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Therapeutic Efficacy of Malachite Green-Based Photodynamic Therapy in Acute Myeloid Leukemia

Akut Miyeloid Lösemide Malahit Yeşili-Bazlı Fotodinamik Tedavinin Terapötik Etkinliği

Sercin Ozlem-Caliskan[1], Omer Faruk Duran[2], Cem Aslan[2], Husne Ozen[2], Metin Caliskan[3], Rahsan Ilikci-Sagkan[3]

[1]Usak University School of Medicine Department of Biopyhsics, Usak, Turkey
[2] Student at Usak University, School of Medicine, Usak, Turkey
[3]Usak University School of Medicine Department of Medical Biology, Usak, Turkey

Abstract

Aim: Acute myeloid leukemia (AML) is a disease characterized by relapse and treatment resistance in most patients. Therefore, there is a need for targeted therapies in AML. Photodynamic therapy (PDT) is a promising alternative for the treatment of malignant tumors. Also, PDT has the potential to be used individually or complementally in the treatment of leukemia. In this study, it was aimed to investigate possible the effect of malachite green (MG)-based PDT on acute myeloid leukemia cells.

Material and Method: Cells were incubated with 0.19, 0.39, 0.78,1.56, 3.125, and 6.25 µM MG for one hour and irradiated with 46.4 J/cm² of light. The trypan blue test was used to assess the viability of cells, and the change in mitochondrial activity was determined by MTT. Morphological features were determined by Giemsa staining and scanning electron microscopy. Cell cycle and Annexin V/PI assays (measuring fluorescence emitted by staining reagents) were measured by flow cytometry.

Results: With the combination of MG and light, HL60 cell viability was found to be significantly reduced compared to the control group. Giemsa staining and SEM results showed that 3.125 µM MG-based PDT induced various morphological changes in cells typical for apoptosis. Late apoptosis was observed in cells treated with 3.125 µM MG combined PDT according to Annexin/PI staining, further showing that it caused an arrest in the subG1 phase of the cell cycle.

Conclusion: MG-based PDT has the potential to inactivate HL60 cells. Thus, MG-based PDT may ensure a promising approach for treating acute myeloid leukemia cells.

Keywords: HL60 cells, Malachite green, Photodynamic therapy, apoptosis

Öz

Amaç: Akut miyeloid lösemi (AML), çoğu hastada nüks ve tedavi direnci ile karakterize bir hastalıktır. Bu yüzden AML de hedefleme tedavilerine ihtiyaç vardır. Fotodinamik tedavi (FDT), malign tümörlerin tedavisi için umut verici bir alternatiftir. Aynı zamanda FDT, lösemi tedavisinde tek başına veya tamamlayıcı olarak kullanılma potansiyeline sahiptir. Bu çalışmada malahit yeşili (MG) aracılı FDT'nin akut miyeloid lösemi hücreleri üzerindeki olası etkisinin araştırılması amaçlanmıştır.

Gereç ve Yöntem: Hücreler 0,19, 0,39, 0,78, 1,56, 3,125 ve 6,25 µM MG ile bir saat süreyle inkübe edildi ve 46,4 J/cm² ışık ışınına tabi tutuldu. Hücrelerin canlılığını değerlendirmek için tripan mavisi testi kullanıldı ve mitokondriyal aktivite değişikliği MTT ile belirlendi. Morfolojik özellikler Giemsa boyama ve taramalı elektron mikroskobu ile belirlenmiştir. Hücre döngüsü ve Annexin V/PI testleri (boyama reaktifleri tarafından yayılan floresan ölçümü) akım sitometrisi ile ölçüldü.

Bulgular: MG ve ışık kombinasyonu ile HL60 hücre canlılığının kontrol grubuna göre anlamlı derecede azaldığı bulunmuştur. Giemsa boyama ve SEM sonuçları, 3,125 µM MG aracılı FDT'nin,hücrelerde apoptoz için tipik olan çeşitli morfolojik değişikliği indüklediğini göstermiştir. Annexin/PI boyamasına göre 3,125 µM MG kombine FDT ile tedavi edilen hücrelerde geç apoptoz gözlenmiş, ayrıca hücre döngüsünde subG1 fazında bir durmaya neden olduğunu göstermiştir.

