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Cytotoxicity of $PP(Arg)_2$- and $PP(Ala)_2(Arg)_2$-based photodynamic therapy and early stage of apoptosis induction in human breast cancers *in vitro*

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Mitochondria are cell energetic centers where ATP is produced. They also play a very important role in the PDT as intracellular sites of photosensitizer localization. Photosensitizers gathering in mitochondria (like porphyrin derivatives used in this work) are more effective in tumor cell destruction. Moreover, it was assumed that di-amino acid substituents attached to porphyrin ring will strengthen the effectivity of interaction with membrane receptors of examined cells. MTT assay was performed to investigate the influence of $PP(Arg)_2$ and $PP(Ala)_2(Arg)_2$-based PDT on breast cancer cell viability for 24 h, 48 h and 120 h after cell irradiation. Then the influence of $PP(Ala)_2(Arg)_2$- and $PP(Arg)_2$-mediated PDT on early mitochondrial apoptosis induction via measurements of the transmembrane mitochondrial potential changes was examined. Results showed that lower energy doses and maximal nontoxic photosensitizer doses of $PP(Ala)_2(Arg)_2$ and $PP(Arg)_2$ applied in PDT can imply apoptotic cell death. It was confirmed that modification of the protoporphyrin IX by attaching two alanine substituents raised the efficiency of photodynamic therapy.

Key words: photodynamic therapy, transmembrane mitochondrial potential, breast cancer

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INTRODUCTION

Photodynamic therapy is a cancer destruction method (Henderson & Dougherty, 1992; Kessel & Dougherty, 1999; Kessel, 2004; Raymond, 1999; Schmuitmaker, 1996; Sharman *et al*., 1999). The major advantages of PDT are: selectivity, low invasiveness and small side effects important for improving the patients' quality of life. PDT is a photochemical reaction which requires the simultaneous presence of: photosensitizer, light energy source (emission band of light source complementary with absorption band of photosensitizer), and oxygen concentration (as high as possible) (Brown & Brown, 2004; Castano *et al*., 2005a;2005b; Graczyk, 1999; Kubler, 2005; Mchsner, 1997; Oleinick & Evans, 1998).

Porphyrin based photosensitizers and its derivatives are commonly used in PDT (Gibson & Hilf, 1985; Graczyk, 1999; Sternberg & Dolphin, 1998; You *et al*., 2006). Porphyrin photosensitizers such as protoporphyrin IX containing a heterocyclic ring are very abundant due to the ease of structure modification like the photosensitizers applied in this work: $PP(Arg)_2$ and $PP(Ala)_2(Arg)_2$. It was assumed that di-amino acid substituents attached to the porphyrin ring will raise the effectivity of interaction with membrane receptors of studied cells. A new compound group, diamino acid derivatives of protoporphyrin IX were synthesized. This should make cell penetration by these compounds easer and cause more effective cancer cell destruction (Graczyk & Konarski, 1995; Graczyk & Konarski, 1997; Graczyk, 1999). Two arginine substituents should also improve the solubility of these compounds in water (Graczyk, 1999). That is why in this work diarginine derivatives of the examined photosensitizers were used.

All over the world breast cancer is still the most often malignant neoplasm diagnosed in women. Differences can be a result of genetic factors and the synergism of many environmental factors also connected with lifestyle (Bartsch *et al*., 2007; Bissonauth *et al*., 2008; Palermo *et al*., 2007; Suresh, 2007). Half of women with inherited breast cancer have a mutation in the *BRCA1* gene (on chromosome 17) and 1/3 in the *BRCA2* gene (on chromosome 13). These genes play important roles in the mechanisms of DNA repair. It was shown that benign breast diseases like proliferational lesions, papillomatosis of the mammary duct or adenomatous can increase risk for breast cancer (Broeks *et al*., 2007; Loizidou *et al*., 2007).

In these studies the two breast cancer cell lines were chosen: the standard line MCF-7 and more resistant to standard treatment MDA-MB231.

The purpose of this work was to describe the influence of $PP(Arg)_2$- and $PP(Ala)_2(Arg)_2$-based PDT on induction of the early stage of mitochondrial apoptosis — changes in the transmembrane mitochondrial potential. The $PP(Arg)_2$- and $PP(Ala)_2(Arg)_2$-mediated PDT cytotoxicity towards MCF-7 and MDA-MB231 breast cancers were described by cell viability measurements (MTT). The assumption about better properties of the new dialanine derivatives of protoporphyrin IX was also

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Abbreviations: ANOVA, one way analysis of variance; FBS, foetal bovine serum; LSCM, laser scanning confocal microscope; MANOVA, multi way analysis of variance; MCF-7, human breast cancer cell line responsive to androgen therapy; MDA-MB231, human breast cancer cell line non-responsive to androgen therapy; MitoLightTM, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine; MTT, 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide; PBS, phosphate-buffered saline; PDT, photodynamic therapy; PP(Arg)2, diarginine protoporphyrin IX; PP(Ala)2(Arg)2, diarginine dialanine protoporphyrin IX.

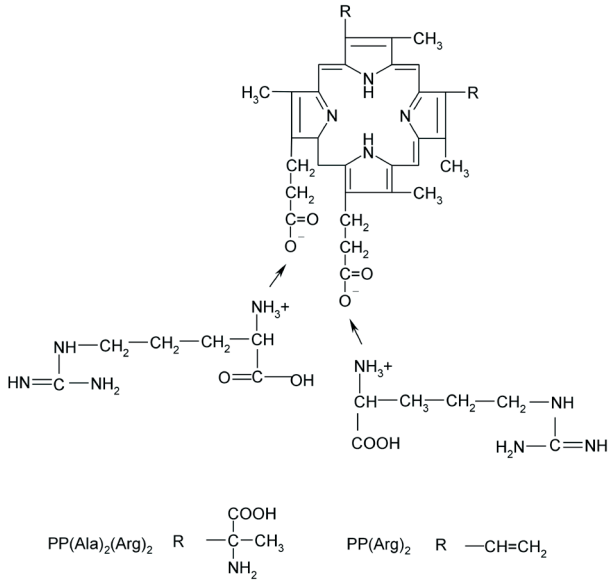

Figure 1. Structures of diarginine ($PP(Arg)_2$) and dialanine ($PP(Ala)_2(Arg)_2$) derivatives of protoporphyrin IX.

checked. All performed experiments should help to optimize the conditions for breast cancer destruction, mostly *via* the mitochondrial apoptosis mechanism.

