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## Expression of Proapoptotic *BAX* and *TP53* Genes and Antiapoptotic *BCL-2* Gene in MCF-7 and T-47D Tumour Cell Cultures of the Mammary Gland After a Photodynamic Therapy with Photolon<sup>\*</sup>

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of article; G – other

### Abstract

**Background.** Breast cancer is the most common malignant tumour in women in the whole world. Despite significant developments in the early diagnosis of breast cancer, there is no effective method which would assure total recovery of the patient. Currently available clinical data and laboratory tests indicate a possibility to introduce photodynamic therapy (PDT) to the supplementary treatment of breast cancer.

**Objectives.** The aim of this study was to assess the influence of PDT with Photolon as a photosensibilizer on the expression of apoptosis associated genes (*BCL-2*, *BAX*, *TP53*) in human breast cancer cell lines, preceded by assessment of survivorship and proliferative activity in the tested cells after PDT.

**Material and Methods.** In the present study human breast cancer cell lines MCF-7 and T-47D were used. Photolon (chlorin e6 complex: PVP 1:1) was used as a photosensitizer. Assessments of survivorship and proliferative activity of cells under the influence of PDT (WST-1 test) were conducted along with the expression of selected genes involved in the process of apoptosis: *BCL-2*, *BAX*, *TP53* (RT-QPCR).

**Results.** PDT limited both survivorship and proliferative activity of breast cancer cells in the two tested lines. In case of T-47D cell line was found increase of *BAX* and *BCL-2* genes expression after PDT and sustained activity of *TP53* gene. Conversely, in MCF-7 cell line a decrease in expression was found for both *BAX* and *TP53* genes, but also an increase of *BCL-2* gene expression.

**Conclusions.** A progressing decrease (24, 48 and 72 h after PDT) in the count of culture cells, which suggests the occurrence of apoptosis initiated by a photodynamic reaction with simultaneous increase of *BCL-2/BAX* index, indicates activation of a different endogenous apoptosis pathway than the one examined, namely pointing to suicidal death of cells after PDT (Adv Clin Exp Med 2015, 24, 1, 37–46).

**Key words:** photodynamic therapy, Photolon, MCF-7, T-47D, apoptosis.

It is estimated that over a million women in the world have breast cancer diagnosed annually [1, 2]. Wide-spread availability of educational programmes and screening tests which allow early detection of breast cancer in cases without clinical symptoms of the disease is aimed at decreasing the high ratio of morbidity related to that type of tumour [1, 3]. Currently used methods of treating of breast cancer are dependent on the degree

of advancement of the disease. The main method of treatment for early tumour lesions within the mammary gland is surgical procedure, which has undergone significant changes in the last years. Instead of a radical amputation of the breast, modified breast-conserving surgery is conducted while the possible removal of the axillary lymph nodes is preceded by a biopsy of the sentinel lymph node [1–3]. Additional supplementary treatment in the

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form of radiotherapy is applied after the procedure in order to reduce the risk of topical recurrence of the tumour as much as possible. Irradiation combined with breast-conserving surgery can be applied to the whole mammary gland or limited to the area where the tumour was removed [2, 4]. However, radiotherapy used in treatment of invasive forms of breast cancer usually takes the form of palliative care. Additionally, the occurrence of metastatic foci in organs distant from the primary tumour requires, in many cases, a systemic approach to treatment, which includes both chemotherapy and hormonal therapy [1]. None of the available methods is fully efficient and some patients who underwent radical treatment still suffer from recurrences [2, 4].

In order to decrease the morbidity rate related to breast cancer and improve the quality of patients' life, it is necessary to apply a multidisciplinary approach involving prevention, diagnosis, anticancer therapy, continuous improvement of the treatment standards, as well as to conduct research for new therapeutic methods [3].

Photodynamic therapy (PDT) is an alternative method of treating tumours and non-cancerous hyperplastic lesions [5]. The advantage of PDT over traditional treatment methods used in oncology is connected to the relatively small damage of tissue which surrounds the pathological lesion, low invasiveness and minimal complications after the therapy [6]. PDT is conducted in two stages, during which a selective elimination of cells occurs in the tissue owing to the co-operation of three basic factors: a photosensibilizer, light and oxygen [7].

The first stage of PDT comprises of a topical or systemic application of a photosensitizing compound, which then accumulates in the tissue undergoing treatment [5, 8]. After that, light with a wavelength matching the absorption spectrum of the photosensibilizer irradiates the tissue and generates high levels of reactive oxygen species and free radicals [5, 8]. Due to the short half-life and limited ability to diffuse the resulting products, the affected area is practically limited to the place where the photodynamic reaction was initiated [8].

