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In Vitro Therapy Investigation for Breast Cancer by B13-Chloroquine Application

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Abstract

Cancer disease still remains to be strong treat for public health. New treatment approaches and agents with low side effects are needed for the treatment of breast cancer. Based on these, herein was aimed to investigate the cytotoxicity of a combination comprising a ceramidase inhibitor (B13) and an autophagy inhibitor (chloroquine) on human breast cancer cell line. The antiproliferative activity was tested by Sulforhodamine B and ATP viability assays. For ultrastructural and morphological changes and apoptotic signs of MCF-7 cells were used TEM and confocal microscopy techniques. Results showed the high cytotoxic and antiproliferative activities of the combination along with the ultrastructural and morphological changes indicating apoptosis. B13+Chloroquine combinations found to be effective on inducing cell death on MCF-7 cells and antiproliferative and cytotoxic effects on cells. Consequently, the new combination is suggested as good candidate for further investigations to be an anti-cancer agent.

Keywords: MCF-7, combination therapy, cytotoxicity, B13, chloroquine.

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Meme Kanserinde B13-Klorokin Uygulamasıyla İn vitro Tedavi Araştırması

Özet

Kanser hastalığı hala halk sağlığı için ciddi bir tehdit olmaya devam edmektedir. Meme kanseri tedavisi için yeni tedavi yaklaşımlarına ve yan etkisi düşük ajanlara ihtiyaç duyulmaktadır. Bunlara dayanarak, burada bir seramidaz inhibitörü (B13) ve bir otofaji inhibitörü (klorokin) içeren bir kombinasyonun insan meme kanseri hücre hattı üzerindeki sitotoksisitesinin araştırılması amaçlanmıştır. Antiproliferatif aktivite, Sulforhodamine B ve ATP canlılık deneyleri ile test edilmiştir. MCF-7 hücrelerinin ince yapısal ve morfolojik değişiklikleri ve apoptotik belirtileri için TEM ve konfokal mikroskopi teknikleri kullanılmıştır. Sonuçlar, kombinasyonun yüksek sitotoksik ve antiproliferatif aktivitelerinin yanı sıra apoptozu gösteren ince yapısal ve morfolojik değişikliklere neden olduğunu göstermiştir. B13+Klorokin kombinasyonunun MCF-7 hücrelerinde ölümü tetiklediği, antiproliferatif ve sitotoksik etkilere neden olduğu saptanmıştır. Sonuç olarak, yeni kombinasyonun antikanser bir ajan olarak ileri araştırmalar için iyi bir aday olduğu ortaya konulmuştur.

Anahtar kelimeler: MCF-7, kombine terapi, sitotoksisite, B13, klorokin.

1. Introduction

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Breast cancer incidence and mortality rates remain unchanged despite the developments on its treatment [1, 2]. Due to the difficulties on traditional cure, it is important to develop effective treatment agents for cancer treatment [3, 4, 5]. Preclinical studies support the use of chloroquine as anti-cancer agent with important antineoplastic effects and opportunity for usage for treatment of many aggressive and metastatic cancers [6, 7]. B13, has been indicated for its anti-cancer activity in different cells lines with mechanism of inhibiting acid ceramidases [8, 9, 10]. Herein, we aimed to investigate the characteristics of changes on the morphology and ultrastructure of breast cancer MCF-7 cells applied with B13+chloroquine combination.

2. Material and method

2.1. Materials

MCF-7 (ATCC® HTB-22™) cells were purchased from the American Type Culture Collection (Manassas, USA). B13 (D-NMAPPD), Chloroquine, fetal bovine serum, penicillin streptomycin, dimethyl sulfoxide (DMSO), Sulforhodamine B (SRB), Trichloroacetic acid were obtained from Sigma-Aldrich (St. Louis, USA) and Roswell Park Memorial Institute medium (RPMI-1640) was from GIBCO (Grand Island, USA).

2.2. Cell culture

In the cell culture study, MCF-7 cell line was grown in RPMI-1640 medium containing penicillin–streptomycin (100 units/mL-100 µg/mL) and fetal bovine serum (10%) at 37°C and 5% CO_2 in humidified incubator until they become confluent. In all of the experiments cell culture flasks with at least 85% confluency.