MATERIALS AND METHODS

Chemicals. Diarginine protoporphyrin IX and dialanine derivatives of PPIX were synthesized at The Biochemistry and Spectroscopy Laboratory, at The Institute of Optoelectronics, Military University of Technology (Graczyk & Konarski, 1995; Graczyk & Konarski, 1997; Graczyk, 1999) (Fig. 1). Two arginine substituents in PPIX and $PP(Ala)_2$ ensure solubility of these compounds in water which implies improvement of the photosensitizer solubility (Graczyk, 1999).

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

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

Cell culture. MCF-7 (Human breast cancer responsive to androgen therapy) was received from Ludwik Hirszfeld Institute of Immunology and Experimental Therapy of the Polish academy of Sciences (Wroclaw, Poland). MDA-MB231 cancer cell line (Human breast cancer non-responsive to androgen therapy) came from ATTC (American Type Culture Collection). Cancer cells were grown in complete medium: 100 ml medium supplemented with 10% foetal bovine serum (FBS) and antibiotic solution 1:100, L-glutamine 1:100 and amino acids 1:100 in the case of MDA-MB231, 1 ml of antibiotic solution containing penicillin (10000 U/ml) and streptomycin (4 mg/ml) for MCF-7. Cells were maintained in humidified air containing 5% CO_2 at 37°C, in sterile Nunc cell culture dishes (Kamstrupvej, Denmark).

Complete medium was changed three times a week and cells were washed with PBS. 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 was used to determine cell viability (Nowak-Stępniowska *et al.*, 2011; Plumb, 2003). 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 a purple formazan, insoluble in water. The reduction of MTT to formazan is performed by mitochondrial dehydrogenase which exhibits activity only in viable cells. On the basis of cell ability to reduce MTT, cell viability is determined. Formazan crystals were dissolved in isopropyl alcohol and the concentration of the 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.

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 3h at 37°C in a 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: photosensitizer alone, light alone and PDT) to the control one (cells without photosensitizer, light and PDT) was given in percentage.

Evaluation of the MCF-7 and MDA-MB231 cell viability under the influence of $PP(Arg)_2$ and $PP(Ala)_2(Arg)_2$ in darkness. Human breast cancer cells were seeded into Nunc sterile plates (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 ($PP(Arg)_2$ and $PP(Ala)_2(Arg)_2$) 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 remove the photosensitizer. After time periods: 24 + 120 h, cancer cell viability was analyzed by MTT (24h is the time when cancer cells were being irradiated during PDT experiments, but in analogous experiments without irradiation the cells were stored in the incubator).

Evaluation of the MCF-7 and MDA-MB231 cells 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 a lamp manufactured in Military University of Technology (Warsaw, Poland) as the light source and the light intensity was set to 140 mW. After 120 h cancer cell viability was described by MTT. Applied wave lengths were within the range of the Soret band, and the depth

of the Soret band penetration was sufficient for cell line studies.

Evaluation of the MCF-7 and MDA-MB231 cell viability under the influence of the PDT effect. Cells were seeded according to the procedure described above. The maximal non-toxic photosensitizer doses were applied to the examined cells 6 hours after seeding (for $PP(Arg)_2$ and $PP(Ala)_2(Arg)_2$ with the dose of: 40 and 130 μg/ml for MCF-7 and 50 and 130 μg/ml for MDA-MB231, 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. 120h after irradiation, further actions were performed in accordance with the procedure described above.

Statistical analysis. MTT assay experiments were carried out in 6 parallel samples 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, time after irradiation and type of cancer cell line) and their interactions with viability of the investigated cancer cells. To estimate the significant differences among the parameters, the Tukey post-hoc test was performed, in which the significance level was set at $P<0.05$. In the case of photosensitizer concentration studies, the one-way analysis of ANOVA, followed by the Dunnett post-hoc test was performed. The significance level was set at $*P<0.05$ as a 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 Laser Scanning Confocal Microscope. A mitochondrial indicator, MitoLight™ (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine chloride) was used to evaluate the changes in transmembrane mitochondrial potential in MCF-7 and MDA-MB231 under influence of different factors. In healthy cells (polarized mitochondria) the dye accumulates and forms J-aggregates giving red fluorescence. In apoptotic cells (depolarized mitochondria) monomeric dye gives green fluorescence (the dye stays in the cytoplasm) (Misiewicz *et al.*, 2003; Misiewicz *et al.*, 2004; Misiewicz-Krzemińska *et al.*, 2009; Reers *et al.*, 1991; Smiley *et al.*, 1991).

Cells of the investigated line were seeded to 8-well microplates at a density of 1.5×10^4 cells/ml. PDT experiments were carried out with cancer cells where concentrations of $PP(Arg)_2$ and $PP(Ala)_2(Arg)_2$ determined in MTT assay were 40 and 130 μg/ml for MCF-7 and 50 and 130 μg/ml for MDA-MB231, respectively; the energy doses of 10 and 30 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 MitoLight solution (1 μl/ml dye) was added to each well. Incubation time was 15 min at 37°C in a humidified atmosphere containing 5% CO_2. the next, dye solution was removed and 20 μl of PBS was added. The changes of mitochondrial transmembrane potential of the studied cancer cell line under the 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, a blue dye laser was used (Argon laser 488 nm). To observe monomeric fluorescence (green fluorescence) a 505–525 nm filter was used. Meanwhile, red fluorescence from aggregates was collected through a BP560 filter.