Sonuç: MG aracılı FDT, HL60 hücrelerini inaktive etme potansiyeline sahiptir. Bu nedenle, MG bazlı FDT, akut miyeloid lösemi hücreleri için umut verici bir yaklaşım sağlayabilir.

Anahtar Kelimeler: HL60 hücreleri, Malahit yeşili, Fotodinamik tedavi, apoptoz

Corresponding *(İletişim)*: Sercin Ozlem-Caliskan, Usak University School of Medicine Department of Biopyhsics, Usak, Turkey
E-mail *(E-posta)*: srn.ozlem@gmail.com
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INTRODUCTION

Photodynamic therapy (PDT) is a non-invasive method based on the simultaneous combination of a photosensitizer and light and the generation of reactive oxygen species in cells in the presence of molecular oxygen.[1] PDT consists of the combination of three different components: photosensitizer, light, and molecular oxygen. These components, which are non-toxic individually, form cytotoxic reactive oxygen species when combined. Once activated by visible light, the photosensitizer can react in two photooxidative pathways classified as type I and II.[2] Type I reactions involve the formation of free radicals via a photosensitizer. These radicals react with oxygen to form reactive oxygen species, including hydroxyl radicals, hydrogen peroxide, and superoxide anions. In a type II reaction, on the other hand, the photosensitizer transfers energy directly to oxygen to produce $^1O_2$, which induces apoptosis. Both reactions can lead to cell and oxidative damage, including necrotic, autophagic, and apoptotic cell death.[3,4]

PDT is an approved method for use in many countries for various types of cancer, Barrett's esophagus, age-related macular degeneration, actinic keratosis,atherosclerotic vascular disease, etc.[5] PDT has several advantages over traditional therapeutic methods, including repetitive treatment potential, minimal side effects, and potential for combination with other forms of treatment, including radiotherapy and chemotherapy.[6]

According to the course of the disease, leukemia can be classified as acute or chronic leukemia. Acute leukemia is usually characterized by overgrowth and rapid accumulation of immature malignant blood cells, whereas chronic leukemia is usually characterized by slower overgrowth of mature blood cells, and its progression may take months or even years.[7] Although current treatments for leukemia are mainly chemotherapy, radiation therapy, and allogeneic stem cell transplantation, these treatments can lead to serious late effects such as a drug resistance, high risk of infection, graft-versus-host disease,and cytotoxicity to normal cells.[8] Therefore, seeking alternative approaches has become the focus of research. PDT is a promising alternative to chemotherapy or radiation therapy for the treatment of malignant tumors.[9] One of the advantages of PDT is that the maximum cumulative dose in both radiation therapy and chemotherapy does not cause cumulative toxicity in the patient.[10] It accumulates in photosensitizing cancer cells at higher concentrations compared to normal cells.[11] With photosensitizer-based PDT, it can be applied locally to a certain area by selectively illuminating the lesion without damaging normal tissues.[12]

Malachite green (MG) is a triphenylmethane cationic dye obtained from dimethylaniline and benzaldehyde with an absorption band of approximately 617 nm and used for staining.[13] Triarylmethane dyes have received attention as anticancer and antimicrobial agents due to their structural properties and selective localization.[14] To the best of our knowledge, no studies have been conducted in HL60 cells to determine morphological imaging (Giemsa staining and SEM), cell viability, mitochondrial activity, and apoptotic effects (Annexin V-PI staining and SubG1 peak analysis) of MG-based PDT. This study aimed to determine the effects of MG-based PDT on acute myeloid leukemia cells in terms of cell viability, mitochondrial activity, morphology, and apoptosis.

