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Effects of verteporfin-mediated photodynamic therapy in breast cancer cells

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Photodynamic therapy works with a photosensitizer that is stimulated when exposed to a light source of a specific wavelength and produces a form of oxygen that can be used in cancer treatments. In this study, we investigated the effect of laser on apoptosis on breast cancer cell lines (MDA-MB-231) treated with verteporfin in cell culture media. Verteporfin added MDA-MB-231 cells were incubated without light for 24 hours after applying laser light at a wavelength of 695 nm at an intensity of 50 J/cm2 at various times. Anti-proliferative effects were evaluated by immunoreactivity of anti-Bcl-2 and anti-Bax antibodies by immunocytochemical staining. When anti-Bax/Anti-Bcl-2 ratio are compared, the ratio of 1.5 in the control group cells decreases in short-term laser applications, while it approaches normal values in the 7th min after long-term laser application and reaches a very high value in the 9th min. Therefore, our results suggest that verteporfin-mediated PDT may be a potential combined therapy strategy against breast carcinoma by increasing apoptosis.

Keywords: Bax, Bcl-2, Laser, MDA-MB-231, Photosensitizer

Breast cancer is the leading cause of cancer-related deaths worldwide and is the most common type of cancer among women. Despite advances in molecular classification and treatment, breast cancer incidence and mortality remain high[1]. New therapeutic strategies are needed because of the resistance to the current treatment modalities of metastatic, triple- negative breast cancer cases that lack estrogen receptor, progesterone receptor, and human epidermal growth factor receptor. Nowadays, it is seen that photodynamic therapy (PDT), which is one of the alternative treatments, has become widespread in the treatment of cancer without damaging healthy tissues. The method of PDT is based on the application of a substance that sensitizes the tissue called photosensitizer (PS), which is treated with the light of a specific wavelength that accumulates to some extent in the pathological tissue. PDT is based on the principle of the effect of photochemical reaction on the cells resulting from the interaction of the photosensitive molecules with light. The reactive oxygen products produced by PDT can have a direct harmful effect on the cells or they may cause damage to the cells due to organelle damage or cell membrane damage[2]. The response to PDT at the cellular level depends on the localization of the photosensitizing agent. The localization of the photosensitizing agents towards cytosol and lysosomes increases the effect[3].

Depending on their characteristics, a photosensitizing agent is generally localized to organelles such as the plasma membrane, lysosomes, mitochondria, Golgi apparatus, or endoplasmic reticulum (ER)[4]. PDT is thought to be the three main tumor destruction mechanisms. Due to the localization and activation of photosensitivity agents in the tumor tissue, reactive oxygen species (ROS) can directly kill malignant tumor cells. Oxidative stress caused by increased intracellular ROS concentration usually progresses through dangerous processes such as lipid peroxidation, oxidative modification of nucleic acids and proteins, however, it plays a signal and regulatory role in the organism[5]. The direct cell killing effect of PDT on both the tumor parenchyma and stroma occurs due to the formation of ROS on the presence of oxygen[6]. On the other hand, PDT may target the tumor vessel structures and prevent the supply of oxygen and essential nutrients. Damaging existing vessel formation or inhibiting new vessel formation has detrimental consequences for tumor proliferation, and based on this, anti-angiogenic therapeutics are clinically applied for the treatment of cancers[7]. The third mechanism of PDT-induced tumor destruction is the initiation of an inflammatory response followed by host tumor immunity. PDT-induced

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oxidative stress can upregulate the expression of heat shock proteins (HSP), transcription factors associated with inflammation and release of inflammatory cytokines[8]. After PDT, HSPs such as HSP70 have been shown to be upregulated or expressed on the cell surface[9]. HSP may bind to tumor antigens and interact with Toll-like receptors (TLRs), which are the main means of activating antigen- presenting cells (APC). In addition, these interactions regulate the expression of the inflammatory and immune response genes. Other damage- associated molecular patterns (DAMPs) observed after PDT are lipid fragments and arachidonic acid metabolites, membrane degradation products such as adenosine triphosphate (ATP), or ER protein calreticulin[10]. Increased expression and activation of transcription factors such as nuclear factor κB (NF-κB) and activator protein 1 (AP1) are also an important mechanism in inducing an inflammatory response following PDT-induced oxidative stress. Numerous studies with different PSs have increased NF-κB and AP1 transcription factors after PDT[11].