RESULTS

The effect of energy dose on cell viability depending on the type of cancer cell line was examined first. The influence of energy doses (5, 10, 30, 50, 80 and 120 J/cm^2) on cancer cell viability MCF-7 and MDA-MB231 120h after irradiation is shown in Table 1. Statistical analysis shows that neither energy dose nor type of cell line are statistically significant factors.

The influence of $PP(Arg)_2$ and $PP(Ala)_2(Arg)_2$ concentration without irradiation on viability of MCF-7 and MDA-MB231 cancer cells in darkness for 120h (this time correlates with 120h after irradiation in PDT experiments) is shown in Table 2. Statistical analysis showed that photosensitizer concentration, type of photosensitizer, type of cell line and their interactions are significant factors. The results indicate that the higher the photosensitizer concentration the lower the viability of MCF-7 and MDA-MB231 cells (significant differences). Moreover, mean viability of examined cell lines was lower under influence of $PP(Arg)_2$ and higher in the case of $PP(Ala)_2(Arg)_2$. Post-hoc analysis gives significant differences between the results for $PP(Arg)_2$ and $PP(Ala)_2(Arg)_2$. $PP(Arg)_2$ caused greater cell viability decrease on MDA-MB231 than on MCF-7 line, however, $PP(Ala)_2(Arg)_2$ caused a greater one on MCF-7 than on MDA-MB231. Statistical analysis revealed significant differences between results of: $PP(Arg)_2$ on MDA-MB231 and: $PP(Ala)_2(Arg)_2$ on MDA-MB231, $PP(Ala)_2(Arg)_2$ on MCF-7, $PP(Arg)_2$ on MCF-7 and of: $PP(Ala)_2(Arg)_2$ on MDA-MB231 and $PP(Arg)_2$ on MCF-7.

Additionally it was shown that mean viability under the influence of the examined photosensitizers was lower in almost the whole concentration range in the case of MDA-MB231 (significant differences between results for MCF-7 and MDA-MB231).

Experiments show that photosensitizers without irradiation interact with studied cancer cells in darkness. It was also noticed that small concentrations of photosensitizers stimulate growth of cancer cells in comparison to controls (Nowak-Stępniowska *et al.*, 2011). Moreover for each of the examined photosensitizers ($PP(Arg)_2$, $PP(Ala)_2(Arg)_2$) it was possible to determine significant concentration border beyond which cell viability decreased. On the basis of results significant maximal non-toxic concentrations of $PP(Arg)_2$ and $PP(Ala)_2(Arg)_2$ were determined and are presented in Table 3 (maximal

Table 1. The influence of energy dose on cell viability rate depending on the cell line 120 hours after irradiation. Values are means ± S.D. (standard deviation).

	MCF-7	MDA-MB231
Energy dose [J/cm^2]	Cell viability rate [%]	Cell viability rate [%]
5	103±6	103±7
10	102±6	103±9
30	102±5	101±7
50	100±4	100±10
80	99±6	98±10
120	97±6	98±6

*significant differences in comparison to the control were set at $P<0.05$

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

MCF-7			MDA-MB231	
Concentration of photosensitizer [mg/ml]	$PP(Arg)_2$ Cell viability rate [%]	$PP(Ala)_2(Arg)_2$ Cell viability rate [%]	$PP(Arg)_2$ Cell viability rate [%]	$PP(Ala)_2(Arg)_2$ Cell viability rate [%]
10	104±8	108±10*	110±9	103±6
20	102±6	108±8*	104±7	104±9
30	100±5	102±5	101±6	102±7
40	100±9	102±6	101±4	100±10
50	94±8*	100±4	100±5	100±10
60	91±12*	102±8	91±10*	99±10
70	92±10*	102±2	88±9*	99±8
80	89±9*	100±2	84±10*	99±8
90	84±10*	100±4	80±13*	99±8
100	83±11*	100±4	74±9*	99±10
110	±83±8*	100±4	67±8*	100±9
130	79±8*	100±3	60±12*	100±11
150	74±*	89±11*	56±7*	80±8*
200	65±9*	85±9*	52±9*	73±10*
400	58±10*	52±8*	45±6*	70±8*
800	52±11*	33±7*	38±3*	52±8*
1600	40±13*	12±2*	35±5*	36±8*

*$P < 0.05$ significant differences in comparison to control.

non-toxic concentration in comparison to the control — Dunett post-hoc test).

Taking into consideration the determined maximal non-toxic concentrations of photosensitizers, both breast cancer cell lines MCF-7 and MDA-MB231 were found to be more sensitive to $PP(Arg)_2$ than $PP(Ala)_2(Arg)_2$.

In Figures 2–5 the influence of photodynamic reaction using $PP(Arg)_2$ and $PP(Ala)_2(Arg)_2$ on viability of MCF-7 and MDA-MB231 cells is shown depending on time after irradiation. PDT reaction was carried out in conditions described by MTT: wide range non-toxic energy dose (Table 1) and maximal non-toxic concentrations of examined photosensitizers (Tables 2 and 3) were used. Statistical analysis showed that energy dose, type of photosensitizer, time after irradiation and their interactions are significant factors.

On the plots it can be seen that the higher the energy dose the lower the significant cell viability of MCF-7 and

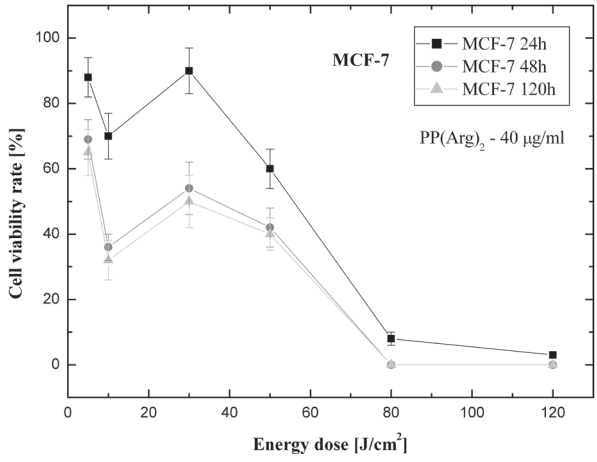

Figure 2. $PP(Arg)_2$-mediated PDT effect on MCF-7 cell viability rate: (■) 24 h, (●) 48 h and (▲) 120 h after irradiation.
The maximal non-toxic concentrations were applied. Cells were incubated with photosensitizer for 24 h in darkness (Materials and Methods). The energy dose control probes were shown in Table 1. The $PP(Arg)_2$ control probes were shown in Table 2. Values are means ±S.D. (standard deviation).