MATERIAL AND METHODS

Cell Culture

HL60 myeloid leukemia cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 1 mmol/L L-glutamine, and 1% penicillin-streptomycin, and incubated at 37°C in a 5% humidified CO_2 incubator.

Photosensitizer

In this study, the MG, which is in the cationic structure, was used as a photosensitizer. It was dissolved in PBS. Concentration experiments were performed for MG at doses of 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.56, 0.39, and 0.19 µM. Since no alive cells were observed above 6.25 µM; 0.19, 0.39 ,0.78,1.56, 3.125, and 6.25 µM MG concentrations were used throughout this study. Cells ($1x10^5$) were exposed to MG for 1 hour at 37°C in the dark.

Experimental Design

Four different groups were formed in the PDT study using different MG concentrations. 1-Control group: No MG or No light; 2-Light Control: HL60 cells were exposed to light for 30 minutes; 3-MG group: HL60 cells were exposed to MG in all concentrations for one hour with no light; 4-MG-based PDT group: HL60 cells were exposed to MG for one hour and then exposed to light for 30 minutes

Photodynamic Therapy

After the cells were exposed to MG for one hour, they were centrifuged at 1000 rpm for five minutes, and free MG was removed from the medium. After the cells were washed three times with PBS, fresh PBS was added. A LED50 device with spectral ranges at λ=420 nm and 780 nm was used for irradiation, and a 550 nm longpass filter was used for the wavelength of MG. The light output was measured with a power meter (Newport, USA), and the cells were exposed to light at a distance of 10 cm for 30 minutes with an irradiance of 25.8 mW/cm² and a fluence of 46.4 J/cm².

Cell Viability and Mitochondrial Activity Assay

Cell viability was determined using the trypan blue exclusion method. At the end of treatment, 0.4% trypan blue dye was added to the 50 µL cell suspension. The number of viable cells was calculated by using the following formula: percentage of viable cells=[1.00-(number of blue cells÷total number of cells)]*100. The 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay was used to assess the mitochondrial activity of HL60 cells. MTT reagent was added to all groups 24 hours after the treatment and

incubated for 4 hours. At the end of the four-hour period, a solubilization buffer was added, and the cells were incubated overnight at 37°C. HL60 cells were evaluated with a microplate reader at 550 to 600 nm. Percentage of mitochondrial activity was calculated according to the following formula: %=[(Sample OD value- Blank OD value) / (Control OD value-Blank OD value)] × 100.

Giemsa Staining

Three samples were prepared from each group. The samples were dried on slides and fixed in methanol for 10 minutes. Then, the slides were stained with May-Grunwald for 1 minute, washed with distilled water, and kept at room temperature for 5 minutes with Giemsa staining (1:1). All samples were then backwashed with tap water and visualized with a light microscope.

Scanning electron microscope (SEM)

Cells in the control, light control, MG, and MG-based-PDT groups were fixed in 2.5% glutaraldehyde. The cells were then allowed to dry after a series of ethanol dilutions. After the samples were coated with palladium-gold, they were observed under a JSM 5600 model scanning electron microscope.

Annexin/PI staining

The apoptosis induced by MG-based PDT was analyzed by flow cytometry (BD Accuri C6 Plus, USA). Apoptotic cells represent green fluorescence. Late apoptotic cells represent both green and red fluorescence. Living cells show low fluorescence. After the treatments, 5 µl of PI and 1 µl of Annexin V-FITC were added to the cells and incubated for 15 minutes in the dark.Percentages of late and early apoptotic cells were determined for apoptosis.

SubG1 Peak Analysis

SubG1 peak analysis is a method used to detect cells that have lost some of their DNA in the late phase of the apoptosis. SubG1 analysis of treatment groups was performed using the BD Cycletest Plus DNA kit (BD Biosciences, USA). Post-treatment cells were centrifuged at 1000 rpm for 5 minutes. Trypsin buffer containing 250 µl solution was added to each sample. Then, 200 µl of solution containing Trypsin inhibitor and RNase buffer was added to the cells and incubated for 10 minutes. After 10 minutes of incubation, 200µl of buffer containing PI staining solution was added to the cells and incubated in the dark (10 minutes). The percentage of cells in SubG1 (apoptotic peak) was determined with the BD Accuri C6 Plus software.

