ANTI-TUMOR EFFECT OF THE INHIBITOR OF CHOLESTEROL SYNTHESIS PATHWAY – LOVASTATIN AND PHOTODYNAMIC THERAPY IN COLORECTAL CANCER IN VITRO

EFEKT ANTYNOWOTWOROWY INHIBITORA SZLAKU SYNTEZY CHOLESTEROLU – LOWASTATYNY I TERAPII FOTODYNAMICZNEJ W RAKU JELITA GRUBEGO IN VITRO

Beata Joanna Osiecka[1], Mateusz Olbromski[2], Elżbieta Wojdat[3], Stanisław Kwiatkowski[1*], Sylwia Borska[2]

[1] Uniwersytet Medyczny im. Piastów Śląskich we Wrocławiu, Katedra Patomorfologii, 50-368 Wrocław, ul. Marcinkowskiego 1

[2] Uniwersytet Medyczny im. Piastów Śląskich we Wrocławiu, Katedra i Zakład Histologii i Embriologii, 50-368 Wrocław, ul. Chałubińskiego 6A

[3] Polska Akademia Nauk, Instytut Immunologii i Terapii Doświadczalnej im. Ludwika Hirszfelda, 53-114 Wrocław, ul. Weigla 12

*e-mail: stkwiatek@gmail.com

ABSTRACT

Photodynamic therapy (PDT) is a promising treatment method for non-oncological and oncological diseases. PDT requires the use of photosensitizer, light, and cell oxygen. A selective, cytotoxic effects can be achieved in cancer cells, thanks to formation of radical oxygen species (ROS). Recent data indicate, that PDT with aminolevulinic acid (ALA) is successful in treatment of colorectal cancer. However, anti-cancer effects PDT *in vivo*, can vary in PDT *in vitro*, due to low level of oxygenation of tumor cells. Experimental research confirms the role of cholesterol in oncogenesis and indicates, that in colorectal cancer the cholesterol synthesis is disrupted; among others lovastatin belong to inhibitors 3-hydroxy-3-methyl-glutharyl-coenzyme A (HMG-CoA) enzyme of cholesterol synthesis pathway. Anti-tumor potential of statins: antiproliferative and pro-apoptotic, were demonstrated in various tumors *in vitro* and *in vivo*. Colorectal cancer, which often has genes mutations that are related to programmed death cells, shows resistance to chemotherapy. The objective of the research was to investigate the effect of antiproliferative and cytotoxic lovastatin and ALA-PDT (in low dose) working separately and in combination method (lovastatin and PDT) − for apoptosis induction, in metastatic colon cell line SW620. Our finding showed, that lovastatin inhibits the growth of cancer cells, depending on the dose and incubation time. ALA-PDT in low dose works on cell cytotoxic, yet do not initiate apoptosis. Whereas, preincubation of cells with lovastatin (IC50) increases the cytotoxic effect after PDT and induces apoptosis in cancer cells SW620, as demonstrated using an assay of Annexin V for flow cytometry. Our research demonstrates for the first time, the effectiveness of ALA-PDT connection with an inhibitor of cholesterol synthesis pathway – lovastatin, in increasing the cytotoxicity and induction of apoptosis in colon cancer cells, that are resistant to chemotherapy.

Keywords: lovastatin, aminolaevulinic acid, photodynamic therapy, apoptosis

STRESZCZENIE

Terapia fotodynamiczna (ang. photodynamic therapy, PDT) jest obiecującą metodą w leczeniu nowotworowych i nienowotworowych schorzeń. Działanie PDT wymaga użycia fotouczulacza, światła i tlenu komórkowego, dla uzyskania wybiórczego cytotoksycznego efektu w komórkach patologicznych. Efekt ten jest wynikiem powstania wolnych rodników (ROS). Badania pokazują, że PDT z kwasem aminolewulinowym (ALA) jest skuteczna w leczeniu raka jelita grubego. Jednak efekty PDT *in vivo*, ze względu na niski poziom oksygenacji guza, mogą odbiegać od działania PDT *in vitro*. Dane eksperymentalne potwierdzają rolę cholesterolu w procesie onkogenezy i wskazują na zaburzony szlak syntezy cholesterolu, m.in. w raku jelita grubego. Statyny, np. lowastatyna, należą do inhibitorów 3-hydroksy-3-metylo-glutarylo-koenzymu A (HMG-CoA) − enzymu szlaku syntezy cholesterolu. Potencjał antynowotworowy statyn, antyproliferacyjny i proapoptotyczny, wykazano w nowotworach *in vitro* i *in vivo*. Rak jelita grubego, cechujący się mutacjami genów związanych z apoptozą, wykazuje oporność na standardowe chemioterapeutyki. Celem pracy było zbadanie efektu antyproliferacyjnego i cytotoksycznego lowastatyny i ALA-PDT (w niskiej dawce) działających osobno, oraz w metodzie połączonej (lowastatyna i PDT) – na indukcję apoptozy w przerzutowej linii raka jelita grubego SW620. Wyniki pokazały, że lowastatyna hamuje wzrost komórek w związku zależnym od dawki i czasu inkubacji. ALA-PDT w niskiej dawce działa cytotoksycznie, ale nie inicjuje apoptozy. Natomiast preinkubacja komórek raka z lowastatyną (IC50) zwiększa efekt cytotoksyczny po PDT i indukuje apoptozę w komórkach, co wykazano w teście z aneksyną V na cytometrze przepływowym. Nasze wyniki po raz pierwszy demonstrują skuteczność metody łączonej: ALA-PDT i inhibitora syntezy szlaku cholesterolu – lowastatyny, w zwiększeniu cytotoksyczności i indukcji apoptozy w komórkach raka jelita grubego, opornego na chemioterapię.