There are three known biological mechanisms which allow PDT to be a mediator in the destruction of tumorous tissue: cellular, vascular and immunological. They are also related to the area where the photosensitizer accumulates the most [7, 8]. *In vitro* research with use of various cell lines, breast cancer among them, confirm the direct cytotoxic influence of PDT [9–12]. Both necrosis and apoptosis were observed in cultures affected by a photosensitizer and then irradiated [12]. It was proved that the key factors which influence the

final effect of PDT (death of cells) are: type and concentration of the applied photosensibilizer [5], keeping an adequate time span between application of the photosensibilizer and irradiation of the tumour cells [13, 14], as well as the dose of exciting radiation applied to the cultures [12]. However, what seem to play a particularly significant role for the effect of PDT are the properties of the cells in which the photodynamic reaction takes place. One of the features which distinguish normal and tumorous cells is change in the profile of expression of genes involved in the process of apoptosis that encode the pro- and antiapoptotic factors. In many cases, resistance of tumorous cells to apoptosis is the main reason why the applied treatment is not efficient. A large part of breast tumours, that is approximately 35% of all cases, is related to mutations in *TP53* suppressor gene, whose protein product is responsible for the reconstruction of the DNA and is involved in the regulation of the cell cycle [15]. P53 protein (encoded by *TP53*) is also a transcription factor which influences the expression of many genes and, as such, is responsible for keeping the right balance between expression of an antiapoptotic *BCL-2* gene and a proapoptotic *BAX* gene. Results of research on the application of various breast cancer cell lines (e.g. SKBR-3 [16], MCF-7 [17], MDA-MB-468 [17] and MCF-10A [7]) published in the available literature show a direct influence of PDT on the pro- and antiapoptotic factors belonging to the *BCL-2* family which regulate the internal pathway of apoptosis (dependent from the mitochondria).

If PDT is to become one of the main methods of treatment of breast cancer, it requires optimization of the above described parameters. Many research centres are currently focusing on assessing the effectiveness of various photosensitizers used during photodynamic reactions on *in vitro* breast cancer cells, including those from metastatic foci [9–14].

Photolon® – a photosensibilizer used in the research, a chlorin e6 (Ce6) and polyvinylpyrrolidone (PVP) compound with ratio of 1 : 1, is a second generation hydrophile photosensitizing compound synthesized by Belmedpreparaty pharmaceutical company (Minsk, Belarus). As it was created by joining Ce6 and PVP, Photolon features better stability and solubility in water and, therefore, also better bioavailability than Ce6 alone [18]. Comparisons of the Ce6-PVP complex with pure Ce6 also demonstrated a higher ability of the compound to accumulate in neoplastic tissue and faster removal time from the body [18].

The aim of the study was to assess the influence of PDT with Photolon on the expression of genes involved in the process of apoptosis:

antiapoptotic *BCL-2* gene and proapoptotic *BAX* and *TP53* genes, with prior assessment of the influence PDT on survivorship and proliferative activity in the tested breast cancer cells of MCF-7 and T-47D lines.

## Material and Methods

### Cell Cultures

Two cell lines of human breast cancer, MCF-7 and T-47D, were used in the *in vitro* tests. Those cell lines were purchased from the American Type Culture Collection (ATCC). The culture of MCF-7 line was grown in the Eagle's Minimum Essential Medium (EMEM) medium, while the base growth medium for T-47D line of cells was RPMI-1640. Both media were enriched with an addition of fetal bovine serum (FBS) and antibiotics: penicillin and streptomycin. Bovine insulin with concentration of 0.01 mg/mL was added to both cultures. The cells were grown in the temperature of 37°C, the atmosphere with 5% of CO<sub>2</sub> concentration and controlled water vapour saturation.

### PDT

Photolon® – a chlorin e6 (Ce6) and polyvinylpyrrolidone (PVP) compound with a ratio of 1 : 1 and a concentration of 0.01 mg/mL in the growth medium was used to photosensitize the cells. The photosensibilizer was added directly to the media in which MCF-7 and T-47D cell lines were grown. The cells were incubated with Photolon for 1 h in darkness and then irradiated with a laser (PDT-662, Kriomed) with a wavelength of  $\lambda = 662$  nm and output power of 1–500 mW.

### Survivorship and Proliferative Activity

MCF-7 and T-47D breast cancer cell lines were used for the assessment of survivorship and proliferative activity. The cell cultures were grown in plates with 96 wells. The result of the photodynamic reaction was assessed in cultures incubated with Photolon and irradiated with doses of 2.5 J/cm<sup>2</sup>, 5 J/cm<sup>2</sup>, 10 J/cm<sup>2</sup>. Additional trials were conducted in order to analyse the influence of the photosensibilizer and laser light alone.

The assessment of survivorship and the proliferative activity of MCF-7 and T-47D breast cancer cells was conducted with the use of WST-1 test by Roche Diagnostics GmbH. In order to assess the survivorship of the cells, a growth medium without

FBS was used, whereas a medium with 10% FBS concentration was applied to assess proliferative activity. The test was run after 24, 48 and 72 h after irradiation of the test cells. Absorbance measurements were conducted with a Biogenet microplate reader.

