 10 J/cm^2.
D — $Hp(Arg)_2$ (110 μg/ml)-mediated PDT 24 hours after irradiation of 30 J/cm^2.
E — $Hp(Arg)_2$ (110 μg/ml)-mediated PDT 24 hours after irradiation of 50 J/cm^2.
F — $PP(Arg)_2$ (60 μg/ml)-mediated PDT 24 hours after irradiation of 5 J/cm^2.
G — $PP(Arg)_2$ (60 μg/ml)-mediated PDT 24 hours after irradiation of 10 J/cm^2.
H — $PP(Arg)_2$ (60 μg/ml)-mediated PDT 24 hours after irradiation of 30 J/cm^2.
I — $PP(Arg)_2$ (60 μg/ml)-mediated PDT 24 hours after irradiation of 50 J/cm^2.

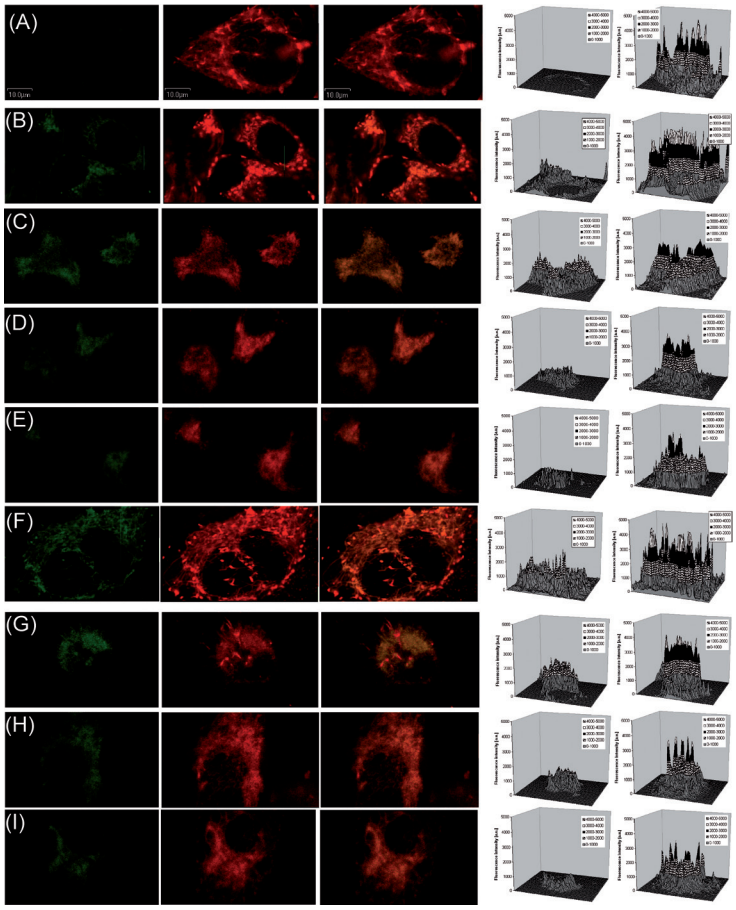

Figure 7. The changes of transmembrane mitochondrial potential in LNCaP cell line under influence of $Hp(Arg)_2$ and $PP(Arg)_2$-mediated PDT 48 hours after irradiation.
Cells were stained with apoptosis detection kit MitoLight™. On the left side there are images (magnification x 1200) of the following: green, red fluorescence and fluorescence of both signals put together. Green fluorescence represents dye monomers in apoptotic cells with depolarized mitochondrial membrane, while red one — dye aggregates bound to polarized mitochondrial membrane in healthy cells. On the right side there are plots of green and red fluorescence intensity of the representative cell.
A — control probe which represent also green and red fluorescence level of other control probes (cells without photosensitizer and not irradiated, cells under influence of $Hp(Arg)_2$ and $PP(Arg)_2$, cells only irradiated with: 5, 10, 30 and 50 J/cm^2).
B — $Hp(Arg)_2$ (110 μg/ml)-mediated PDT 48 hours after irradiation of 5 J/cm^2.
C — $Hp(Arg)_2$ (110 μg/ml)-mediated PDT 48 hours after irradiation of 10 J/cm^2.
D — $Hp(Arg)_2$ (110 μg/ml)-mediated PDT 48 hours after irradiation of 30 J/cm^2.
E — $Hp(Arg)_2$ (110 μg/ml)-mediated PDT 48 hours after irradiation of 50 J/cm^2.
F — $PP(Arg)_2$ (60 μg/ml)-mediated PDT 48 hours after irradiation of 5 J/cm^2.
G — $PP(Arg)_2$ (60 μg/ml)-mediated PDT 48 hours after irradiation of 10 J/cm^2.
H — $PP(Arg)_2$ (60 μg/ml)-mediated PDT 48 hours after irradiation of 30 J/cm^2.
I — $PP(Arg)_2$ (60 μg/ml)-mediated PDT 48 hours after irradiation of 50 J/cm^2.