Słowa kluczowe: lowastatyna, kwas aminolewulinowy, terapia fotodynamiczna, apoptoza

1. Introduction

Colorectal cancer is one of the most prevalent type of cancer in the population, while having a relatively unfavourable prognosis. There are many risk factors associated with Western world lifestyle that contribute to it, including the presence of numerous chemicals in the environment, a diet based on processed food containing saturated fats, smoking, and minimal physical activity. Incidence of colorectal cancer is most often observed in the elderly people challenged with developed atherosclerosis, high cholesterol and cardiovascular events [1].

Results of experimental studies corroborate the role of cholesterol in oncogenesis, indicating the faulty cholesterol synthesis pathway in many cancers including colorectal cancer. Recent studies suggest that this pathway play an important role in proper functionality of the cell. The mevalonate (MVA) pathway produces various end products, such as non-sterol isoprenoids, geranylgeranyl transferase (GGTP), farnesyl transferase (FPP) which play an important role in protein synthesis, cell proliferation, cytoskeletal organization and cell signalling pathway. It has been observed that in various cancer types, e.g. leukaemia, lung carcinoma, liver cancer, and colorectal cancer, the activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase is increased.

Statins are drugs that inhibit HMG-CoA reductase. By blocking the conversion of HMG-CoA into mevalonate they limit cholesterol biosynthesis. Therefore, they are widely used for the treatment of cardiovascular events and hypercholesterolemia. It is for the pleiotropic effects of statins, concerning inhibition of mevalonate pathway and inhibition of protein prenylation, that makes them emerge as a group of compounds that might be potentially used in prevention of cancer and oncological therapy.

Current experimental studies emphasise the role of statins in the inhibition of growth of various types of tumors, pointing towards their cytostatic, cytotoxic, and even pro-apoptotic properties. Depending on the cell type, statins trigger different molecular events. It has been shown that statins have antiproliferative effects on the cells of pancreatic adenocarcinoma, colorectal cancer, melanoma, lung carcinoma, neuroblastoma, and glioblastoma. *In vitro* studies proved that via inhibition of MVA, statins inhibit isoprenylation of such proteins as Ras and Rho which mutations are encountered in many

neoplasms with frequency as high as 50% in colorectal cancer, 90% in pancreatic adenocarcinoma and 50% in thyroid cancer.

A cytotoxic effect of statins such as lovastatin or simvastatin has been proved in the activation of caspases during the induction of apoptosis on HL-60 leukaemia and prostate cancer cell lines. Pro-apoptotic properties of statins are also associated with the decrease of cholesterol concentration in cell membrane and a change in the conformation of lipid rafts [2]. It is thought that the apoptotic effect of statins stems from their impact on various signalling pathways dependent on MAP kinases (MAPK), which was observed *in vitr*o on leukaemia cell lines. Results of statins activity are very diverse and depend on cancer type, administered dose, time of activity, and statin type [3]. The mechanisms of their anti-cancer activity are, however, poorly understood and are a subject of intense experimental investigation.

A traditional method of colorectal cancer treatment is surgical resection followed by chemo- or radiotherapy. Conventionally applied chemotherapeutics are not always effective, and oftentimes they exhibit high organ-specific toxicity and a lack of specificity towards cancerous tissue. A significant cause of cancer treatment failure lies in the phenomenon of multidrug resistance (MDR). Moreover, tumour cells develop mechanisms that dysregulate apoptotic pathways resulting in resistance to cytotoxic agents. Suppression of apoptosis is frequently associated with increased expression of anti-apoptotic proteins, and decreased expression of pro-apoptotic proteins [4]. Therefore, there have been attempts to utilize different therapy strategies aiming to increase anti-cancer outcomes in a selective way that would be free of side effects.

Photodynamic therapy (PDT) is a contemporary form of light therapy which has been utilized clinically in diagnosis and treatment of various noncancerous and cancerous disorders. Its therapeutic activity lies in photocytotoxic reactions triggered with a photosensitizer and a light of certain wavelength necessary to excite it. The photosensitizer is a chemical compound that accumulates selectively in pathological cells. The mitochondria are the most suited localisation for the photosensitizer to obtain optimal cytotoxic efficacy due to PDT. As a consequence of exciting with light reactive oxygen species (ROS) are created, one of them being singlet oxygen, which triggers oxidative stress that destroys the cell from the inside. It is worth mentioning that not every type of cancer is equally sensitive to PDT with any type of photosensitizer – there are cancer types resistant to PDT. The possible ways of cell death triggered by PDT include apoptosis, necrosis, autophagy or combination of the above. It is claimed that shorter period of light excitement induces apoptosis whereas longer excitements cause necrosis. PDT activity does not affect the tumor cells exclusively – it also indirectly contributes to the occlusion of blood vessels present in the tumor microenvironment and the activation of immune system.