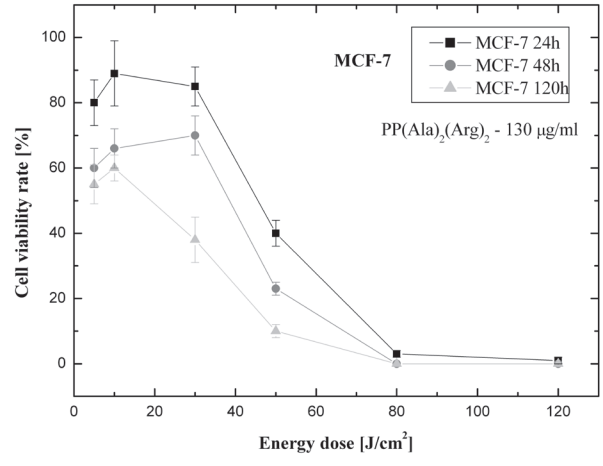

Figure 3. $PP(Ala)_2(Arg)_2$-mediated PDT effect on MCF-7 cell viability rate: (■) 24 h, (●) 48 h and (▲) 120 h after irradiation.
The maximal non-toxic concentrations were applied. Cells were incubated with photosensitizer for 24 h in darkness (Materials and Methods). The energy dose control probes were shown in Table 1. The $PP(Ala)_2(Arg)_2$ control probes were shown in Table 2. Values are means ±S.D. (standard deviation).

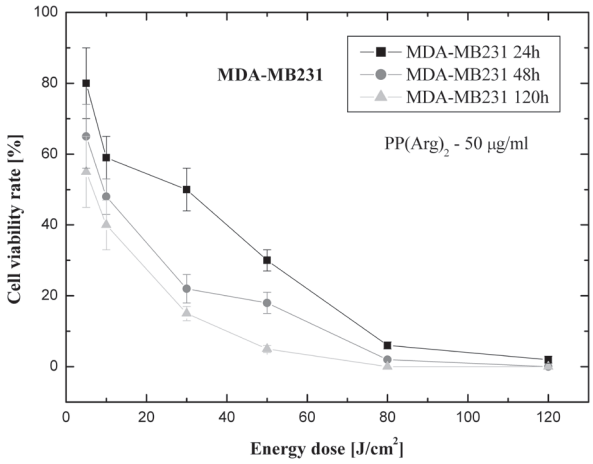

Figure 4. PP(Arg)$_2$-mediated PDT effect on MDA-MB231 cell viability rate: (■) 24 h, (●) 48 h and (▲) 120 h after irradiation.
The maximal non-toxic concentrations were applied. Cells were incubated with photosensitizer for 24h in darkness (Materials and Methods). The energy dose control probes were shown in Table 1. The PP(Arg)$_2$ control probes were shown in Table 2. Values are means ±S.D. (standard deviation).

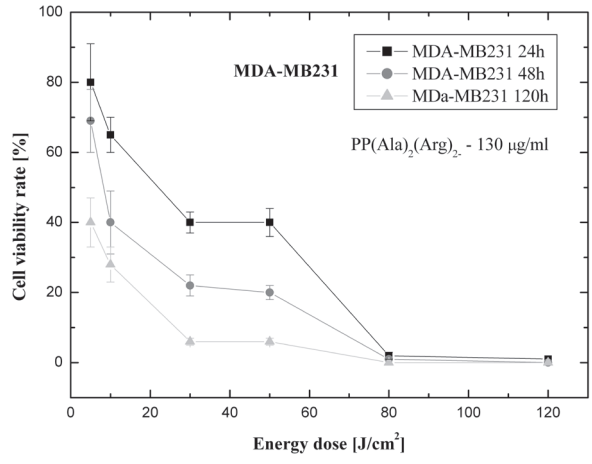

Figure 5. PP(Ala)$_2$(Arg)$_2$-mediated PDT effect on MDA-MB231 cell viability rate: (■) 24 h, (●) 48 h and (▲) 120 h after irradiation.
The maximal non-toxic concentrations were applied. Cells were incubated with photosensitizer for 24h in darkness (Materials and Methods). The energy dose control probes were shown in Table 1. The PP(Ala)$_2$(Arg)$_2$ control probes were shown in Table 2. Values are means ±S.D. (standard deviation).