DISCUSSION

Type of the photosensitizer, cytotoxicity and cell death mechanism are *inter alia* key parameters in deciding about effectiveness and then potential applications of PDT. Porphyrin photosensitizers contain a heterocyclic ring, where various substituents, which define their properties, can be attached to it. Protoporphyrin and hematoporphyrin are very abundant thanks to the easiness of structure modification like the photosensitizers used in the work (Gomer, 1990; Pass, 1993; Graczyk & Konarski, 1995; Noodt *et al*., 1996; Ochsner, 1997; Żołądek *et al*., 1997; Sternberg & Dolphin, 1998; Graczyk, 1999; Li *et al*., 1999; Kessel & Luo, 1999; Graczyk & Kwaśny, 2002; Castano *et al*., 2005a; 2005b; Kwitniewski *et al*., 2005; You *et al*., 2006; Misiewicz-Krzemińska *et al*., 2009). Usually, due to photosensitizers toxicity, their cytotoxicity in darkness must be examined.

In present work, the effect of PP(Arg)$_2$- and Hp(Arg)$_2$-based PDT on LNCaP cell line was studied. Cell viability under influence of photosensitizers, energy doses and PDT depending on time after irradiation, and following the change in transmembrane mitochondrial potential as an early stage of mitochondrial apoptosis were examined (Palmer *et al*. 2000; Misiewicz *et al*. 2003).

The cell viability assay was used first to evaluate the cytotoxicity of PP(Arg)$_2$ and Hp(Arg)$_2$ towards LNCaP and next to find maximal non-toxic concentrations which were applied in PDT (Ryter & Tyrrell, 1999; Kwitniewski *et al*., 2009). It was shown that photosensitizers interact with LNCaP cells in darkness even without irradiation (Kwitniewski *et al*., 2005; Kwitniewski *et al*. 2009). For each of investigated time and photosensitizers it was possible to determine a border, beyond which cell viability was going to decrease (Nowak-Stępniowska *et al*., 2011). Moreover, it was also noticed that small concentrations of studied photosensitizers cause stimulation of the cancer cell growth in a type of photosensitizers and time dependent manner (Graczyk, 1999; Ryter & Tyrrell, 1999; Kwitniewski *et al*., 2009). So the safe doses of photosensitizer used in PDT can not be any lower than the ones observed, due to stimulation cancer cell growth effect and because of the lower effectiveness of PDT (Nowak-Stępniowska *et al*., 2011).

Next MTT assay was performed to find the level of cell viability decrease under influence of PP(Arg)$_2$- and Hp(Arg)$_2$-mediated PDT for 24 h, 48 h and 120 h after cell irradiation. It was shown that cell viability was going to decrease with time for both studied photosensitizers (using of the maximal non-toxic doses firstly determined). Moreover, Hp(Arg)$_2$-based PDT effect caused greater cell viability descrease (especially noticed for 120 h after irradiation).

Cytotoxicity level of over 70% decide about necrotic cell death (Morgan & Oseroff, 2001). The MTT results show that the higher energy doses (80 and 120 J/cm^2) cause fast and high decrease of the cell viability just 24 h after irradiation (Noodt *et al*., 1996; Li *et al*., 1999; Otsuki, 2000; Shi, 2001; Plaetzer *et al*., 2003; Buytaert *et al*., 2007), which can indicate mainly necrotic cell death (Noodt *et al*., 1996; Dellinger, 1996; Ochsner, 1997; Oleinick *et al*., 1998; Graczyk, 1999; Oleinick *et al*., 2002; Castano *et al*., 2005a; 2005b).