Currently, PDT is utilized in the treatment of pre-cancerous lesions and superficial melanoma, endoscopically accessible cancers like urinary bladder cancer, digestive tract cancer, lung cancer located in the bronchi, and as a palliative method in the treatment of more advanced cancers. One of its advantages is the possibility to combine it with other therapies, minor side effects, the possibility of multiple application and non-invasiveness. On the other hand, the limitations of PDT include insufficient penetrability of the light through a cancerous tissue where the photosensitizer accumulated at. Moreover, the most commonly used compound in PDT, 5-aminolevulinic acid (ALA), the precursor of the actual photosensitizer, has a hydrophilic nature, which hinders its ability to penetrate through cell membrane and incorporate into a cell. The responsiveness of cancerous cells to ALA-PDT is still unclear and a subject to many experimental investigations. In order to enhance therapeutic efficacy, a combination of various therapeutic methods is currently being tested. The literature concerning the combination of interaction between PDT and statins is scarce.

In this paper, we present a novel approach that makes use of a combination of ALA-PDT (in low dose) and lovastatin in the SW620 colorectal cancer metastatic cell line. These cells have undergone multiple gene product modifications that dysregulate apoptotic pathways resulting in a resistance to cytotoxic stimuli. The aim of the study was to examine whether a combination of PDT with an inhibitor of cholesterol synthesis – lovastatin – can exert stronger cytotoxic effect and anti-cancer activity in colorectal cancer cells. Moreover, we sought to assess the anti-proliferative impact of lovastatin acting alone in SW620 cell line. Our results indicate the possibility of synergistic combination of the two methods as a new modality for anti-cancer therapy in the future.

2. Materials and methods

2.1. Cell line

We used human colon cancer cell line: SW620. The cell lines were obtained from the Institute of Immunology and Experimental Therapy, Polish Academy of Science. Cells were grown in DMEM (Gibco) medium, supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) Sigma 2 mmol/L glutamine and 10% fetal bovine serum (FBS) as monolayers, in 25 cm^2 Falcon flasks. The cells were maintained in a humidified atmosphere at 37 °C and 5% carbon dioxide. Cells were kept sub-confluent, media were changed. Next, cells were removed from culture flasks by trypsynization, then washed with phosphate-buffered saline (PBS) and prepared for the experiments.

2.2. Reagents

Lovastatin (Lova) in inactive form, was purchased from Merck Research Laboratories. The drug was converted to lactone, active form by dissolving in ethanol, adding of 0.1 M NaOH, heating at 50 °C for 2 hours, neutralizing the solution with 0.1 M HCl to pH 7,2 and finally adjusting with distilled water to a volume of 20 ml. Stored as a 5 mg/ml stock solution at 4 °C.

5-aminolevulinic acid (ALA) – precursor of sensitizer, from Sigma-Aldrich, was used in the experiments, in a concentration – 1 mM.

2.3. Cell viability MTT assay

In order to obtain desired results (cytotoxicity below 50% post PDT), preliminary studies were conducted at different doses of ALA concentrations and light illuminations.

Viability of cells was determined by the thiazolyl blue tetrazolium bromide assay (MTT), (Sigma-Aldrich), which is based on the reduction of a yellow soluble tetrazole to an insoluble purple formazan, which occurs only in living cells. Briefly, the cells were seeded on 96-well plates (1x1000 cells/well). This allowed to attach cells to the plate. Next:

a) the medium was removed and replaced with fresh medium with ALA, for 4 hours of incubation, and then cells were irradiated. MTT assay was evaluated 24 hours after PDT. Control SW620 cells were treated: with ALA and no light, with light only, and without any procedure.
b) in experiment with Lova only, cells were exposed to different concentrations of Lova (0−100 μM) in serum complete medium, for 24h and 48h. Next, cells were subject to cell proliferation MTT assay. For a main experiment we used Lova (MTT 50).
c) in combined experiment (Lova + ALA-PDT), cells SW620 were preincubated with Lova for 24 hours, before illumination. Next, the cells were replaced with fresh medium with ALA, incubated for 4 hours, and then irradiated. After illumination, the cells were washed twice in PBS, replaced with fresh medium and incubated for 24h. Next, cells were treated according to manufactures instructions for MTT assay. The absorbance was determined by spectrophotometer at 570 nm (Labsystem Multiscan MS 352). Mitochondrial activity of cells was evaluated by percentage of viable cells relative to control group cells (without light, ALA and Lova, respectively). All cytotoxic experiments were repeated for three times.

2.4. Photodynamic treatment

In the experiments, cells were incubated with precursor of photosensitizer ALA in a complete medium, in the dark, for 4 hours. Then, medium was replaced on the medium without phenol red and serum, and cells were irradiated with red light. After illumination, the cells were incubated in fresh, standard medium, at 37 °C in an atmosphere of humidified air with 5% CO_2.

All irradiations were performed using a halogen lamp (Penta Teclas, Switzerland) at an excitation wavelength of 630 ± 20 nm, selected with a bandpass filter. Fluence rate was: 50 m/Wcm^2 and total light dose: 3, 5, and 7 J/cm^2.

2.5. Determination of apoptosis using the Annexin V Assasy by flow cytometry

In order to examine whether low dose of PDT (cytotoxicity below 50%) and dose of Lova (IC_{50}) induce apoptosis in SW620 cells, apoptosis was examined 2 hours post illumination.

Apoptosis analysis was measured independently: after PDT, and after combined treatment (ALA+PDT).