Statistical Analysis

The results were calculated as mean±standard deviations (SDs). All data analyses were performed using SPSS 25 (San Diego, CA, USA). One-way ANOVA analysis of variance was used for data analysis, followed by Tukey post hoc test. A p value of <0.05 was considered a significant difference in all tests

RESULTS

Damage to the outer cell membrane was assessed by using the trypan blue exclusion test. Metabolically active cells with intact plasma membranes that could not divide were evaluated by the MTT test. It was determined that the cells in the group with MG-based PDT were significantly lower at 0.78, 1.56, 3.125 and 6.25 µM concentrations compared to the control group. The observed IC50 of MG-based PDT was 3.125 µM for HL60 cells. It was determined that the cells in the group with MG-based PDT were significantly lower at 0.78, 1.56, 3.125 and 6.25 µM concentrations compared to the control group. According to the trypan blue exclusion test, the light group did not show cytotoxic effects on HL60 cells.No effect was found on HL60 cells treated with light and MG groups. These results results indicated that MG-based PDT decreased the viability of HL60 cells with both the trypan blue exclusion test and the MTT (**Figure 1A** and **B**).

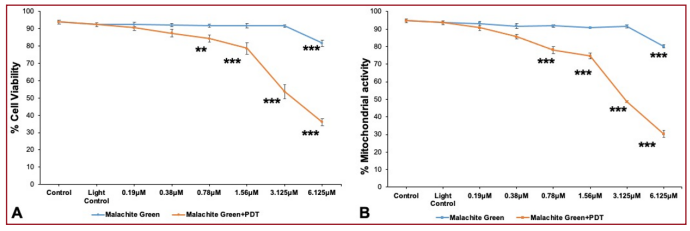

Figure 1. A. Evaluation of cytotoxicity after treatment with control, light control, MG and MG-based PDT. B. Evaluation of mitochondrial activity after treatment with control, light control, MG and MG-based PDT. The data represent the means±standard deviations (SDs) of 3 independent experiments. ** indicates statistically significance compared to control group ($p<0.001$);*** indicates statistically significance compared to control group ($p<0.0001$)

Giemsa staining and SEM were employed to assess morphological alterations in the HL60 cells exposured by MG-based PDT. In the 3.25 µM group, leukemia cells treated with MG-based PDT exhibited features not found in control cells such as chromatin condensation, irregular cytoplasmic contours (**Figure 2**).

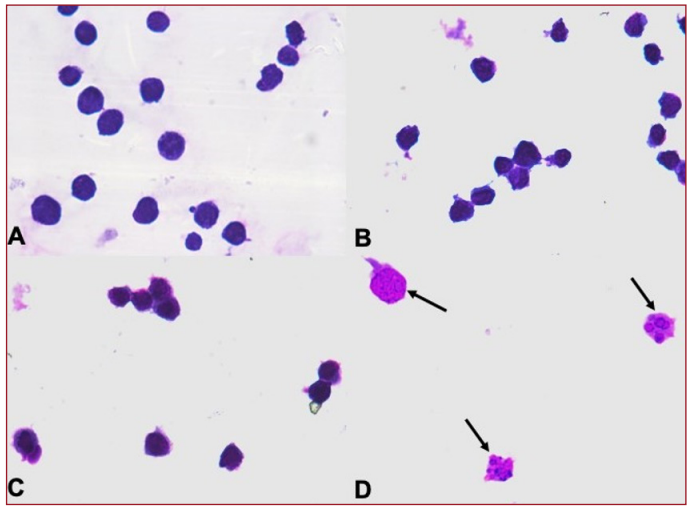

Figure 2. Morphology of HL60 cells with Giemsa staining for all experimental groups (x100). A. Control group; B. Light Control; C. 3.125 µM MG; D. 3.125 µM MG + PDT. Black arrows indicate apoptotic bodies and damaged cells

SEM analysis of the control, MG and light groups cells, revealed normal cell structure without cell damage. However, in the treatment group, some cells had shrunk in volume and showed typical apoptotic properties such as apoptotic bodies, holes, ırregular cytoplasmic contours, and broken cell membranes (**Figure 3**).