2.3. Sulforhodamine B method (SRB)

When performing the Sulforhodamine B method (SRB), cells were planted in 96-well cell culture dishes at a density of 2500-7500, 100 µl per well. Cells were treated with B13 compound and chloroquine (between 1.5-100 µM doses) for 24 hours. Treated cells were fixed in situ with 50% (w/v) cold TCA and stained with 0.4% (w/v) SRB in 1% acetic acid for measurement. At the end of the treatment period, the SRB dye bound to the cells will be removed using unpuffed 10 mM Tris and the optical density (absorbance) was measured at 530nm on a plate reader (Synergy HTX, Bio-Tek, USA). Viability percentages were calculated in comparison to the untreated cell.

2.4. ATP viability method

ATP level measurement is based on luminescence technology and is much more sensitive than other viability methods, however, even at low cell counts, it is more sensitive and reliable than colorimetric tests as there is an excellent correlation between the viable cell count and the RLU (relative light unit) values read on the device. For this purpose, B13 and chloroquine treatment (between 1.5-100 µM doses) were applied to MCF-7 cells in 96-well cell culture dishes with a density of $5x10^3$ cells/well for 24, 48 and 72 hours. ATP content in treated cells and control cells was measured using a luminometer (Synergy HTX, Bio-Tek, USA) with a measurement time of 1 second with the luciferin-luciferase bioluminescence reaction shown below. The results were analyzed as Relative Light Unit (RLU).

$$ATP + Luciferin + O_2 \rightarrow\rightarrow\rightarrow\rightarrow\rightarrow AMP + 2Pi + CO_2 + Photon\ (RLU)$$

Viability percentages of the samples were calculated according to the RLU values that were obtained from the control cells. Viability calculation formula is as follows:

$$Viability\ (\%) = [100 \times (Sample\ RLU) / (Control\ RLU)]$$

2.5. Confocal microscopy

The morphological changes that B13 and chloroquine agents can cause in MCF-7 cells were examined by confocal microscopy method. In preparation of MCF-7 cells to be examined under a confocal microscope, $3x10^5$ cells were incubated with IC_{50} concentrations of B13 and Chloroquine on sterilized coverslips in 6-well plates. At the end of the incubation period, the medium was removed and the cells were fixed in glutaraldehyde by washing in phosphate buffer (PBS). After fixation, cells were washed again with PBS and double stained with acridine orange and phalloidin dyes [11]. The morphological changes of the cells were examined using a confocal microscope (Leica TCS-SP5 II) in Leica Confocal Software Version 2.00 and visuals were obtained.

2.6. Determination of ultrastructural changes by TEM

Ultrastructural changes of untreated MCF-7 cells with a density of $1x10^6$/mL and MCF-7 test cells exposed to IC_{50} concentrations of B13 and Chloroquine were examined under a transmission electron microscope (TEM). At the end of the incubation period, MCF-7 cells were fixed in glutaraldehyde overnight at +4°C, then subjected to secondary fixation in osmium tetraoxide by washing with buffer. Fixed cells were dehydrated in ethyl alcohol series (50%, 70%, 90%, 96% and absolute ethyl alcohol) and cells were exposed to propylene oxide followed by blocking in resin. The blocks were polymerized in an oven at 60°C for 48 hours. The resulting blocks were cut into sections (80-100 nm) and placed on copper grids. Samples were stained in uranyl acetate and lead citrate. Samples were visualized under a transmission electron microscope (TEM) at 120 kV (Biotwin FEI, USA) and subtle structural changes were detected.

2.7. Statistical analysis

Cytotoxicity test results will be tested and determined by one-way analysis of variance (ANOVA). All tests will be tested at α 0.05 significance level. Statistical analysis will be evaluated with GraphPad 8.0 computer package programs.