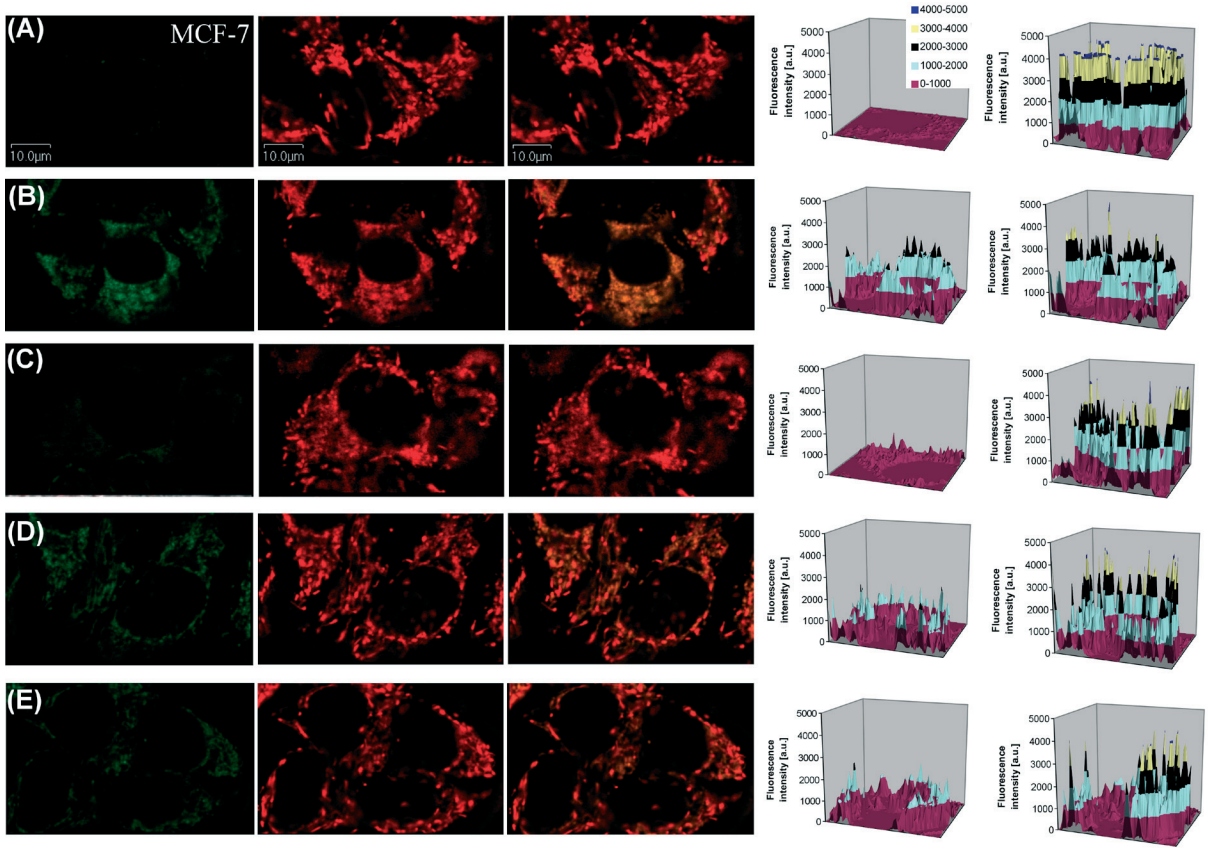

Figure 6. The changes of transmembrane mitochondrial potential in MCF-7 cell line under the influence of PP(Arg)$_2$- and PP(Ala)$_2$(Arg)$_2$-mediated PDT 24 hours after irradiation.
Cells were stained with apoptosis detection kit MitoLight™. On the left side there are images (magnification x 1200) of the following: green, red fluorescence and fluorescence of both signals put together. Green fluorescence represents dye monomers in apoptotic cells with a depolarized mitochondrial membrane, the red one — dye aggregates bound to the 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 also represents green and red fluorescence level of other control probes (cells without photosensitizer and not irradiated, cells under influence of PP(Arg)$_2$ and PP(Ala)$_2$(Arg)$_2$, cells only irradiated with: 10 and 30 J/cm^2). **(B)** PP(Arg)$_2$ (40 μg/ml)-mediated PDT 24 hours after irradiation of 10 J/cm^2. **(C)** PP(Arg)$_2$ (40 μg/ml)-mediated PDT 24 hours after irradiation of 30 J/cm^2. **(D)** PP(Ala)$_2$(Arg)$_2$ (130 μg/ml)-mediated PDT 24 hours after irradiation of 10 J/cm^2. **(E)** PP(Ala)$_2$(Arg)$_2$ (130 μg/ml)-mediated PDT 24 hours after irradiation of 30 J/cm^2.

Table 3. Maximal significant non-toxic concentrations of investigated photosensitizers depending on the cell line for 120 h.

Cell line/photosensitizer	MCF-7	MDA-MB231
$PP(Arg)_2$ [mg/ml]	40	50
$PP(Ala)_2(Arg)_2$ [mg/ml]	130	130

MDA-MB231 after PDT using photosensitizers and in the case of each examined time after irradiation (significant differences). It was noticed that mean cell viability of both lines was lower in the case of $PP(Ala)_2(Arg)_2$-based PDT than $PP(Arg)_2$-mediated PDT. Differences between results for $PP(Arg)_2$- and $PP(Ala)_2(Arg)_2$-based PDT were statistically significant (post-hoc analysis).

Moreover, mean viability of MDA-MB231 after PDT for 24 h, 48 h and 120 h after irradiation was lower than for MCF-7. Significant differences were observed among results of 24 h, 48 h and 120 h for both PDT on MCF-7 and on MDA-MB231. Differences between interactions results of MCF-7 and MDA-MB231 cells were found to be significant in the cases: PDT for 24 h on MDA-MB231 and PDT for 24 h,48 h on MCF-7; PDT for 48 h on MDA-MB231 and PDT for 24h,120h on MCF-7; PDT for 120 h on MDA-MB231 and PDT for 24 h,48 h,120 h on MCF-7 (post-hoc analysis).

Taking into consideration the determined maximal non-toxic concentrations of examined photosensitizers on both cell lines, $PP(Ala)_2(Arg)_2$ exhibits the lowest toxicity in darkness and $PP(Arg)_2$ the highest one. Statistical analysis exhibits significant differences among results of: $PP(Arg)_2$-PDT on MDA-MB231 and $PP(Arg)_2$-PDT on MCF-7, $PP(Ala)_2(Arg)_2$-PDT on MCF-7, $PP(Ala)_2(Arg)_2$-PDT on MDA-MB231 and of: $PP(Ala)_2(Arg)_2$-PDT on MDA-MB231 and $PP(Arg)_2$-PDT on MCF-7, $PP(Ala)_2(Arg)_2$-PDT on MCF-7 (post-hoc analysis).

To sum up, photodynamic effect using $PP(Ala)_2(Arg)_2$ in examined conditions was more effective in decreasing cell viability of MCF-7 and MDA-MB231 in comparison to $PP(Arg)_2$-mediated PDT.