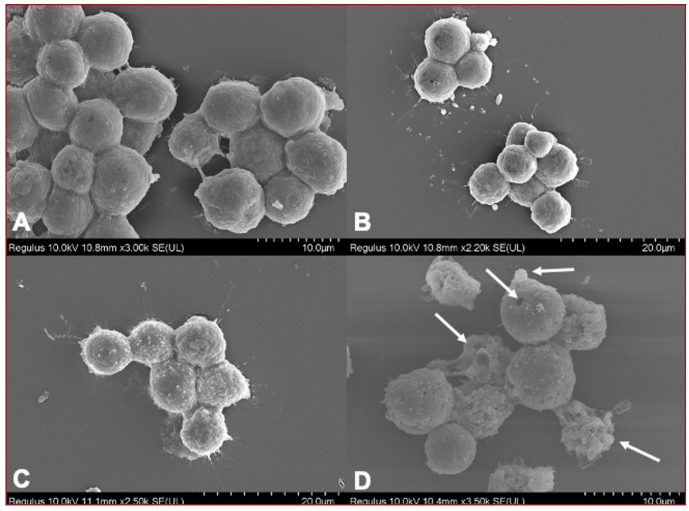

Figure 3. SEM images of HL60 cells. A. Control; B. Light control; C. treatment with 3.125 µM MG in the dark; D. treatment with 3.125 µM MG + light. White solid arrows indicate apoptotic cell with holes, shrunking cell with apoptotic bodies and broken cell membranes

To determine the mechanism of cell death induced by MG-based PDT, HL60 cells were evaluated using Annexin V-FITC and PI staining. Q1 in the histogram only represents dead cells stained with PI (Annexin V–/PI+); Q2 represents late apoptotic and necrotic cells stained with both Annexin V and PI (Annexin V+/P+); Q3 represents viable cells that were not stained with both Annexin V and PI (Annexin V–/PI–); and Q4 represents early apoptotic cells stained with Annexin V (Annexin V+/PI–) only. 93.23% of the control group cells were detected in Q3. A similar cell population was observed in Q3 only in the MG group compared to the control group (91.23%). In the MG (3.125 µM)-based PDT group, the rate of early apoptotic cells (Q4) was determined to be approximately 15.4%. Treatment of HL60 cells with MG-based PDT caused an increase in the percentage of late apoptotic cells (Q2) compared to the control group (0.7-30.36%). In addition, the percentage of late apoptotic cells in the MG-based PDT group was approximately 19 times higher than in the light-control group. When the percentage cell values obtained after Annexin V/PI staining were compared in terms of late apoptotic, early apoptotic, and viable cells, it was found that MG-based PDT showed a significant difference compared to the control group (***p< 0.0001). These results demonstrate that MG-based PDT induces late apoptosis in HL60 cells (**Figure 4**).

The control, light-control, MG only, and MG-based PDT groups were stained with PI 24 hours after treatment and analyzed by flow cytometry. DNA content in cells has a direct relationship with the amount of fluorescence intensity, and DNA degradation in apoptotic cells translates to a lower PI intensity than that of cells in the G1 phase (subG1 peak). 24 hours after the application, it was determined that 37.6% of the cells treated with 3.125 µM MG-based PDT were in the subG1 peak region, but only 2.1% of the cells in the control group were in the subG1 peak region, while 2.2% and 3.5% of the light control and MG-only groups were in the subG1 peak region, respectively. The results of this analysis showed that apoptosis was induced in cells treated with 3.125 µM MG-based PDT (**Figure 5**).