Cells (20000 cells/well) were cultured in 6-well tissue culture plates (EuroClone, Italy), 20 000 cells/well. Cells were divided into 3 groups: (1) control (untreated cells); (2) cells exposed only to PDT; and (3) cells treated by combined method: 24h preincubation lovastatin 30 μM and then exposed to ALA-PDT. Additionally, apoptosis induction was evaluated after incubation of SW620 cells with lovastatin (IC_{50}) alone up to 48h. Cells were harvested by the addition of trypsin, centrifuged for 5 min at 1000x, and finally washed with PBS. Cells were stained according to the kit's protocol, and were analyzed by a BD FACS Canto II Flow Cytometer (BD Biosciences, San Jose, CA, USA) with use of Annexin V-FITC Apoptosis Detection Kit (BD Bioscience, San Jose, USA). The determinations were performed in 3 duplicates.

3. Results

3.1. Cytotoxicity of ALA on SW 620 cell line

SW 620 cells were incubated for 24 hours with increasing concentrations of a drug (1 mM, 3 mM, and 5 mM ALA), and further their viability was checked with MTT test. It has been observed that 1 mM concentration of ALA did not cause significant effect (MTT 99%), however concentrations 3 mM and 5 mM, have caused a significant decrease in viability up to 91% MTT 88% MTT, respectively. Basing on this data 1 mM ALA has been chosen for further experiments.

3.2. Cell viability SW620 line post irradiation

In the preliminary study, control SW620 cells were subjected to the following doses of illumination: 3 J/cm^2, 5 J/cm^2, and 7 J/cm^2, and then were assessed for viability after 24h using MTT test. It was observed that the more the cell were illuminated the less viable they were. The application of 3 J/cm^2 does not affect cells viability (100%), however using 5 J/cm^2 yielded cells viability of 93%. Using 7 J/cm^2 caused cells viability to decrease to 87%. In control cells, which were not illuminated, no cytotoxic effect was observed.

3.3. Cell viability SW620 post PDT

In order to assess photocytotoxicity, SW620 cells were incubated for 4h with ALA (1 mM), and then illuminated with the rate of 50 mW/cm^2. Initially, cells were illuminated using two doses of light: 3 J/cm^2 and 5 J/cm^2, and viability was confirmed using MTT test. Exposure to light dose of 3 J/cm^2 lowered cells viability to 76%. In the case of 5 J/cm^2 viability of cells decreased to 59% after illumination, with dose of 7 J/cm^2 to 38%. Obtained results showed that the photodynamic effect with ALA causes cytotoxicity of SW620 cells and increases with the doses of light density. For an actual experiment, the dose of 5 J/cm^2 was selected – one that caused cytotoxicity below 50% (see Fig. 1).

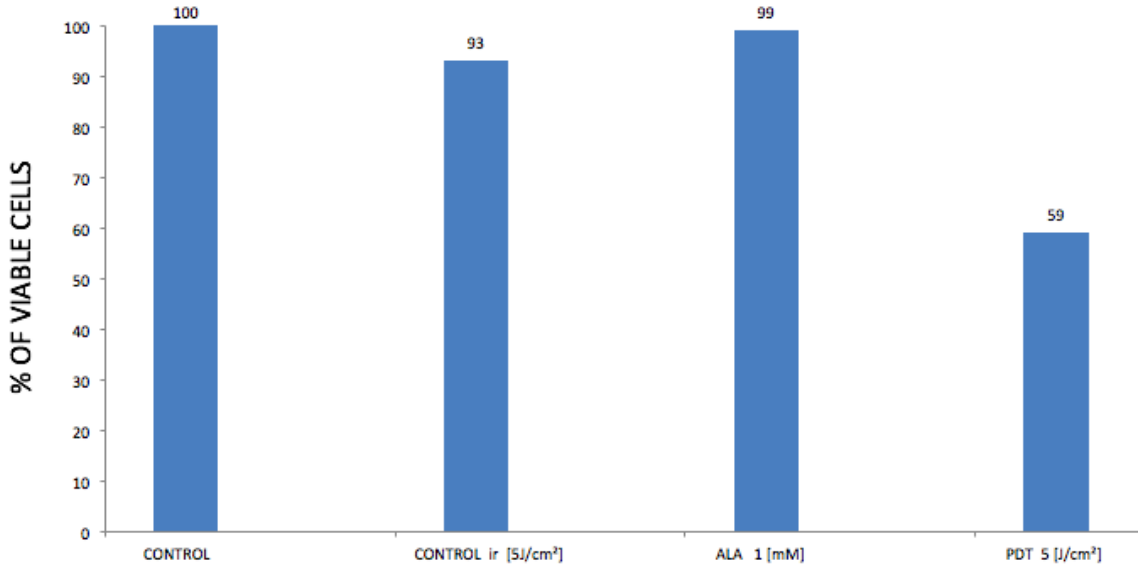

Fig. 1. Percent of viable cells determined by the MTT assay. Photodynamic therapy by means of 5-aminolevulinic acid (5-ALA) decreased the viability of SW620 cells. Cells were treated with equal concentration of the above precursor for 4h (5-ALA) and then irradiated with 5 J/cm^2 at 630 ± 20 nm (ir = irradiation).
Cell viability was measured after 24 h following irradiation.