Significant cell viability decrease under influence of PDT using 80 and 120 J/cm² and examined photosensitizers especially on MDA-MB231 line suggest necrotic cell death. However, the lowest used energy dose of 5 J/cm² does not give satisfying results what MTT results also showed (Nowak-Stępniowska *et al.*, 2011). Taking into consideration these causes, the changes of transmembrane mitochondrial potential were carried out only for chosen, lower energy doses.

In Figures 6–9 there were shown images of transmembrane mitochondrial changes of MCF-7 and MDA-MB231 cells under influence of PDT evaluated with laser scanning confocal microscope (on the left). There are attached plots of green and red fluorescence intensity of the representative cell to the images (on the right side). Green fluorescence represents mitochondrial membrane depolarization (monomeric dye).

Transmembrane mitochondrial potential changes under influence of photodynamic effect using $PP(Arg)_2$ (Fig. 6b and c), $PP(Ala)_2(Arg)_2$ (Fig. 6d and e) on MCF-7 cell line 24 h after irradiation was shown in Fig. 6. Figure 6a presents results of control probe. Control cells have polarized membrane and dye aggregate giving dominant red fluorescence and trace green one. In experiments, the changes of the red fluorescence intensity in comparison to control probes were not observed (Fig. 6b–e). Thus, green fluorescence intensity decides about changes of transmembrane mitochondrial potential. $PP(Ala)_2(Arg)_2$-based PDT 24 h after irradiation give greater green fluorescence (Fig. 6b–e) than $PP(Arg)_2$ on MCF-7 cell line. In case of $PP(Arg)_2$- and $PP(Ala)_2(Arg)_2$-mediated PDT green fluorescence intensity was greater using energy dose of 10 J/cm² than 30 J/cm². 48 hours after irradiation (Fig. 7b and c) green

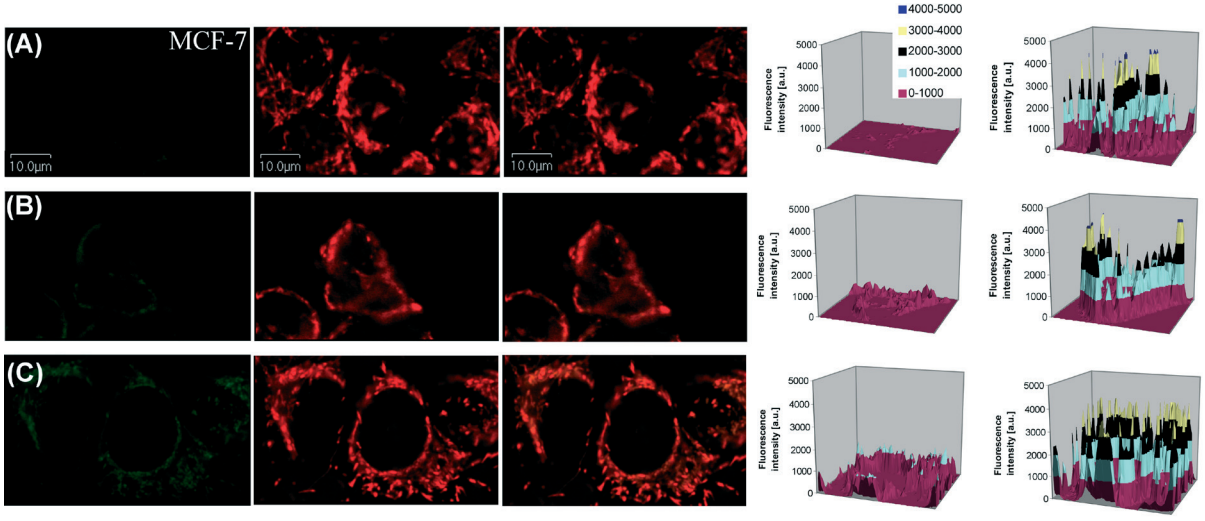

Figure 7. The changes of transmembrane mitochondrial potential in MCF-7 cell line under the influence of $PP(Arg)_2$- and $PP(Ala)_2(Arg)_2$-mediated PDT 48 hours after irradiation.
Cells were stained with apoptosis detection kit MitoLight™. On the left side there are images (magnification x 1200) of the following: green, red fluorescence and fluorescence of both signals put together. Green fluorescence represents dye monomers in apoptotic cells with a depolarized mitochondrial membrane, the red one – dye aggregates bound to the 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 also represents green and red fluorescence level of other control probes (cells without photosensitizer and not irradiated, cells under influence of $PP(Arg)_2$ and $PP(Ala)_2(Arg)_2$, cells only irradiated with: 30 J/cm²). **(B)** $PP(Arg)_2$ (40 µg/ml)-mediated PDT 48 hours after irradiation of 30 J/cm². **(C)** $PP(Ala)_2(Arg)_2$ (130 µg/ml)-mediated PDT 48 hours after irradiation of 30 J/cm².