Verteporfin (VP), a third- generation PS, has been widely used to treat macular degeneration and has been proposed to be used for cancer treatment in recent years. The mouse liver cancer model and *in vitro* colon cancer research has demonstrated that VP is effective in cancer treatment. However, the mechanisms by which VP inhibits cancer growth are not yet well known[12]. The therapeutic efficacy of PDT depends on the properties of light used to activate PS. The light has to penetrate the skin and the tissue to reach the target area[13]. Lasers are widely used in PDT because of their potency and can be interstitially distributed to deep-seated tumors and attached to optical fibers that can be used with the application of diffusion tips. Verteporfin is characterized by an intense absorption band in the red region of the visible spectrum which is capable of penetrating the tissue deeper than other visible wavelengths[14].

PDT has been suggested to induce β-cell Lymphoma 2 (Bcl-2) and activate proapoptotic mechanisms[15]. Changes in the Bcl-2 family after PDT have been demonstrated in cell lines and cancer cells. In a study to investigate the effects of verteporfin on apoptosis in NB4 human leukemia cell line, expression levels of Bcl-2, and Bcl-2 associated X (Bax) proteins showed significant changes after verteporfin administration. Consistently, increased Bax and decreased Bcl-2 expression were observed in VP treatment groups compared to the control group[16].

PDT has been proposed to be used in cancer treatments and breast cancer cases. Although the clinical success of promising stage III trials of PDT has been approved for cancer treatment, it is still not widely used in clinical practice. However, the dose of verteporfin to be used and the mechanism of laser energy are not fully known. The aim of this study was to investigate the effect of laser on apoptosis on breast cancer cells treated with verteporfin in cell culture media.

Materials and Methods

The study was carried out in Manisa Celal Bayar University Faculty of Medicine, Department of Histology and Embryology.

Cell culture

MDA-MB-231 breast cancer cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 supplemented containing 10% fetal bovine serum, 1% penicillin-streptomycin, 1% L-Gluthamine in an environment with 5% CO2 at 37°C.

Determination of IC_{50} of verteporfin by MTT (Methylthiazolyldiphenyl-tetrazolium bromide) Assay

MDA-MB-231 cells were seeded at 5000 cells per well of a 96-well culture plate. Cells were incubated at 37°C and 5% CO2 for 24 h and then exposed to increasing doses of verteporfin for 72 h. Then 10 μL of MTT was added to each well and incubated for 4 h. Drug concentration (IC_{50} -The half maximal inhibitory concentration) resulting in 50% inhibition of cell proliferation was calculated after measurement at a wavelength of absorbance density 490 nm.

Verteporfin and laser application to cells

After MDA-MB-231 breast cancer cells were seeded on 8-well cell culture slides, the verteporfin dose with the determined IC_{50} value was added to each well. Verteporfin was activated by stimulating the cells with a laser beam at a wavelength of 695 nm at an intensity of 50 J/cm^2. Diode laser was applied to each group for 3, 5, 7, and 9 min. The laser beam diameter was determined according to the eye diameter of the 8-well cell culture slides on which cell transplantation was performed.

Indirect immunohistochemical staining of cells

After 24 h of incubation, the cells were fixed with 10% paraformaldehyde. The cells were washed 3 times with phosphate- buffered saline (PBS) for 5 min, then kept on ice for 15 min in triton-X solution to increase

membrane permeability. Subsequently, 3% H_2O_2 was applied for 5 min to inhibit endogenous peroxidase activity of the cells. The cells were washed 3 times with PBS solution for 5 min and treated with 1 h blocking solution. After the blocking solution was removed from the cells, the primary antibodies were incubated with Bcl-2 and Bax overnight at +4°C. The next day, cells washed 3 times with PBS solution were stained with anti-mouse biotin-streptavidin secondary antibody for 30 min. Cells were stained with 3, 3′-diaminobenzidine tetrahydrochloride hydrate (DAB) for 5 min to determine the visibility of the immunohistochemical reaction. After Mayer's hematoxylin background staining, cells washed with distilled water were covered with mounting medium.