DISCUSSION

There are several issues with the use of the treatments that are commonly used to treat leukemia. The side effects of high-dose radiotherapy on normal cells are evident.[15] Chemotherapy has similar side effects and drug resistance possibilities.[16] Hematopoietic stem cell transplantation is one of the most used methods in the treatment of leukemia. There are significant risks in terms of recipient and donor for this treatment.[17] Not all therapeutic approaches are effective in destroying leukemia cells completely. PDT is a minimally invasive or non-invasive therapeutic technique for diseases and especially cancer.[18] Clinical studies reveal that PDT can prolong survival and significantly improve quality of life in patients with inoperable cancer.[19] Several previous studies have shown that PDT can be applied to leukemia cells with different photosensitizers.[20-22] This application has a cytotoxic effect on leukemia cells and allows them to differentiate into phagocytic cells.[23] The effectiveness of PDT varies depending on the photosensitizer applied, the light source, and the type of treatment.[24] The severity of the phototoxic effect is related to the dose of the drug and irradiation. The ideal agent to use in clinical applications was reported to be one whose applied dose is reduced as much as possible and whose skin toxicity is low to avoid side effects.[25]

Recently, ex vivo applications such as immunotherapy and replacement therapy have been used in leukemia patients. PDT application is a non-invasive method with fewer side effects. Recent studies have shown that this treatment is also effective in various leukemia cell lines.[26] It can be used to remove leukemic cells from the bone marrow intended for autologous transplant.

The HL60 cell line and other leukemia lines are widely used in chronic myeloid leukemia studies. Salmeron et al. reported that phenelonone-based PDT showed strong antitumor cell activity in HL-60 promyelocytic leukemia cells, and that the formed free oxygen radicals induced apoptosis.[27] In another study Sun et al. reported that ALA-based PDT induced DNA damage and apoptosis in K562 leukemia cells.[28] Cisariková et al. showed that PDT mediated by acridin-3,6-dialkyldithiourea hydrochlorides increased cytotoxicity and arrested cell cycle in the subG0 phase in the mouse leukemia line L1210.[29] In another study was reported that nanoparticle-ZnPc-based PDT caused apoptosis in leukemia cells and that PDT could be an excellent alternative for leukemia treatment.[30] Xu et