### Assessment of Expression of Genes Selected Among Those Participating in Apoptosis

MCF-7 and T-47D line breast cancer cell cultures grown on Petri dishes with 5.5 cm diameter were used to assess the influence of PDT on the expression of genes selected among those participating in apoptosis: proapoptotic *BAX*, *TP53* and antiapoptotic *BCL-2*. The cell cultures were incubated for 1 h with a photosensitizer and irradiated with a dose of 10 J/cm<sup>2</sup>. Additional trials were conducted in order to analyse the influence of the photosensibilizer and laser light alone on the expression of those genes.

RNA was extracted from the cells with the use of TRIZOL® (Invitrogen) reagent, according to the method designed by Chomczynski and Sacchi. The quantitative assessment of extracts was conducted on the basis of absorbance measurements at the wavelength of  $\lambda = 260$  nm (while assuming that 1.0 OD<sub>260</sub> corresponds to 40 µg RNA in 1 mL of the extract). Spectrophotometric measurements were made using a HP 8452A spectrophotometer (Hewlett Packard).

In order to establish the number of mRNA copies of *BAX*, *TP53* and *BCL-2* genes in the extracts from the total RNA, RT-QPCR in real time method was used. DNA Engine OPTICON™ fluorescence detector (MJ Research) with a QuantTect® SYBR® Green RT-PCR kit (Qiagen) and specific complementary starters to amplify the sequence were used to run the reaction. Starter sequences were designed with use of Primer Express™ Version 1.0 computer software (ABI Prism), based on the data from MEDLINE (<http://www.ncbi.nlm.nih.gov/>).

### Statistical Analysis

Data distribution for each of trials met the requirements of normal distribution (assessed with Wilk-Shapiro test), which gave the basis for a statistical analysis conducted with use of parametric tests. The control group and one of the tested groups were compared using *t*-test, provided that the variances of those trials were homogeneous or using *t*-test with separate estimation of variance (i.e. Cochran-Cox test) if they were not.

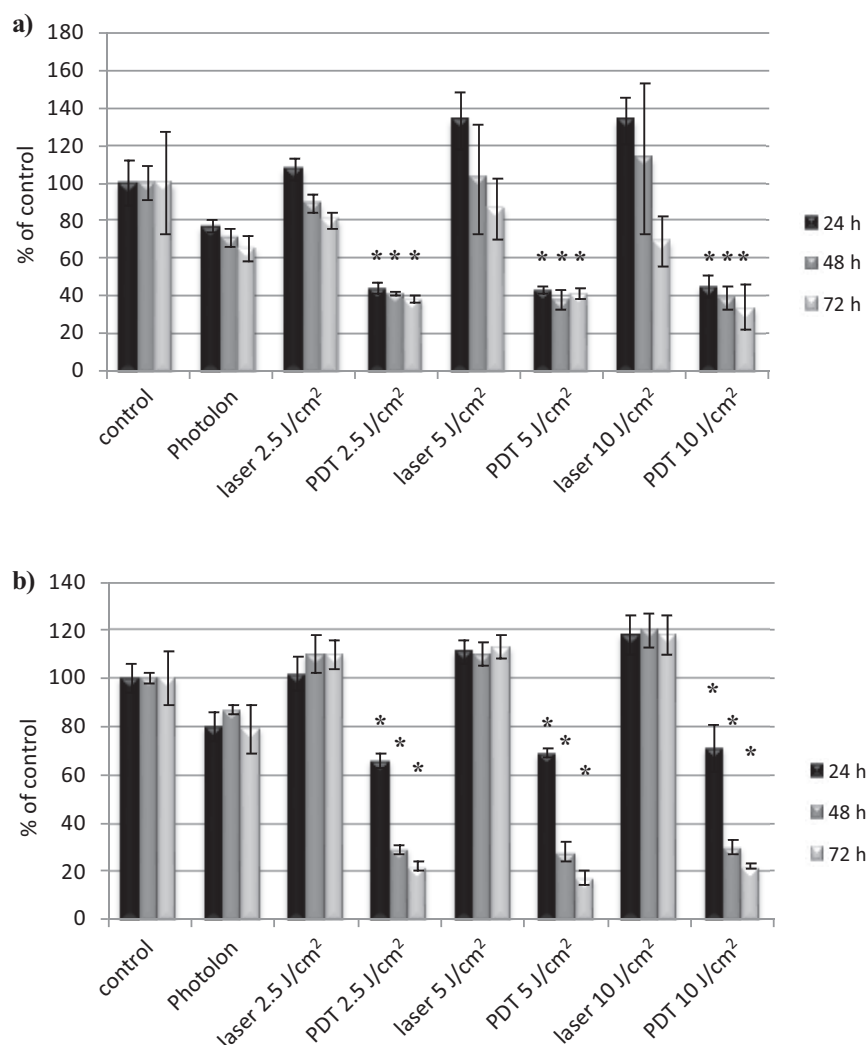
Subsequently, the control group was compared with three tested groups using Dunnett's test, after prior confirmation (with F-test in variance analysis) of statistically significant differences in analysed means. Assumptions of variance homogeneity were checked with use of Levene's test. All tests were performed with the significance level of  $\alpha = 0.05$ , using STATISTICA 8.0 software.