Statistical analysis

The immunohistochemical staining results were evaluated by H-score. After the staining rate was graded semiquantitatively (Staining rate, 0 = staining in less than 1% of cells; 1+ = staining in 1-10% of cells; 2+ = staining in 11-50% of cells; 3+ = staining in 51-80% of cells; 4+ = by staining in more than 80% of cells; staining intensity 0 = no staining; 1 = pale; 2 = intermediate; 3 = intensive), the total score was calculated with the formula "(1 + staining intensity/3) × staining rate". The differences between the groups and the findings were determined by one-way ANOVA test and P <0.05 was considered statistically significant.

Results and Discussion

The safe and effective dose of verteporfin administered to breast cancer cells was calculated to be 0.5 mg/kg using the therapeutic window and was administered with 490 J laser with light energy. In a study by Jiang Y *et al*. verteporfin was administered to MDA-MB-231 cells at doses of 0, 4, 8, 12 and 16 μmol/mL and the minimum inhibitory concentration was 4 μmol/mL[17].

VP has been suggested to reduce cancer cell proliferation[18]. However, the mechanism of action of VP on the proliferation of cancer cells is not fully known, it is known that verteporfin down regulates Yes-associated protien 1 - Transcriptional enhanced associate domain (YAP-TEAD) complex in epithelial carcinomas and prevents uncontrolled proliferation. This mechanism facilitates cancer treatment by specifically affecting cancer cells without damaging healthy cells[19,20].

A study by Liang Dong *et al.* they showed that verteporfin applied in bladder cancer stopped the growth and invasion of cancer cells by suppressing the Hippo signaling pathway[21]. In addition, treatment with VP has been shown to cause downregulation of cyclin D1 and cyclin E1, modulation of Bcl-2 family proteins, and Poly ADP ribose polymerase (PARP) activation. It has been reported that VP has an inhibitory effect on angiogenesis and vasculogenesis by suppressing angiogenin ribonuclease A family member 2 (Ang2), matrix metalloproteinase-2 (MMP2), and cadherin, and alpha smooth muscle actin (α-SMA) expression *in vitro* and *in vivo*[22].

Bcl-2, a regulator classified as an oncogen mediating cell death, acts as an anti-apoptotic protein. Decreased anti-apoptotic Bcl-2 protein in pathogenetic processes contributes to cancer treatment by increasing apotosis, while overproduction is known to accelerate cancer development.

Bcl-2 can form heterodimers and can be used as an anti-apoptotic regulator. In contrast, Bax, another regulator of apoptosis, binds Bcl-2 protein and suppresses it. This, in turn, triggers cell death. In this study, it was seen that there was a decrease in Bcl-2 activity compared to the control group in MDA-MB-231 cells treated with Verteporfin and laser in the 3rd min. Bcl-2 levels returned to normal at the 5th min and increased significantly in the 7th and 9th min. It was thought that the effect of photodynamic therapy on Bcl-2 started to show from the 3rd min but this effect was not applied in the 5th min. The Bcl-2 family of proteins is known to be a key regulator of apoptosis and an important determinant of cell fate. Bcl-2 exhibits antiapoptotic function and is known to exert its effect by disrupting the integrity of the outer mitochondrial membrane. In vertebrates, they release intermembrane space proteins such as cytochrome c to promote caspase activation in cytochrome. It has been reported that Verteporfin significantly inhibits expression of c-MYC, cyclin D1, YAP, Cysteine-rich angiogenic inducer 61 (CYR61) and Connective tissue growth factor (CTGF), as well as significantly inhibiting cell proliferation, invasion and migration by downregulating the expression of their target genes CYR61 and CTGF[23]. Verteporfin is known to have a reducing effect on Bcl-2. In a study of CAL27 cells treated with Verteporfin, it was shown that transcription and translation of Bcl-2 and c-MYC decreased[17].