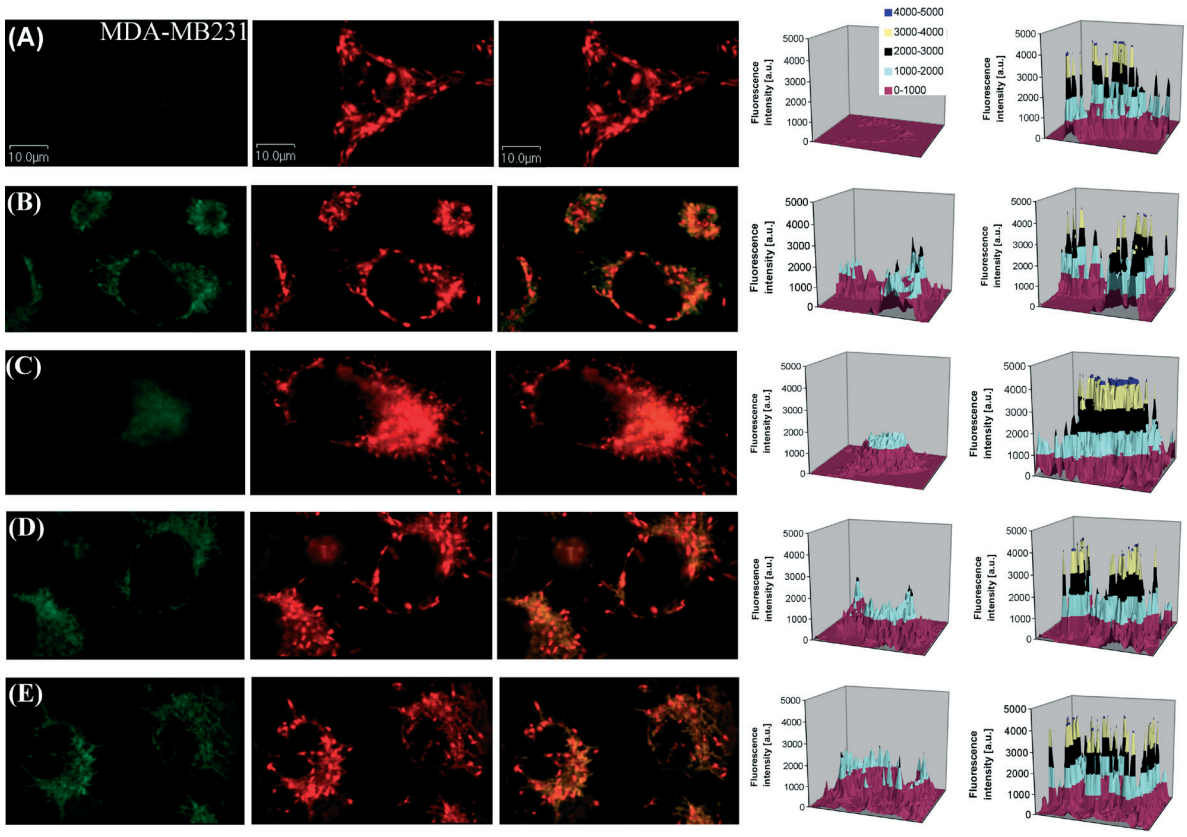

Figure 8. The changes of transmembrane mitochondrial potential in MDA-MB231 cell line under the influence of PP(Arg)$_2$- and PP(Ala)$_2$(Arg)$_2$-mediated PDT 24 hours after irradiation.
Cells were stained with apoptosis detection kit MitoLight™. On the left side there are images (magnification x 1200) of the following: green, red fluorescence and fluorescence of both signals put together. Green fluorescence represents dye monomers in apoptotic cells with a depolarized mitochondrial membrane, the red one – dye aggregates bound to the 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 also represents green and red fluorescence level of other control probes (cells without photosensitizer and not irradiated, cells under influence of PP(Arg)$_2$ and PP(Ala)$_2$(Arg)$_2$, cells only irradiated with: 10 and 30 J/cm^2). **(B)** PP(Arg)$_2$ (50 µg/ml)-mediated PDT 24 hours after irradiation of 10 J/cm^2. **(C)** PP(Arg)$_2$ (50 µg/ml)-mediated PDT 24 hours after irradiation of 30 J/cm^2. **(D)** PP(Ala)$_2$(Arg)$_2$ (130 µg/ml)-mediated PDT 24 hours after irradiation of 10 J/cm^2. **(E)** PP(Ala)$_2$(Arg)$_2$ (130 µg/ml)-mediated PDT 24 hours after irradiation of 30 J/cm^2.

fluorescence intensity was lower in case of PP(Arg)$_2$- and PP(Ala)$_2$(Arg)$_2$-based PDT 24 hours after irradiation and for PP(Ala)$_2$(Arg)$_2$-based PDT greater one than for PP(Arg)$_2$-mediated PDT. It shows that membrane depolarization was proceeding.

Generally, PP(Ala)$_2$(Arg)$_2$-based PDT 24 hours after irradiation give greater green fluorescence (Fig. 8b–e) on MDA-MB231 line than PP(Arg)$_2$-based PDT. For PP(Ala)$_2$(Arg)$_2$-mediated PDT green fluorescence intensity is equal for 10 and 30 J/cm^2 and in the case of PP(Arg)$_2$-based PDT one is greater for 10 J/cm^2 than 30 J/cm^2.

48 hours after irradiation green fluorescence intensity in the case of PP(Arg)$_2$-based PDT is lower than 24 h after irradiation and for PP(Ala)$_2$(Arg)$_2$-mediated PDT greater than 24 h after irradiation (Fig. 9b and c). The greatest apoptosis induction was observed for the greatest change of transmembrane mitochondrial potential occurring 24 h after irradiation. Additionally, it was noticed that 48 hours after irradiation cells of the MDA-MB231 line have just been more damaged under influence of lower energy doses in comparison the MCF-7 cells, according to the MTT results — lower cell viability in the case of MDA-MB231 line.

DISCUSSION

It is known that not only high cytotoxicity is important in PDT, but also mechanism of cell death. Large protein mass from destroyed necrotic cancer cells cause fast and irreversible blocking of renal tubules implying life threatening side effects (Graczyk, 1999). However, an apoptosis allows to destroy cancer cells causing small inflammatory under influence of PDT (Buytaert *et al.*, 2007; Oleinick *et al.*, 2002; Robertson *et al.*, 2009). That is why, it is essential to destroy cancer cells mainly via apoptotic mechanisms with some part of necrosis to strengthen the inflammatory induction (Oleinick & Evans, 1998; Plaetzer *et al.*, 2003). Furthermore, porphyrin photosensitizers due to mitochondrial localization can successfully destroy cancer cells *via* apoptosis (Agonis *et al.*, 2011; Kessel *et al.*, 1997; Kessel & Luo, 1998; Kessel & Luo, 1999; Moan *et al.*, 1989; Morgan & Oseroff, 2001; Oleinick & Evans, 1998; You *et al.*, 2006).