## Results

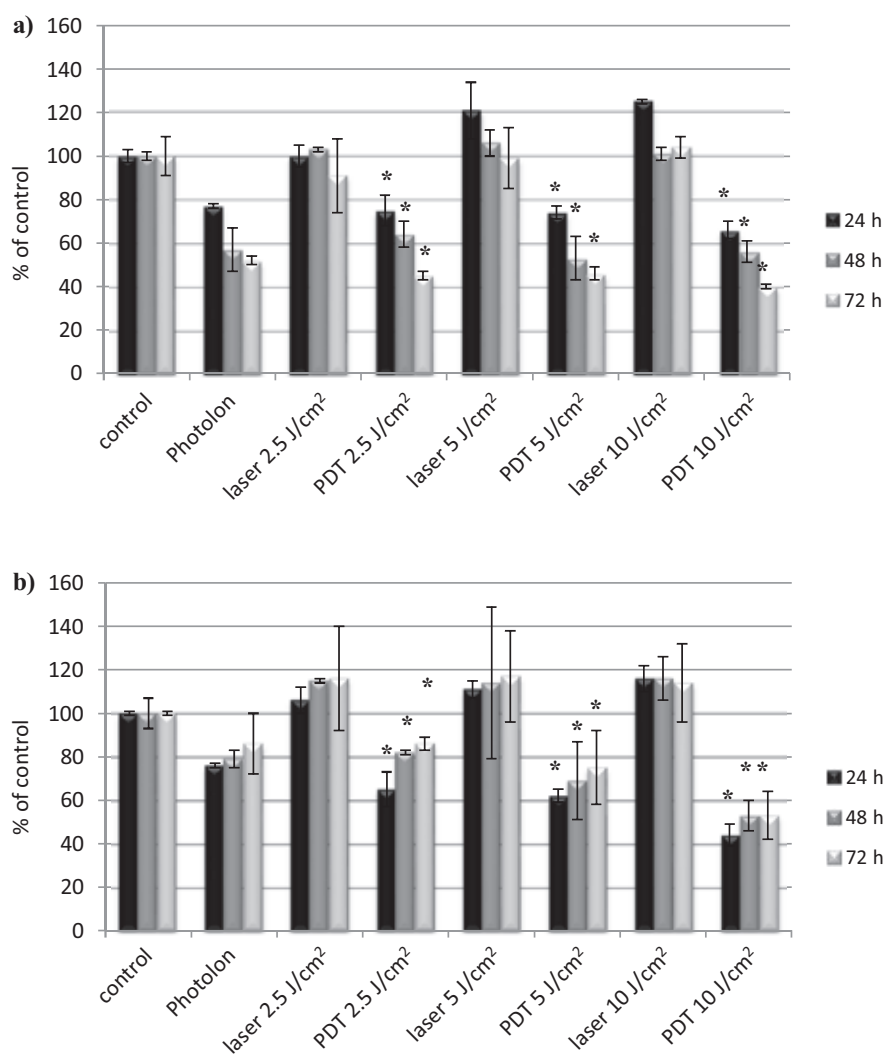
Photodynamic therapy causes a decrease in the cell count in both cultures of cell lines (Fig. 1, 2). Those changes are observed in case of all applied irradiation doses ( $2.5 \text{ J/cm}^2$ ,  $5 \text{ J/cm}^2$ ,  $10 \text{ J/cm}^2$ ). Statistically significant differences are observable as soon as 24 h after irradiation ( $p < 0.001$  in all tested cultures after 24 h). In case of those cultures in which the growth factors were eliminated prior to irradiation (media without a serum additive), which resulted in limited proliferation of the cells, subsequent tests conducted after 24, 48 and 72 h since PDT yielded cytotoxic effects of

PDT – a decrease of live cell counts in those cultures. In case of MCF-7, the cell count in the culture amounted to approx. 40% as soon as 24 h since PDT (when compared to the control group) and such amount was sustained in the following days of the tests (Fig. 1a). In case of T-47D line of cells, the cell count decreased to approx. 75% of the initial number after the first 24 h and it took another 48 h (72 h in total since the end of irradiation) to reach the value of approx. 40% (when compared to the control group) (Fig. 2a). It proves that the cell destruction process progressed with time. On the other hand, an analysis of the cell count in FBS cultures (presence of a growth factor in the medium allowed the cells to multiply) showed a significant decrease in the proliferative potential of MCF-7 after PDT in comparison to the control culture (Fig. 1b). A slight increase in the cell count of T-47D after 48 and 72 h since the irradiation (when compared to 24 h after it) in all cases of PDT ( $2.5 \text{ J/cm}^2$ ,  $5 \text{ J/cm}^2$ ,  $10 \text{ J/cm}^2$ ) suggests the sustained ability of those cells to divide (Fig. 2b), with a simultaneous, abovementioned decrease in the survivorship of a part of those cells (Fig. 2a).