In this study, it was observed that Bcl-2 activity of verteporfin and laser-treated MDA-MB-231 cells decreased compared to the control group at 3th min, normalized at 5th min, and increased significantly at 7th and 9th min (Figs. 1 & 2).

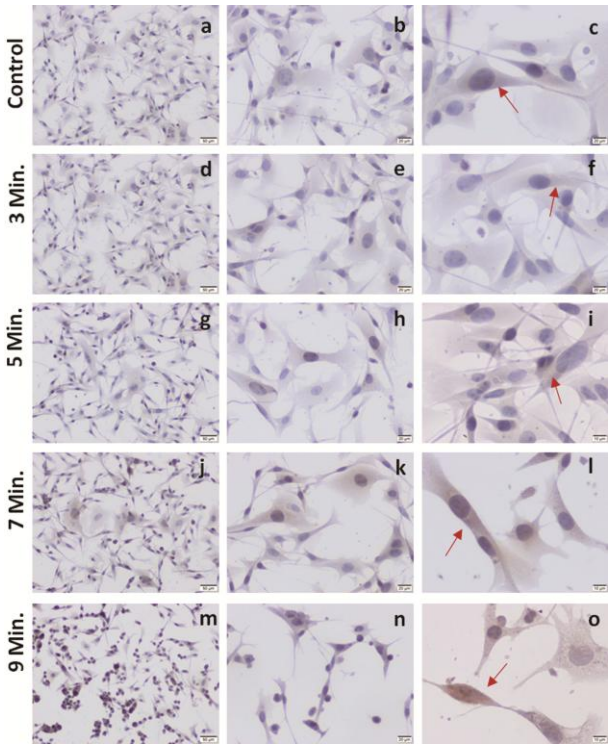

Fig. 1 — The immunoreactivity of Bcl-2 on MDA-MB-231 cells untreated (a, b, c) and treated for 3 min. (d, e, f), 5 min. (g, h, i), 7 min. (j, k, l) and 9 min. (m, n, o) laser with verteporfin. Arrows indicate Bcl-2 positive cells with brown cytoplasmic staining (hematoxylene, Scale bar of a, d, g, j, m: 50 μM; b, e, h, k, n: 20 μM; c, f, i, l, o: 10 μM)

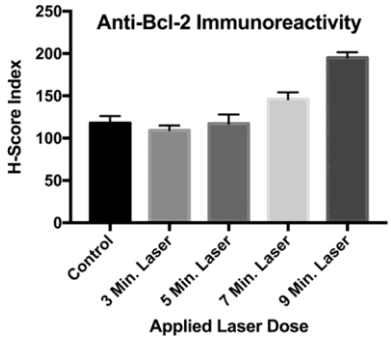

Fig. 2 — Distribution of anti-Bcl-2 immunoreactivity according to laser dose applied to MDA-MB-231 cells. Experiments were performed in triplicate and repeated three times with similar results. Bars display mean ± SD. One-way ANOVA (P <0.05) was performed using GraphPad Prism 7® to test the differences of anti-Bcl-2 immunoreactivity between control and 3, 5, 7, 9 min laser treated clams

It is known that Bax undergoes structural changes in order to trigger apoptosis in cancer tissue, and it acts by interacting with the organelle membrane, especially the mitochondrial membrane. As a result of inhibition of bax expression by gene silencing, it has been shown