3.4. Cell viability SW620 post incubation with lovastatin

Experiments showed that the viability of cells after 24h of incubation drops insignificantly, independently of lovastatin concentration. Doses of 10 µM and 30 µM of lovastatin caused the decrease of viability of 95% and 92%, respectively. At the concentration of 100 µM, the viability of cells decreased to 89% after 24h of incubation. Only a longer incubation, that of 48h, yielded a significant gradual decrease of cells viability along with an increase of molecule concentration. The dose of lovastatin of 30 µM causes cells viability to drop to 50%, while that of 100 µM to 39%. The obtained results point to an impact lovastatin has on the viability of SW620 cells in a time and dose dependent manner (see Fig. 2).

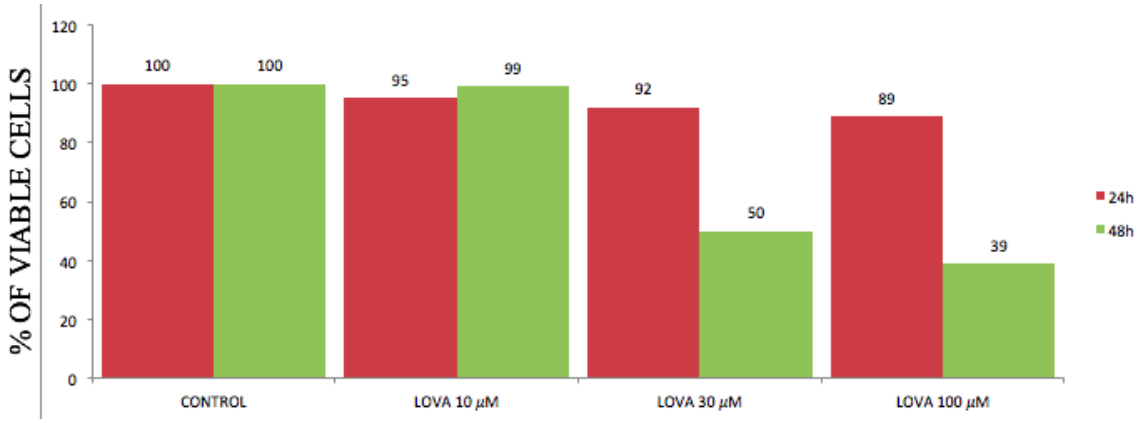

Fig. 2. Effect of lovastatin on the cell viability in SW620 cells. Cells were treated with lovastatin (0−100 µM) for 24h and 48h, and the proportion of surviving cells was measured by the MTT assay

3.5. Cell viability SW620 post incubation with Lova + PDT

The viability of SW620 cells preincubated with lovastatin (30 µM) for 24h and then subjected to photodynamic reaction (5 J/cm^2). A decrease of 43% cells viability was observed. A combination of those two therapies – ALA and PDT caused a greater cytotoxic effect than after applying those methods

alone. The incubation of cells with ALA and lovastatin without light illumination, did not affect the cells viability (see Fig. 3).

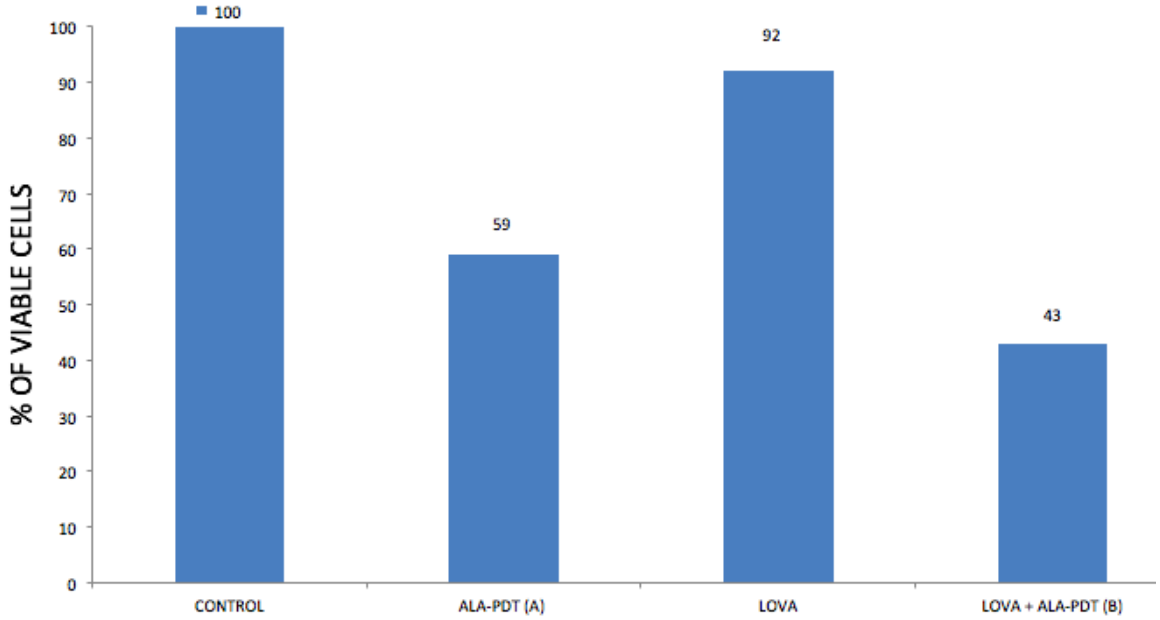

Fig. 3. Effect of combination treatment with lovastatin and ALA-PDT in low dose on the cell viability – the proportion of surviving cells was measured by the MTT assay. Cells SW620 were treated with 30 μM lovastatin (preincubation) and then irradiated PDT. A – after PDT alone. B – after combined method.