An analysis of the expression of genes involved



**Fig. 1.** Survival (a) and proliferative activity (b) of MCF-7 cells after incubation with photosensitizer, irradiation with laser light at different doses and PDT – presented as % of control; \* test probability for the results of variance analysis ANOVA and Dunnett's test for control cell cultures and cell cultures after PDT,  $p \leq 0.05$ .



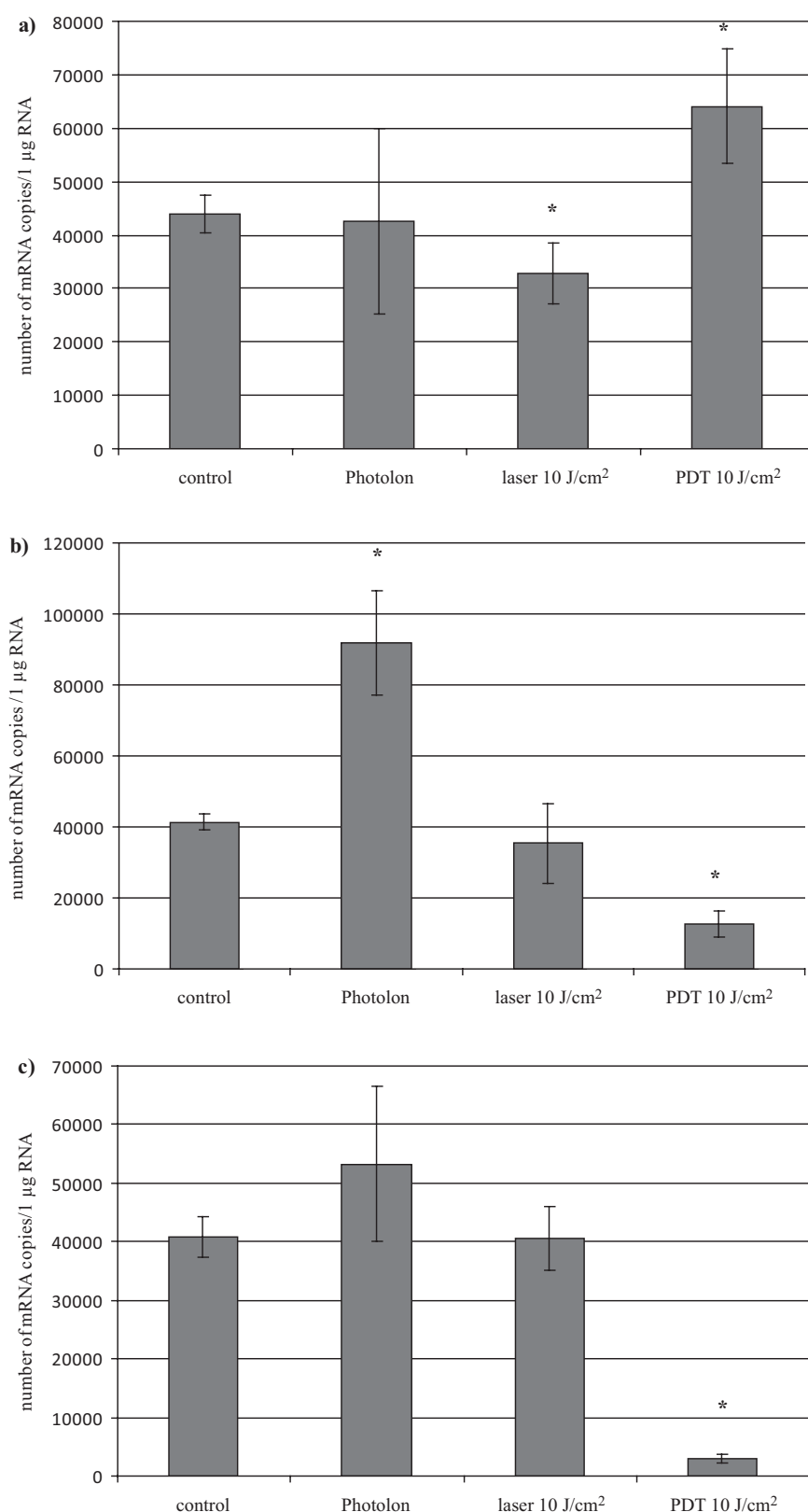
**Fig. 2.** Survival (a) and proliferative activity (b) of T-47D cells after incubation with photosensitizer, irradiation with laser light at different doses and PDT - presented as % of control