3. Results

3.1. Sulforhodamine B cytotoxicity results

Concentrated stock solutions of B13, Chloroquine and B13+Chloroquine compounds were prepared in DMSO. The MCF-7 cell line was incubated on a 96-well plate with different concentrations of B13, Chloroquine and B13+Chloroquine for 24 hours. According to the results of this experiment, IC_{50} concentrations were calculated and antiproliferative activity graphs were created. In these findings, it was observed that the viability of MCF-7 cells decreased dose-dependent after B13 administration. In MCF-7 cells, where the combination of B13+Chloroquine was applied for 24 hours. IC_{50} value for this combination could not be calculated. As a result of the Sulforhodamine B method (SRB), the concentrations (IC_{50} value) suppressing the growth of MCF-7 cells were determined as 19.3 µM for B13 substance, while this value was 61 µM for chloroquine. The viability of MCF-7 cells decreased after B13 administration depending on the concentration. It was found that cell viability decreased as the applied substance concentration increased. The inhibition graph formed by the viability values calculated from the absorbance obtained from the SRB test of MCF-7 cells where B13 and chloroquine are applied for 24 hours is given in Figure 1.

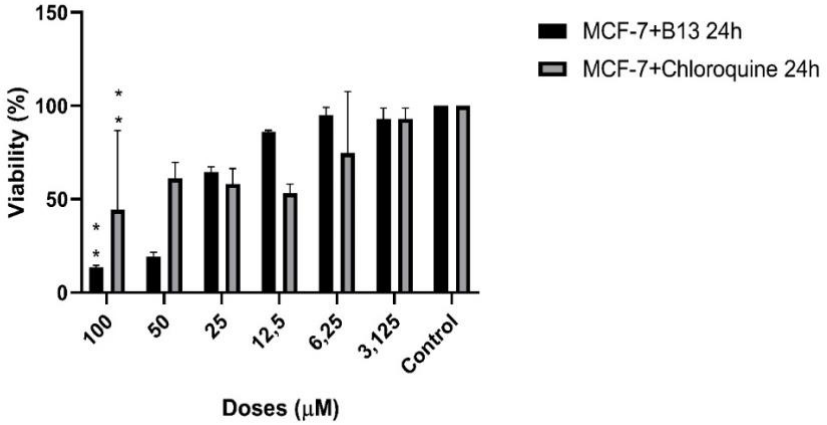


Figure 1. Viability inhibition in MCF-7 by B13 and chloroquine administration. (**; p: 0.0024) (**; p: 0.0046).

3.2. ATP bioluminescence cytotoxicity test results

ATP bioluminescence cytotoxicity test is defined as one of the fastest, most sensitive and easiest methods among viability tests performed in multi-well plates. In our study, 24 hours IC_{50} value for B13 was determined as 8,84 µM in this test results. This value was determined as 56.25 µM for Chloroquine and B13+Chloroquine combination 21.32 µM and the dose-dependent viability graph was shown Figure 2A. B13, Chloroquine and B13 + Chloroquine combination concentrations applied to MCF-7 cells for 48 hours showed that the IC_{50} value of B13 could not be calculated. In the same application period, the IC_{50} value determined for chloroquine was determined as 18.99 µM, while this value of the B13+Chloroquine combination was determined as 4.96 µM. Concentration-dependent viability suppression graph is presented in Figure 2B. MCF-7 cells were treated with varied concentrations of B13, Chloroquine and B13 + Chloroquine

combined compound for 72 hours. As a result of this test, the determined IC_{50} values of B13, chloroquine and B13 + Chloroquine combined compound are detected as 5.49 µM, 20µM and 12.75 µM respectively and the dose-dependent viability graph is shown in Figure 2C.