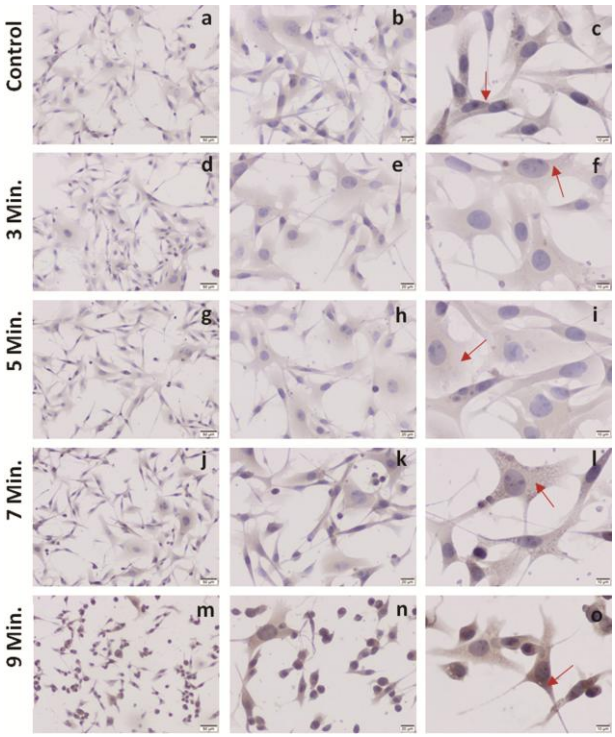

Fig. 3 — The immunoreactivity of Bax on MDA-MB-231 cells untreated (a, b, c) and treated for 3 min. (d, e, f), 5 min. (g, h, i), 7 min. (j, k, l) and 9 min. (m, n, o) laser with verteporfin. Arrows indicate Bax positive cells with brown cytoplasmic staining (hematoxylene, Scale bar of a, d, g, j, m: 50 μM; b, e, h, k, n: 20 μM; c, f, i, l, o: 10 μM)

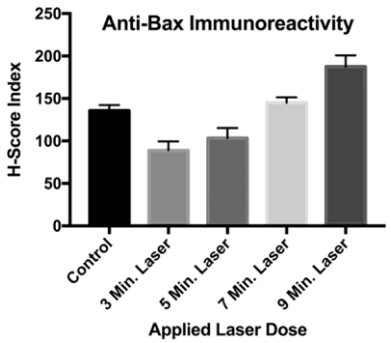

Fig. 4 — Distribution of anti-Bax-2 immunoreactivity according to laser dose applied to MDA-MB-231 cells. Experiments were performed in triplicate and repeated three times with similar results. Bars display mean ± SD. One-way ANOVA (P <0.05) was performed using GraphPad Prism 7® to test the differences of anti-Bax-2 immunoreactivity between control and 3, 5, 7, 9 min laser treated clams

that Bax has no effect on mitochondrial depolarization and cytochrome c release and does not contribute to mitochondrial outer membrane permeabilization (MOMP) after PDT. It has been reported that Bax activation is not necessary for mitochondrial outer membrane permeabilization but is required for dynamin

Table 1 — Quantitative regulation of Bcl-2/Bax ratio in MDA-MB-231 breast cancer cells in all groups

	Control	Laser			
Time/Min	--	3 Min	5 Min	7 Min	9 Min
Energy	--	108 j/s^2	180 j/s^2	252j/s^2	324j/s^2
Bax	135, 7±6, 49	89±10, 49	103, 5±11, 81	145, 2±6, 23	187, 6±13, 17
Bcl-2	86, 1±5, 66	82±4, 07	88, 1±5, 06	96, 4±5, 25	107, 3±4, 81
Bax/Bcl-2	1.57±0, 08	1.08±0, 12	1.17±0, 16	1.51±0, 09	1.74±0, 05

related protein 1 (Drp1) mediated mitochondrial fission during apoptosis caused by Photofrin – PDT[24].

The correlations between Bax protein, p53, and caspase-3 expression are probably known to be associated with an active apoptotic mechanism in breast cancer cells expressing Bax protein. The ratio of anti-pro-apoptotic molecules constitutes a mechanism for accelerating pore formation in the mitochondrial outer membrane, determining the susceptibility threshold to apoptosis for the intrinsic apoptotic pathway, which leads to loss of mitochondrial integrity and release of cytochrome.

In the immunohistochemical examination of the breast cancer cell line treated with verteporfin and PDT, it was found that Bax expression of the cells was higher in the control group, whereas the immunoreactivity was low in the 3 and 5 min laser treated cells, whereas it increased gradually in the 7 and 9 min laser- treated cells (Figs. 3 & 4).