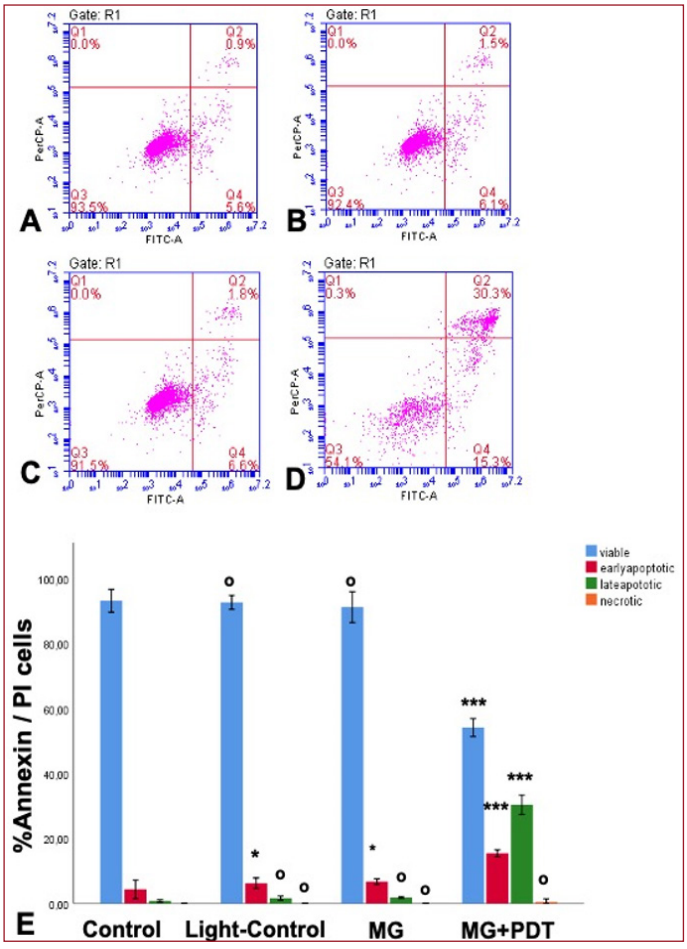

Figure 4. A. Control, untreated HL60 cells; B. Control, treated with only light; C. HL60 cells treated with 3.125 µM MG; D. HL60 cells treated with light combined with 3.125 µM MG; E. Graphical presentation of the percentage of the Annexin V positive apoptotic cell. The data represent the means±standard deviations (SDs) of 3 independent experiments. *indicates statistically significance compared to control group ($p<0.05$);***indicates statistically significance compared to control group ($p<0.0001$); °indicates not statistically significant when compared with the control group ($p>0.05$).

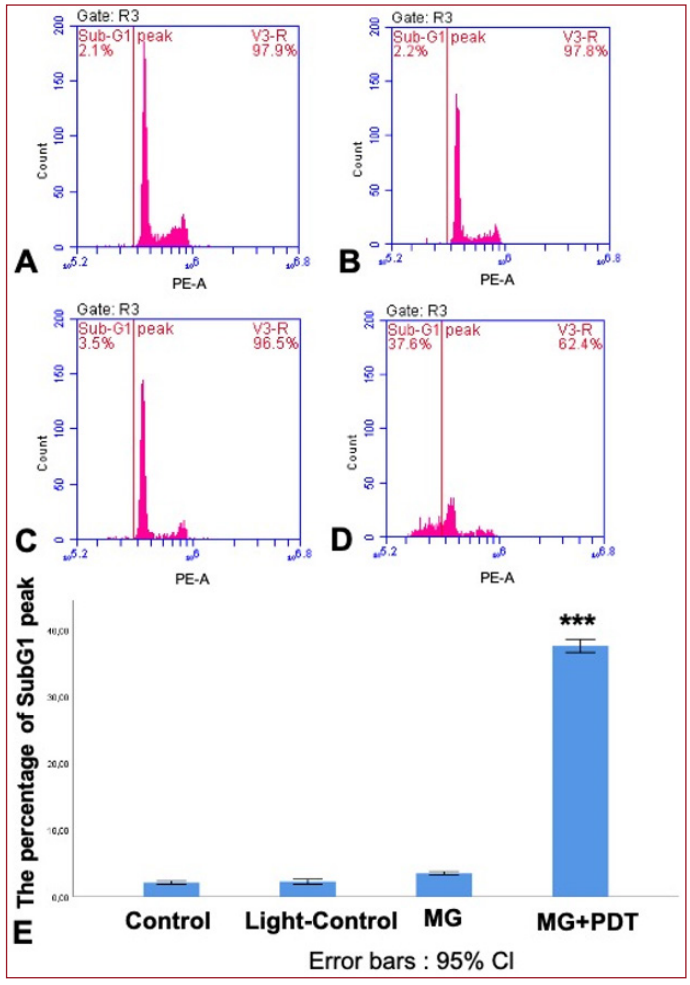

Figure 5. SubG1 apoptotic peak was determined by flow cytometry. Flow cytometry histograms A. Control, untreated HL60 cells; B.Control, treated with only light; C. HL60 cells treated with 3.125 µM MG; D. HL60 cells treated with light combined with 3.125 µM MG; E. Graphical presentation of the percentage of the SubG1 peak apoptotic cell. ***indicates statisticaly significance compared to control group ($p<0.0001$)