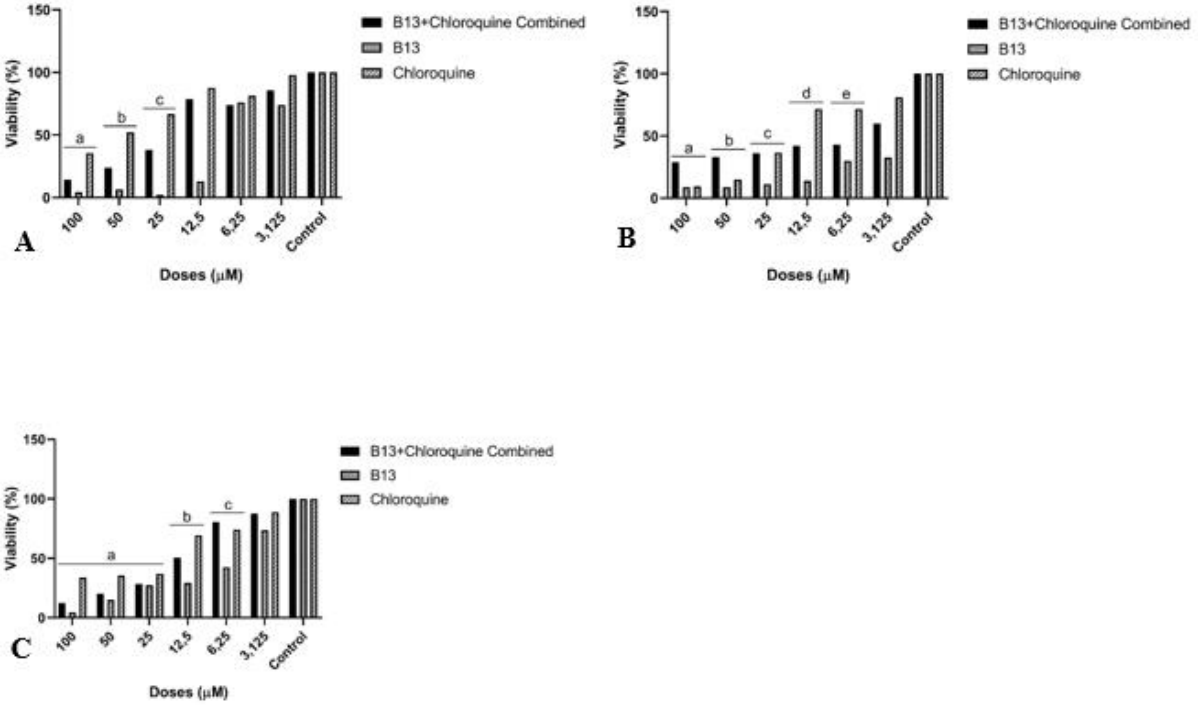


Figure 2**. (A)**Viability inhibition graph of MCF-7 cells exposed 24 hours to B13, Chloroquine and B13 + Chloroquine combination. (p values, a: **:0,0029; b: **:0.0082; c: *:0,0210) **(B)** Viability inhibition graph generated from ATP test findings with 48 hours of B13, Chloroquine and B13+Chloroquine combined compound on MCF-7 cells. (p values; a:***: 0.0006; b:***:0.0008; c: **:0.0025; d:*:0.0166; e:*:0.0358) **(C)** Viability percentages of MCF-7 cells exposed to B13, Chloroquine and B13 + Chloroquine combined compound for 72 hours. (p values: a=****:<0.0001; b=**:0.015; c=*0.0351)

3.3. *Confocal microscopy results*

When the obtained data is evaluated, morphological changes occurring on cells in tha application period of 24 by B13+Chloroquine application are shown in Figure 3. The detected morphological changes were determined as nucleus fragmentation, chromatin condensation, cytoskeleton disruption and hole formation.