in the process of apoptosis (proapoptotic *BAX*, *TP53* and antiapoptotic *BCL-2*) showed in case of MCF-7 line that, in the assumed test environment (concentration of the photosensitizer, incubation time, doze and power of radiation, as well as analysis sampling time): a statistically significant increase of *BCL-2* ( $p = 0.035$ ) expression and a decrease of mRNA copies count for *BAX* ( $p < 0.001$ ) and *TP53* ( $p < 0.001$ ) after 3 h since PDT in comparison to the control group (Fig. 3) and, in case of T-47D, a significant increase in the activity of genes: *BCL-2* ( $p = 0.026$ ) and *BAX* ( $p = 0.025$ ) with sustained activity of *TP53* gene (Fig. 4). The analysis was conducted on the basis of the number of mRNA copies of a given gene when compared to the total mRNA count. A comparison of changes in expression of *BCL-2* and *BAX* in each culture allowed us to establish that, in the case of both MCF-7 and T-47D cell lines, photodynamic therapy performed in the assumed test conditions and after 3 h since the therapy causes an increase of *BCL-2*/*BAX* ratio, where the ratio for MCF-7 reaches the value of approx. 5 and the ratio for T-47D reaches the value of approx. 3, respectively (Fig. 5). It

suggests higher mobilization of MCF-7 cells to protect against activation of the endogenous apoptosis pathway.

## Discussion

Photodynamic therapy (PDT) is one of alternative methods of treatment for such tumours like bladder, esophageal, airway and female reproductive system cancers [19]. Recent years have given us research efforts whose results may in the future allow us to include PDT into the group of main or supplementary methods of treatment for breast cancer. Those efforts include the potential application of PDT in the treatment of primary neoplastic lesions located within the boundaries of the mammary gland, as well as in the metastatic foci in distant organs. Results of tests conducted on rats with implanted cells of human breast cancer MT-1 allowed Burch et al. to prove that PDT can be applied as a therapeutic procedure in the treatment of breast cancer metastasis to the skeletal system