In vitro experiments show interactions between live cell and damaged one due to PDT. In cascade reaction damaged cell can induce apoptosis in undamaged cells. Defining the border to which this reaction is beneficial (border between damaging diseased cell and healthy one)

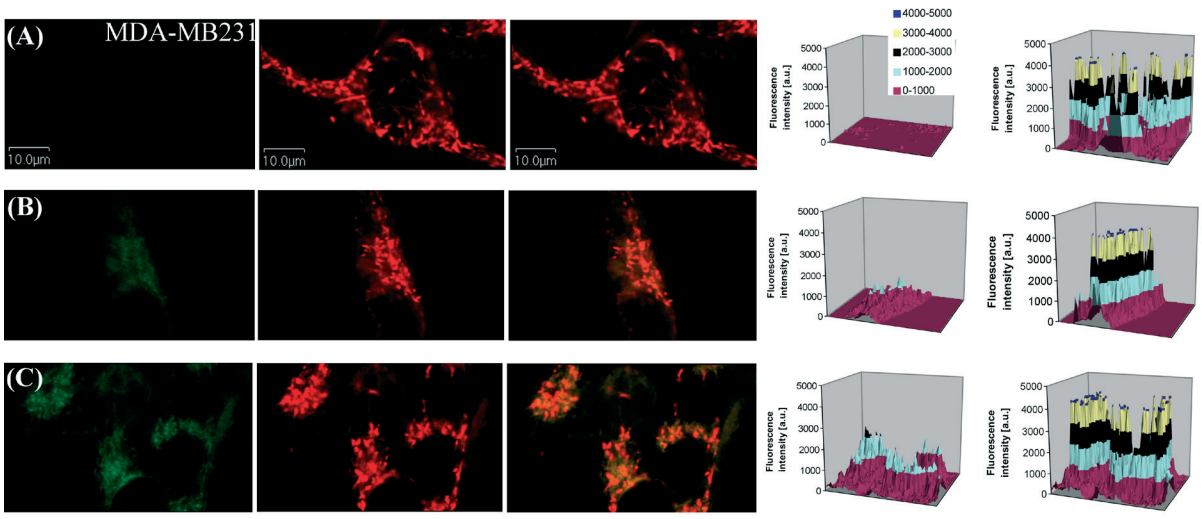

Figure 9. The changes of transmembrane mitochondrial potential in MDA-MB231 cell line under the influence of $PP(Arg)_2$- and $PP(Ala)_2(Arg)_2$-mediated PDT 48 hours after irradiation.
Cells were stained with apoptosis detection kit MitoLight™. On the left side there are images (magnification x 1200) of the following: green, red fluorescence and fluorescence of both signals put together. Green fluorescence represents dye monomers in apoptotic cells with a depolarized mitochondrial membrane, the red one – dye aggregates bound to the 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 also represents green and red fluorescence level of other control probes (cells without photosensitizer and not irradiated, cells under influence of $PP(Arg)_2$ and $PP(Ala)_2(Arg)_2$, cells only irradiated with: 10 J/cm²). **(B)** $PP(Arg)_2$ (50 µg/ml)-mediated PDT 48 hours after irradiation of 10 J/cm². **(C)** $PP(Ala)_2(Arg)_2$ (130 µg/ml)-mediated PDT 48 hours after irradiation of 10 J/cm².

is important task for scientists (Graczyk, 1999; Ochsner, 1997; Oleinick *et al*., 2002).

For that reason there is a need to optimize the physicochemical parameters of PDT for different types of cancers to ensure successful PDT.

In this work the photodynamic effect using $PP(Arg)_2$ and $PP(Ala)_2(Arg)_2$ towards breast cancer cell lines MCF-7 and MDA-MB231 was examined. A representative compound of new generation $PP(Ala)_2(Arg)_2$ — dialanine derivative of protoporphyrin IX was investigated as a potentially more effective drug in comparison to the PPIX to PDT (Graczyk & Konarski, 1995, Graczyk & Konarski, 1997; Graczyk, 1999). The cell viability under influence of PDT depending on time after irradiation (controls: non-toxic energy doses and photosensitizer concentrations) and then cell death mechanism by transmembrane mitochondrial potential — early stage of apoptosis were studied (Misiewicz *et al*., 2003; Misiewicz *et al*., 2004; Misiewicz-Krzemińska *et al.,* 2009).

MTT test was used to evaluate the energy dose and photosensitizer concentrations: $PP(Arg)_2$ and $PP(Ala)_2(Arg)_2$ cytotoxicity on breast cancer cells. Then maximal non-toxic concentrations of examined photosensitizers in PDT experiments were investigated. Breast cancer cell growth in comparison to the control depending on type of photosensitizer and cell line was observed (Nowak-Stępniowsk *et al*., 2011).

Results showed that modified derivative of PPIX — $PP(Ala)_2(Arg)_2$ was more effective than $PP(Arg)_2$ on examined cancer lines. Generally, the cells of MDA-MB231 line were more sensitive to the PDT treatment than the MCF-7 ones. Lower energy dose used in PDT can induce apoptotic cell death is shown in the literature (Oleinick *et al.,* 2002). The high energy doses used induce mainly necrotic mechanisms what is not expected as a result of PDT because of acute inflammatory reaction and blocking of renal tubules in the case of application to humans (Graczyk, 1999; Nowak-Stępniowska *et al*., 2011).

The changes of transmembrane mitochondrial potential depending on type of photosensitizer, energy dose and cancer cell line were examined. The biggest changes of transmembrane mitochondrial potential under influence of $PP(Arg)_2$- and $PP(Ala)_2(Arg)_2$-mediated PDT were observed 24 h and 48 h after irradiation on studied cell lines.

It was generally shown that the photodynamic effect using $PP(Ala)_2(Arg)_2$ was more successful in comparison to the $PP(Arg)_2$ due to generally greater cancer cell viability decrease and also greater transmembrane mitochondrial potential depolarization — an early stage of apoptosis.

To sum up, the photodynamic therapy using an dialanine derivative of protoporphyrin IX can be a successful method of breast cancer treatment or complementary one used after surgery.

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