It has been reported that Bcl-2 is upregulated and Bax is down-regulated in the HT29 human colon cancer adenocarcinoma cell line that is resistant to photodynamic therapy[25]. 5-Aminolevulinic Acid (5-ALA), another photosensitive agent whose PDT effects were investigated, was shown to suppress the level of Bcl-2 mRNA and increase the level of Bax mRNA in cervical and esophageal cancer cells[26]. On the other hand, in phototherapy applications using aluminum phthalocyanine, MCF10A human breast cancer cells has been shown to respond very well to treatment even though Bcl-2 overexpression is observed in it[27]. According to this opposite view, Bcl-2 transfection results in both Bcl-2 and Bax overexpression, and mitochondrial photo-damage selectively causes Bcl-2 reduction but not Bax. Thus, this situation is interpreted as increasing Bcl-2/Bax ratio[28]. Similar results have been published by Srivastava *et al*. Apoptosis was induced by Pc4-PDT in RIF1 and A431 cell lines and increased apoptosis was observed with increasing bax protein in A431 cell line. In this study, Bcl-2/Bax ratio was also increased. The antisense Bcl-2 oligonucleotide in the RIF1 cell line significantly reduced apoptosis after 6 h[29].

In a recent study, it was suggested that the Bcl-2/Bax ratio in breast cancer cells had the ability to reduce apoptosis physiologically by numerical regulation[30]. In this study, when the Bax/Bcl-2 ratios were compared, the ratio of the cells in the control group was 1.5 while it decreased in the short-term applications, but after the long-term application, it approached normal in the 7th min and reached a very high value in the 9th min. In a study by Qiao Z *et al*. when they applied laser (670 nm, 10 J/cm^2) to CPF4 photosensitizer chrolophyll derivative treated MCF-7 breast cancer cells, they found that Bcl-2 protein was downregulated, whereas Bax protein was overexpressed and therefore Bcl-2/Bax ratio decreased. Researchers have reported that the application of photosensitizer 2-devinyl-2-[1-methoxylethyl] chlorin f (CPD4) or PDT alone does not cause apoptosis in cells, whereas apoptosis rate is 41.2 ± 1.41% in CPD4-PDT applications[31].

The Bcl-2 family is known to be a mitochondrial permeability transition pore regulator, inhibiting transitions, and playing an important role in providing membrane potential. Bax is located in the cytoplasm and acts in the opposite direction of Bcl-2 in mitochondrial permeability transition pore regulation. Therefore, it is accepted that Bcl-2/Bax ratio plays a key role in cell survival or apotosis[20,32].

In our study, when the Bax/Bcl-2 ratios were compared, the ratio was 1.5 in the cells of the control group while the rate decreased in the short-term applications, but after the long-term application, it approached normal in the 7th min and reached the very high value in the 9th min (Table 1).

Conclusion

PDT is a treatment performed by applying a photosensitive agent deposited in tumor tissue. After an incubation time that sensitizes the target tissue to light, visible light at a wavelength that matches the absorption of a photosensitive agent is applied to the target tissue. In PDT applications, in the presence of oxygen, light activation of photosensitive agents results in reactive oxygen products and causes photochemical reactions. It

is claimed that the resulting oxygen has a cytotoxic effect and thus causes the destruction of tumor cells.

In this study, we tried to observe the anti-proliferative and apoptotic effects of phototherapy on MDA-MB-231 cells in cell culture medium. For this, after adding verteporfin to the medium of MDA-MB-231 cells, we applied laser light of 695 nm wavelength at 50 J/cm2 intensity at various times. Next, we evaluated the immunoreactivities of Anti-Bax and Anti-Bcl-2 antibodies. In our results, Bax/Bcl-2 ratio increased from the 7^{th} min, and it was observed more clearly after 9 min of laser applications. These results showed us that the enhanced in vitro antitumor effect of PDT combined with verteporfin through Bax/Bcl-2 molecules can regulate intrinsic apoptotic pathways that lead to tumor apoptosis.

Taken together, PDT combined with verteporfin resulted in inhibition of cell proliferation and increased apoptosis *in vitro*. Cancer treatments to be performed with such applications may be an important treatment regimen for the prevention of cancers with local effects while protecting from the systemic effects of general chemotherapeutic agents. Therefore, our results show that verteporfin-mediated PDT is a potential combined treatment strategy against breast carcinoma. However, further studies are needed to develop this finding.

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Conflict of interest

All authors declare no conflict of interest.

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