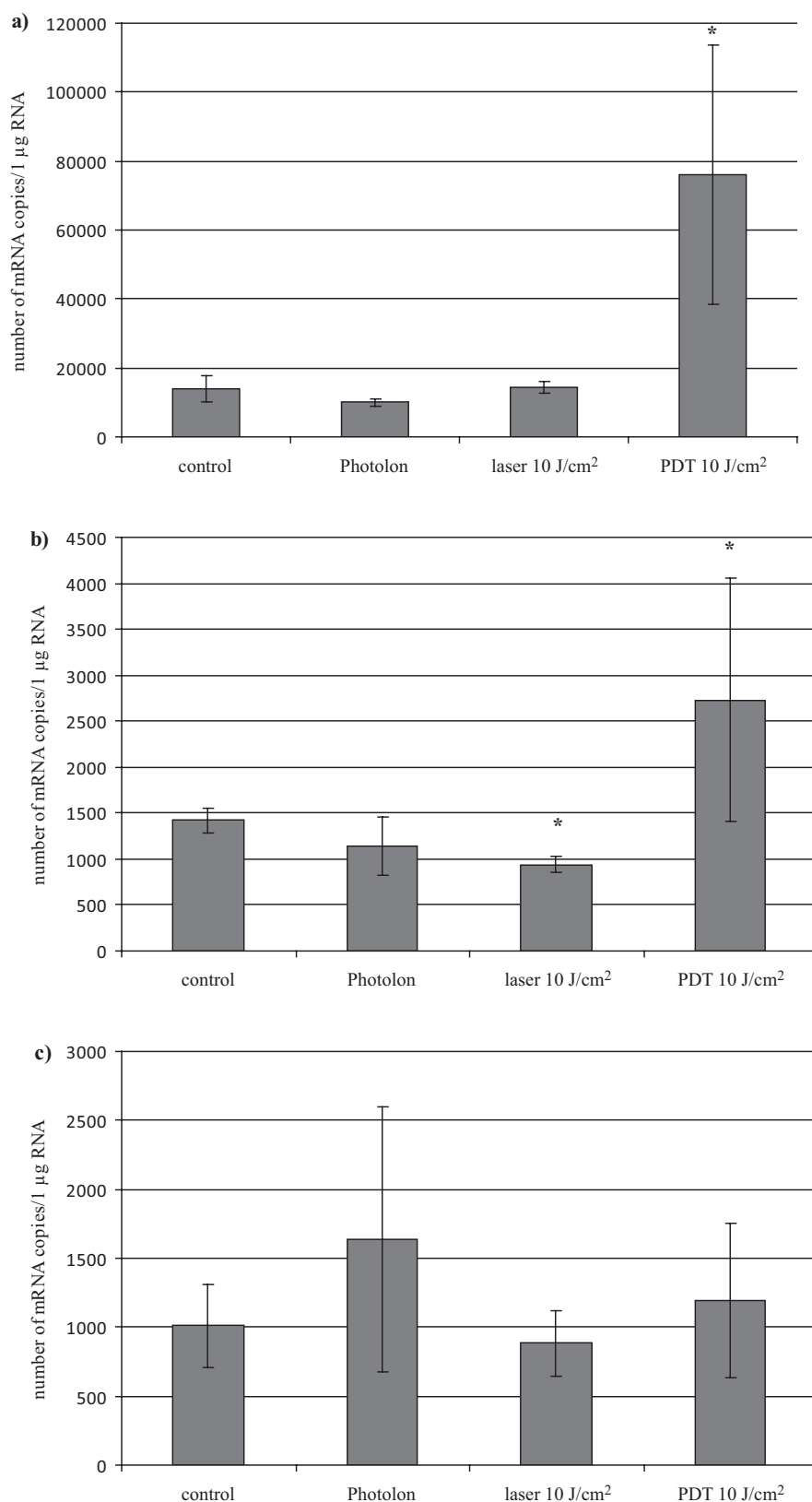


**Fig. 3.** Average number of mRNA copies of genes *BCL-2* (a), *BAX* (b) and *TP53* (c) converted into 1 µg of total RNA in MCF-7 cell cultures; \* test probability for the results of t test and Cochran-Cox test concerning expression of genes *BCL-2*, *BAX* and *TP53*, determined for MCF-7 cells after incubation with photosensitizer, irradiation with laser light and PDT, in comparison to control,  $p \leq 0.05$

[20]. The available literature also contains positive results of clinical tests related to the application of PDT with Photofrin or Purlytin in metastatic breast cancer recurring in the chest wall [2, 21].

Due to the shallow penetration of the light into the tissue, the application of PDT is currently

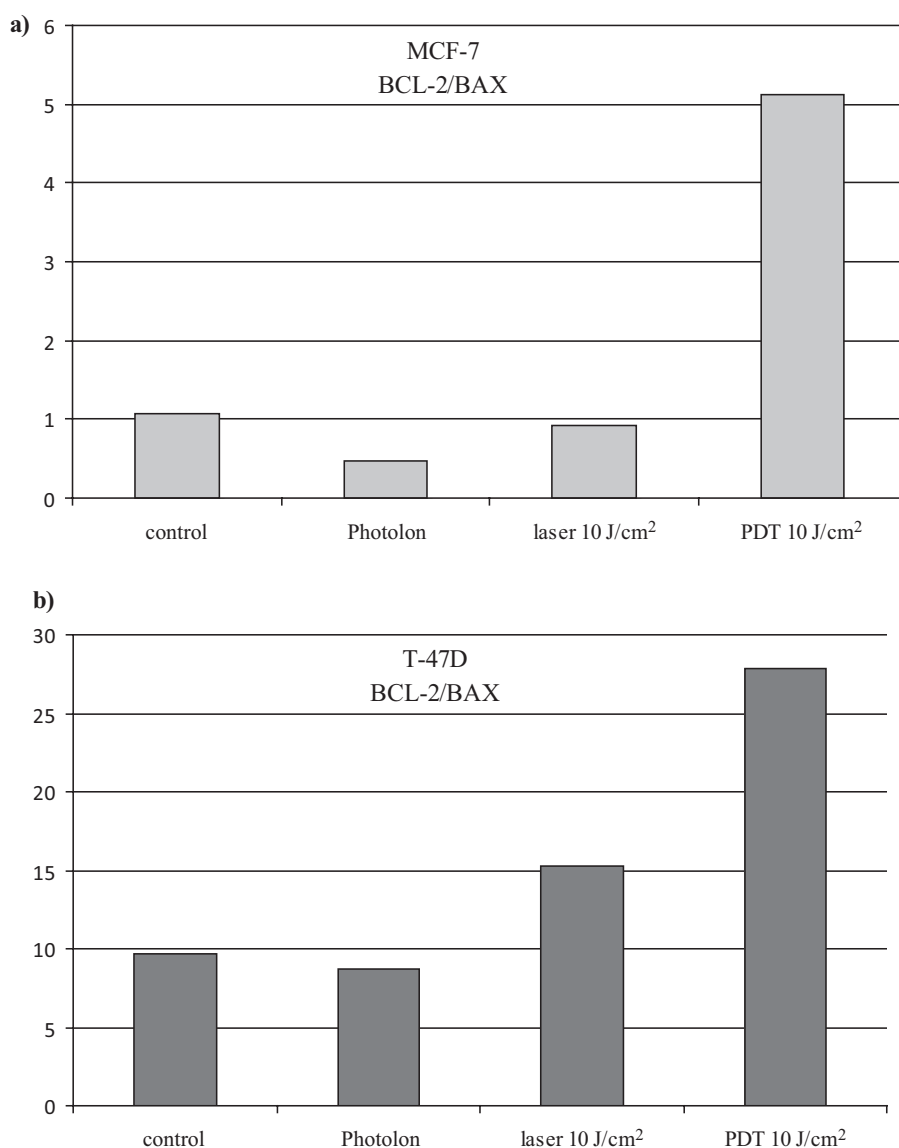
limited only to the treatment of lesions at the surface of the skin, internal organ cavities, gastrointestinal tract, urinary system. However, there are continuous efforts to modify the method in order to use it also to fight deeper lesions, for example quite simple intratissue needles with fibre optics [22].