3.6. Determination of apoptosis using the Annexin V Assasy by flow cytometry

For quantitative assessment of the induction of apoptosis by combined method of: lovastatin and ALA-PDT, we measured the proportion of cells in the sub-G1 phase by flow cytometric analysis. Treatment of the cells with 30 μM lovastatin increased the sub-G 1 DNA content. To further assess apoptosis, Annexin V-FITC dye was utilized to detect the translocation of phosphatidylserine from the inner (cytoplasmic) leaflet of the plasma membrane to the cell surface. Our findings show that 24h incubation cancer cells SW620 with lovastatin (30 μM) and then illuminated by ALA-PDT induced 25.29 % of apoptotic cancer cells SW620 (see Fig. 4).

Our results did not prove the induction of apoptosis in cells after 48h with lovastatin alone (data not show).

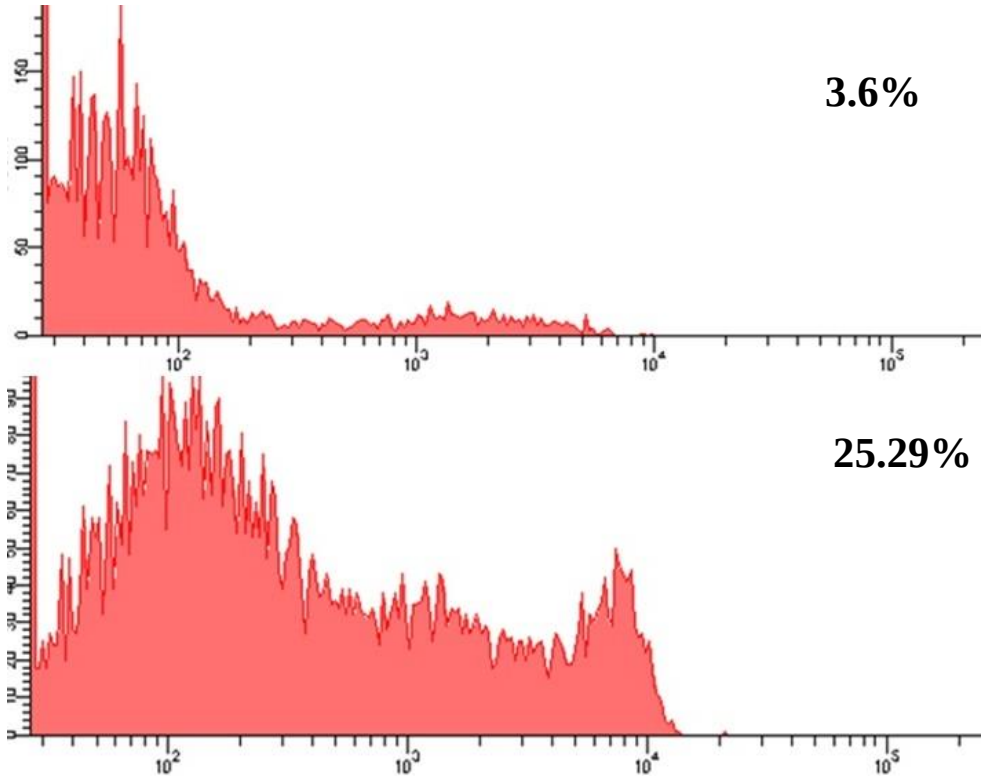

Fig. 4. Representative example of flow cytometry analysis of apoptosis in cells treated with (A) ALA-PDT alone and (B) combined method 30 μM lovastatin (preincubation) and ALA-PDT – in low dose. Cells were stained with FITC-labelled Annexin V. Indicated in the panels are the percentages of positive treated cells (3.6% vs 25.29%). The experiments were performed in triplicate.

4. Discussion

The effectiveness of photodynamic therapy (PDT) in treating cancer has led to the popularity of its use in clinical practice. One of the approaches of currently conducted research is the elucidating of mechanisms underlying the synergy of PDT and various chemical agents or other therapies on anti-cancer effect. However, the impact of statins on PDT in colon cancer remains unclear.

Many studies reported that statins exhibit anti-cancer effect on various types of cancer. Among them is colon cancer which has been shown to be associated with an overexpression of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMG-CoA reductase, HMGCR) – an enzyme involved in the malfunction of cholesterol metabolism. It is stipulated that the extent to which the cholesterol metabolism is dysregulated contributes to the responsiveness of colon cancer to statin activity. The lipophilic statins, lovastatin included, are considered more therapeutically potent as they penetrate the cell membrane relatively more readily via passive diffusion due to their lipophilic character.

In the following study SW620 cell line has been used to determine the responsiveness of colon cancer to lovastatin treatment. We show here that lovastatin has an anti-proliferative effect on colon cancer cells and causes an inhibition of their growth in a time and dose-dependent manner. We were able to show a significant effect of lovastatin after 2 days of incubation (IC_{50}) and at the concentration of 30 μM. Choi and Chung proved that lovastatin at the concentration of 10 μM is sufficient to trigger anti-proliferative effect on C6 glioma cell line [5]. By contrast, in acute myeloid lymphoma (AML) cell line, lovastatin should be administered at the concertation as high as of 70–127 μM to yield an anti-proliferative outcome [6]. In experiments conducted on MCA-38 colon cancer cell line, lovastatin caused dose dependent inhibition of growth: in low doses it had an anti-proliferative effect, and in higher doses it exhibited pro-apoptotic characteristics. Experiments on neuroblastoma cells and acute myeloid leukaemia with P glycoprotein (Pg-P) overexpression showed that they are especially sensitive to Lovastatin activity, which is linked to its impact on the decrease of Pg-P expression.