al. showed that hypericin-based PDT reduces cell viability in leukemia cells and can be developed as an effective treatment for leukemia.[21] Philchenkov et al. demonstrated that ALA- and fotolon®-based PDT resulted in dose-dependent cell death in human T cell lines of acute lymphoblastic leukemia.[31] Zhang et al. reported that ALA-based PDT inhibited cell proliferation in K562 leukemia cells.[32] Ettorre et al. showed that LycoC-based PDT is a good photosensitive agent that can induce apoptosis in HL60 cells.[33] Čunderlíková et al. reported that hexaminolevulinate-based PDT for tumor treatment was effective in photodynamically clearing leukemia cells from bone marrow grafts.[34] Unlike these studies, Chen et al. demonstrated that platinum-based PDT causes cell cycle arrest in different human leukemia cell lines at the G0/G1 stage, induces autophagy-induced cell death, and inhibits cell growth in leukemia cells in a mitochondria-based and caspase-independent manner.[35] Zhang et al. proposed the idea that ALA-based PDT on HL60 cells could be used to inactivate leukemia cells.[36] Di Stefano et al. demonstrated that purpurin-18-based PDT causes rapid apoptotic cell death in human leukemia cell lines at low doses and necrosis at higher concentrations.[37] Grebenova et al. showed that PDT inhibited proliferation and viability and caused an interphase arrest of the cycle of human promyelocytic leukemia HL60 cells and human erythroleukemia cells.[38]

To demonstrate the effects of MG-based PDT on HL60 cells and to develop an ideal PDT protocol, MG-based PDT experiments were performed in this study with different MG concentrations. HL60 cells viability decreased to 53.6% in the concentration of 3.125 µM MG after 30 minutes of irradiation with a light intensity of 25.8 mW/cm². However, the effects were minimal at low MG concentration. The morphological structure was examined by Giemsa staining and SEM analysis. It was observed that MG-based PDT had typical apoptotic cell features such as the formation of apoptotic bodies,and bubble-like protrusions on the HL60 cell surface compared to the MG alone, light-control, and control groups. Annexin-V staining was performed to examine the role of MG-based

PDT in cell apoptosis. Annexin V staining, which binds to phosphatidylserine passing from the plasma membrane's cytoplasmic surface to the plasma membrane's outer surface and is one of the indicators of apoptosis, is a major technique for detecting early-stage apoptosis. Late apoptotic and necrotic cells can be detected by adding propidium iodide to Annexin V-stained cells. In this study, 15.4% of apoptotic cells observed in HL60 cells were determined to be early apoptotic, while 30.36% were late apoptotic. This suggests that MG-based PDT treatment is effective in the action of membrane phospholipid phosphatidylserine in these cells, and that some of them are in the final stage of apoptosis. The results of the SubG1 analysis show that MG-based PDT induces a subG1 phase arrest in the cell cycle of the HL60 cells. These results suggest that MG-based PDT application may have important potential as a therapeutic option for leukemia.

CONCLUSION

PDT has been used clinically for the last 25 years as an alternative therapy in cancer treatment. Given the studies conducted, this subject is the current focus of research, and new studies emerge every day. Therefore, this study, with its multidisciplinary subject, is original research that will lead to promising new research topics. Although autologous bone marrow transplantation is one of the methods used in the treatment of leukemia, tumor cell contamination in autografts affects transplantation success. The effect of photosensitizers on penetrating malignant cells using PDT could be used as an auxiliary method in bone marrow purification. This is a study that includes an in vitro experiment. There is no study in the literature focusing on the mechanisms by which MG-based PDT affects leukemia cells. The results shown in this study provided insights about the effects of PDT on HL60 cells and created a new perspective for an alternative therapy against cells. This suggests that apoptosis may influence cell death after MG-based PDT administration in HL60 cells. This study is significant in that it will provide the basis for future ex vivo studies.

ETHICAL DECLARATIONS

Ethics Committee Approval: Since the methodological structure of the study is a "cell culture study", it does not require ethics committee approval in accordance with the World Medical Association Declaration of Helsinki "Ethical Principles for Medical Research on Humans".

Referee Evaluation Process: Externally peer-reviewed.

Conflict of Interest Statement: The authors have no conflicts of interest to declare.

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Author Contributions: All of the authors declare that they have all participated in the design, execution, and analysis of the paper, and that they have approved the final version.

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