As it has been previously mentioned, the most effective destruction of cancer cells occurs mainly via apoptosis mechanism (some part of necrosis is however necessary to strengthen the induction of inflammatory, and activation of immunological system) (Buja *et al*., 1993; Luo *et al*., 1996; McConkey, 1998; Plaetzer *et al*., 2003; Kessel, 2006). Apoptosis cell death as a result of PDT treatment depends on many factors like: intercellular localization of photosensitizer, type of cell line, type of photosensitizer and the energy dosage (Kessel *et al*., 1997; Kowaltowski & Castilho, 1997). The mitochondria are very important organelles for cell life, and the place where different apoptotic signals from inside and outside meet (Green & Reed, 1998). That is why photosensitizers, which locate *inter alia* in mitochondria, most effectively destroy cancer cells (Juzeniene & Moan, 2007a; 2007b). Photosensitizers, especially porphyrins, locate *among others* in mitochondria and its membranes, and dye *via* mitochondrial pathway of apoptosis (Hilf *et al*., 1984; Graczyk, 1999; Morgan, 2001; Plaetzer *et al*., 2003; You *et al*., 2006; Juzeniene & Moan, 2007a; 2007b). Apoptotic cell death can be induced under influence of low energy doses in PDT (Buja *et al*., 1993; Dellinger, 1996; Oleinick *et al*., 2002; Yow *et al*., 2006; Buytaert *et al*., 2007) through change of the mitochondrial membrane potential, which is an early stage of apoptosis (Palmer *et al*., 2000; Misiewicz *et al*., 2003).

Moreover, it was shown that photosensitizers accumulate in different quantities in the cells, so the photodynamic effect can be different for them. Taking into consideration this aspect of therapy, it is better to induce mainly apoptotic cell death due to the apoptotic cascade (Graczyk, 1999; Oleinick *et al*., 2002; Yow *et al*., 2006).

In present work, the change of transmembrane potential was shown to be depended on the type of photosensitizer, energy dose and time after irradiation (24 and 48h) which were studied in details. Experiments were carried out in different time after irradiation due to the cascade apoptosis connected with interactions between damaged and not-damaged cells (Graczyk, 1999; Oleinick *et al*., 2002; Yow *et al*., 2006).

Determination of the mitochondrial membrane depolarization level was performed by confocal microscopy (Shotton, 1989; Reers *et al*., 1991; Smiley *et al*., 1991; Cavanagh, 1993; Lu, 1996; Paddock, 2000; Misiewicz *et al*., 2003; Misiewicz *et al*., 2004). A mitochondrial indicator, which interacts in a different way with the mitochondria of living and apoptotic cell was used for the experiment (Reers *et al*., 1991; Smiley *et al*., 1991; Otsuki, 2000; Shi, 2001; Misiewicz, 2003).

As it was mentioned before, the lower energy doses (5, 10, 30 and 50 J/cm^2) were used in PDT because of their ability to cause higher than expected cell viability decrease level 24 h after irradiation.

The confocal microscopy studies revealed that control cells polarized mitochondrial membrane and dye was forming aggregates giving intensive red fluorescence. PP(Arg)$_2$- and Hp(Arg)$_2$-based PDT depending on the energy dose and time, cause depolarization of mitochondrial membrane. In the case of both photosensitizers, generally, lower energy doses resluts with higher level of green fluorescence intensity. Moreover, it was also shown that the choice of appropiate energy dose is important, because not every low energy dose can effectively induce change in mitochondrial potential and begin apoptosis. Generally, Hp(Arg)$_2$-based PDT was more effective, causing mostly greater mitochondrial membrane depolarization and decrease of the cell viabillity in comparison to PP(Arg)$_2$ one.

Taking into consideration the resluts of MTT assay and the changes of transmembrane mitochondrial potential of the PP(Arg)$_2$- and Hp(Arg)$_2$-mediated PDT we can initially describe the energy dose range which can

probably induce mitochondrial apoptosis cell death, this however, still requires further examination.

To sum up, the transmembrane mitochondrial potential plays a key role as an early stage of apoptosis. The lower energy doses (10–30 J/cm^2) and maximal non-toxic photosensitizer doses of $Hp(Arg)_2$ and $PP(Arg)_2$ applied in PDT are likely to induce apoptosis in LNCaP cell line. Higher energy and photosensitizer ($Hp(Arg)_2$ and $PP(Arg)_2$) doses used in PDT result with a high cell viability decrease. The influence of PDT using other porphyrins and their derivatives on prostate and breast carcinoma will be a purpose of the further work.

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