Various sensitivity to lovastatin activity depends not only on the type of cancerous cells and their

source but also on the genetic mutations those cell bear. Between 30 and 40% of colon cancer cases carry mutations within Ras oncogene. The SW620 cell line used in this study harbours mutation in Ras oncogene. Agarwal et al. proved that HT29 cell line that does not carry Ras mutations are least sensitive to lovastatin and that the presence of mutated Ras oncogene yields high anti-cancer potency of lovastatin [7]. Ras proteins are located in the cell membrane and are involved in control of MAP kinases that control processes such as: cell cycle, proliferation, and apoptosis. Their functioning is closely linked to hydrolysis of GTP and directly dependent on the products of mevalonic pathway (MVA). The cells with a mutated Ras oncogene are being subjected to the activation of PI3/Akt pathway which results in uncontrolled proliferation. Lovastatin by inhibiting MVA blocks Ras protein prenylation. Consequence of this occurrence is the deactivation of MAPK pathway, which leads to proliferation of cancer cells.

The anti-proliferative effect of statins stems from their ability to regulate the expression of proteins involved in cell cycle control. Experiments conducted on Caco-2 cancer cell line showed the inhibition of growth of those cells, which was a consequence of the activity of cyclin-dependent kinases (cdk1 and cdk2) and cyclin D [8]. It was suggested that the inhibitory impact on cyclin D could be a result of the inhibition of cell signalling pathway involving Ras proteins [9]. Jakobisiak and colleagues proved that lovastatin influenced the cell cycle in a time and dose dependent fashion, i.e.: short incubation (24h) stalled cell cycle at G1/S phase, in turn however, days-long incubation stalled the cycle at G2/M phase [10]. In one experiment involving murine breast cancer model lovastatin was shown to be inhibiting cell cycle in cells harbouring mutation in p-53 gene [11]. Considering the fact that nearly a half of cancer types carries a mutated p-53 gene, SW620 cells included, the use of lovastatin as a cell cycle modulator could be a very useful tool in anti-cancer therapy.

Anti-proliferative characteristics of statins that stem in their pleiotropic activity, were used in experimental anticancer therapies with a usage of different therapeutic agents. In experimental studies on cell lines it has been shown that a combination of statins with nonsteroidal anti-inflammatory drugs (NSAID's), inhibitors of the EGFR family, TRAIL receptor ligands, cisplatin or doxorubicin have shown synergy on growth inhibition and enhanced efficacy against colorectal cancer [12, 13]. Furthermore, in other publication it has been demonstrated that atorvastatin and celecoxib led to significant growth suppression and killing of colon cancer cells by induction of apoptosis resulting from caspase activation and modifications associated with small G-proteins. However, mechanisms of synergic therapies including statins and other agents are still poorly understood [14].

Photodynamic therapy is a potentially useful method in treating benign and malignant tumors, as well as in palliative treatment. In order for it to be used, it must be completely safe and pose no risk of inducing tumorigenesis in residual cancer cells that survives the treatment in the pathological tissue. *In vitro* studies conducted in normoxia are not an ideal model for hypoxic tumor microenvironment and thus poorly reflect the way PDT works *in vivo*. Therefore the cytotoxic effect of PDT in hypoxia might be too weak (like low dose PDT) and cause adaptive response of the cells when challenged with the stressor (photooxidation), leading to unfavourable consequences, like a delay or inhibition of apoptosis and an induction of response to pro-survival factors present in the tumor microenvironment [15]. In order to enhance anti-cancer effect and at the same time diminish pro-survival events post PDT, a synergistic combination of various agents and PDT is currently under robust scientific investigation [16, 17, 18].

Important factors that can influence efficiency of PDT are: types of cancer cells, type of photosensitizer, and its intracellular localization and dose of light. Colorectal cancer show resistance to conventional anticancer drugs experimentally and in clinical treatment. The reason for this are mutations in the genes controlling apoptotic machinery. Many studies show that photodynamic therapy with ALA is a very effective way of treating colorectal cancer [19, 20, 21, 22, 23]. Our study show that cancer cells are sensitive to photodynamic treatment which results in a decrease of growth rate (76%, 59%, 38% respectively) and the cytotoxic effect is dependent from a light dosage (3, 5, and 7 J/cm^2) and a concentration of sensitizer. Similar results were obtained by Yang et al. working on the influence of hematoporphyrin (HpD) on viability of LoVo and Colo25 [24]. Cytotoxic effect shown by them correlated with the increase of light dose (2−20 J/cm^2) and photosensitizer (0.5−4 μg/ml).