**Fig. 4.** Average number of mRNA copies of genes *BCL-2* (a), *BAX* (b) and *TP53* (c) converted into 1 µg of total RNA in T-47D cell cultures; \* test probability for the results of *t*-test and Cochran-Cox test concerning expression of genes *BCL-2*, *BAX* and *TP53*, determined for T-47D cells after incubation with photosensitizer, irradiation with laser light and PDT, in comparison to control,  $p \leq 0.05$

The paper contains efforts to assess the molecular effects of PDT with Photolon in neoplastic cells of the mammary gland in *in vitro* conditions. The tests proved that the application of PDT with Photolon leads to the limitation of both the survivorship and proliferative activity of human breast cancer of

MCF-7 and T-47D cell lines. The obtained results confirm what the available literature describes as direct cytotoxic influence of PDT on cells of various breast tumour lines in *in vitro* conditions [9, 10, 11, 14]. However, this does not explain the mechanism of the final effect of PDT, i.e. the death of cells. It



**Fig. 5.** Value of BCL-2/BAX ratio for MCF-7 (a) and T-47D (b) cells after incubation with photosensitizer, irradiation with laser light, PDT and control cell cultures

seems that MCF-7 line cells are more susceptible and die faster after application of PDT. In the case of T-47D line of cells, the destruction is more dispersed in time, which suggests that the cells enter the apoptosis pathway. Moreover, those cells partially regain their ability to divide with the passage of time, yet they never reach that level of activity which can be observed in the control cultures.

The mechanism of the terminal effect of PDT, that is the death of examined cells is still subject to many research efforts. It depends to a significant degree on the conditions in which the photodynamic reaction takes place. Apart from the concentration and type of used photosensibilizer, it is also important to retain a certain time interval between its application and irradiation of the tested cells that determines the intracellular location of a photosensibilizer to a certain extent [8, 13, 14]. In most cases, the accumulation of a photosensibilizer in mitochondria or endoplasmic

reticulum leads to the activation of the apoptotic pathway in the cell. Huge aggregation of a photosensitizing stain within the cell membrane or liposomes creates favourable conditions for necrotic death of it [8]. Marchal et al. made an attempt to explain how intracellular concentration of Foscan<sup>®</sup> photosensibilizer influences the antiapoptotic activity of BCL-2 factor in breast cancer cells of MCF-7 line. After a 3-h long incubation of cells with the abovementioned photosensibilizer, it showed the affinity mainly to the Golgi apparatus and the endoplasmic reticulum. However, after 24 h, Foscan<sup>®</sup> relocated and the endoplasmic reticulum and mitochondria to a lesser degree became its main accumulation focus. That resulted in an assumption that endoplasmic reticulum is the main organelle which becomes damaged in MCF-7 cells after PDT with Foscan<sup>®</sup> is applied [13].

The analysis of participation of the most known and commonly described genes during



apoptosis, i.e. *BAX*, *TP53* and *BCL-2*, proved that in case of MCF-7 cell line, photodynamic therapy with Photolon, in established conditions, causes an increase in expression of *BCL-2* gene and a decrease in expression of *BAX* and *TP53* genes in comparison to the control culture, whereas the breast cancer cells of T-47D line feature an increase of expression of both *BAX* and *BCL-2* genes with an unchanged level of mRNA copies of *TP53* after PDT treatment.

The P53 protein is considered to be one of the most important regulators of the cell cycle and is attributed a great role in the intracellular regulation of cellular response to harmful level of radiation [23]. Clear proapoptotic activity of P53 protein is known, mainly its influence on the expression of various other proapoptotic proteins, such as *BAX* or *FAS*, which may attribute to the eventual death of the cell. However, indisputable and significant contribution of P53 to the induction of apoptosis does not exclude the possibility that apoptosis exists independently from that protein [24]. The available literature on photodynamic therapy quite frequently describes the outcome of such conduct which leads to apoptosis in the cells with blocked P53 protein [25, 26].

Photolon – photosensitizer used in the research described here accumulates in the target tissue quite quickly and reaches the maximum concentration after approx. 3 h since an intravenous

injection or after approx. 0.5 to 4 h after topical application. It is secreted from the body after 24 to 72 h after application [27]. Research on the mechanisms of the photodynamic therapy with the use of Photolon described in the available literature and conducted on an animal model (with a sarcoma) indicate lysosomal damage and necrosis. Those observations were partially confirmed by the works of Coplej et al. who described an increase in the concentration of LDH after PDT-Photolon and no change in the activity of caspase-3 and -7 [29]. However, the results acquired during the current research tests, that concern changes in the cell count in 24-, 48- and 72-h periods after PDT, as well as statistically significant changes in the expression of genes after PDT (especially those where an increase in the number of mRNA copies of tested genes is observed), indicate that life functions of cells are preserved immediately after PDT and their destruction is dispersed in time. That, in turn, may indicate that apoptosis is taking place. On the other hand, an increase in the value of the *BCL-2/BAX* expression ratio in cultures with a simultaneous decrease of the cell counts in the subsequent days of the experiment prove that a different than endogenous apoptosis pathway exists or even that the cells exposed to PDT-Photolon die in another active way.

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