In the following study, we used PDT parameters that elicit a cellular effect that leads to a cell survival higher than 50%. The obtained growth inhibition of SW620 cells post PDT indicates that the used dose

was cytotoxic and damaged the mitochondria. However, apoptosis was not indicated by Annexin V technique. The mechanism of ROS activity following ALA-PDT includes many subcellular targets and goes beyond the photodamage of the mitochondria. Oxidative reactions damage proteins and phospholipids present in the cell membrane. A faulty cell membrane may lead to a general increase of intracellular damage, including those involving mechanisms dependent on secondary signals mobilized within the cell. The lack of apoptosis in SW620 cells was anticipated due to low dose PDT, however the lack of apoptosis after high dose PDT was an interesting finding. We hypothesize that the direct mitochondrial photodamage induced by high dose PDT was still insufficient to trigger apoptosis in only 2 hours after treatment. PDT-induced apoptosis was categorized by Godar as immediate, intermediate and delayed [25].

ALA is a precursor of a main photoactive agent – protoporphyrin IX which is synthesized in mitochondria via heme biosynthetic pathway. However, the response of tumor cells on phototoxicity action depends on the activation of the enzymes from heme biosynthesis pathway, which may be defective in cancer cells [26, 27]. Mitochondria play pivotal role in energy production. The rapid and continuous proliferation of cancer cells requires high level of energy and that is why interruption of mitochondrial function may lead to cell death. It has been speculated that mitochondria are crucial for inducing cell death in PDT as a result of endogenous apoptotic pathway initiation. Cellular photo-damage after ALA does not always lead to efficient cell death by triggering apoptosis. Moreover the type of response may vary depending on damaged targets and level of phototoxicity. Not sufficient dose of PDT may not work cyto-lethally on tumor cells.

In the following work, we showed for the first time that linking cholesterol synthase inhibitor − lovastatin with ALA-PDT, increases anti-proliferative effect on cancer cell line 620. Cytotoxic effect as a result of synergistic therapy increased the level of inhibition of cancer cells (43%) contrary to PDT alone (59%). We obtained this effect after 24 hours treatment of the cells with Lova (IC_{50}), what initiated the mechanism of apoptotic death showed in Annexin V test. Although Lova alone did not induce apoptosis in SW620 cells, even after 48 hours of treatment, it increased cytotoxic stimuli, which were not sufficient to induce apoptosis through low dose of PDT alone. Molecular mechanisms of pro-apoptotic action of statins are very complex, as they can interfere with many proteins of different cellular functions. Statin by inhibiting MVA cause blockage of prenylation of Rho and Ras proteins. Mutations in these proteins are commonly observed in many different human cancer cells (colon cancer 50%, pancreatic cancer 90%). Hoque et al. noticed that lovastatin has pro-apoptotic effect on prostate cancer cell by decreasing the activity of Rho proteins (inactive Rho residues in cytoplasm, active Rho anchors in cellular membrane) [28]. It has been notified that as a result of blocking Rho protein via statins, expression of Bcl-2 anti-apoptotic protein has been lowered. Overexpression of Bcl-2 protein, which is very common in variety of cancers, may prevent the mitochondria of tumor cells, decreasing cytochrome c release or inhibiting apoptosis following PDT [29, 30]. As SW620 cells have mutation in apoptosis inductor factor (AIF), influence of statins on pro- and anti-proapoptotic proteins ratio (Bax/Bcl-2) in a way of increased Bax level, may enhance efficacy of PDT in induction of mitochondrial apoptotic pathway.

Induction of apoptosis in SW620 cells which we obtained as a result of additive therapy of Lova and ALA-PDT may rely on the influence of statins on a decreased membrane cholesterol concentration, conformational change of lipid rafts and activation of membrane receptors Fas/APO1 in cancer cells. Increase of the expression of these receptors and caspase 8 activation may enhance mitochondrial apoptotic pathway and in consequence lead to cancer cell death. Lovastatin may be effective in elevating death signals from upstream events and can cause induction of apoptosis in post PDT cancer cells which have mutations affecting mitochondrial apoptotic machinery. Moreover, lovastatin via influencing cellular membrane, may modulate signalling pathway in direction of nucleus and inhibit NF-KB [31]. This may increase cytotoxicity and be responsible for initiation of apoptotic signals. Usage of lovastatin in combination with PDT seems to be in high importance, as it has been shown that photodynamic action increases the expression of NF-KB factor in the cells illuminated and grown in hypoxia what may occur in PDT *in vivo*. Taking all together pleiotropic action of statins, influencing variety of intracellular alternative pathways, may ignite programed cell death via strong enhancement of photooxidative damage effects on cancer cells following PDT.

In our study, we confirmed the findings of fellow investigators concerning anti-proliferative activity of lovastatin and cytotoxic activity of ALA-PDT, independently of each other in SW620 colon cancer cell line. However, for the first time, we showed that a combination of those two treatments enhances anti-cancer activity in colon cancer and induces apoptosis in SW620 cell line.

In conclusion, the aim of our study was to enhance anti-cancer efficacy and induction of apoptosis by combining two different therapeutic agents which cellular effects complete and potentiate one another. Our finding indicates for the first time that a combined therapy of lovastatin and ALA-PDT can enhance cytotoxic and anti-proliferative effect on colon cancer cells through induction of cell death via apoptosis. However so, further studies are necessary to explain molecular mechanisms underpinning programmed cell death in cancer cells as a result of synergistic activity of statins and photodynamic therapy. Finally, we want to emphasize that our study brings a promising strategy for treatment of cancer types that are resistant to chemotherapeutics and our strategy can be useful to circumvent many cellular factors that inhibit or delaying apoptosis.

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