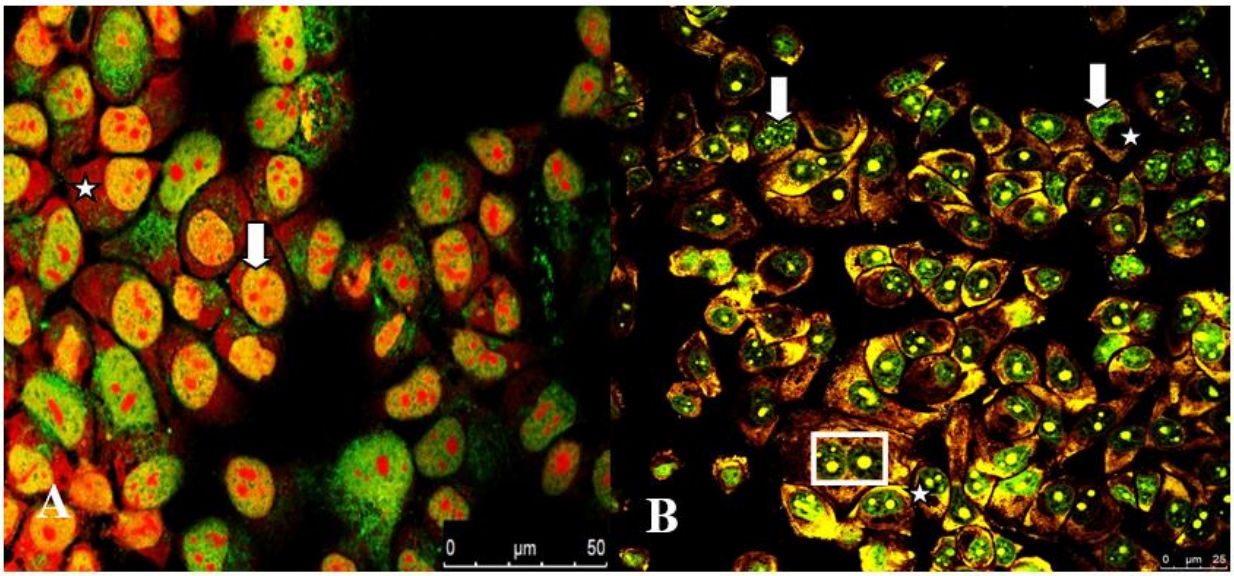


Figure 3**.** Confocal microscopic images of morphological changes of MCF-7 cells. (A) Control group MCF-7 cells: Arrow-free cell nucleus, Asterisk-undamaged cytoskeleton (B) MCF-7 cells in which IC_{50} concentrations of B13+Chloroquine are applied for 24 hours: Arrow-lysed cell nucleus, Asterisk-cytoskeleton disruption, Square-chromatin condensation.

3.4. Electron microscopic analysis of the ultrastructural changes

Electron microscopic analysis of MCF-7 cell line exposed to B13+Chloroquine showed that the ultrastructure of the cell was changed. The detected ultrastructural changes of this cell group were chromatin condensation, blebbings on membranes, shinkage on the nucleus membrane and loss of crystae of mitochondria in comparison with the control cells's unchanged ultrastructure. These detected changes are shown in Figure 4.

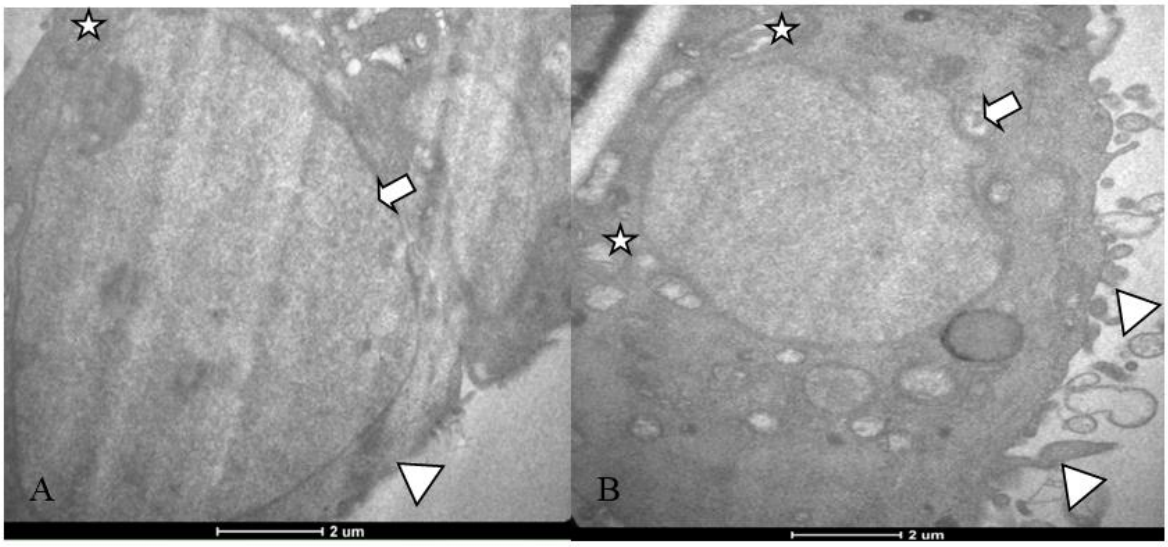


Figure 4. TEM images of ultrastructural changes of MCF-7 cells. (A) Control group MCF-7 cells: Arrow-free cell nucleus, Asterisk-intact mitochondria, Triangular-compact cell membrane. (B) MCF-7 cells in which IC_{50} concentrations of B13+Chloroquine are applied for 24 hours: Arrow-core membrane collapse and chromatin condensation, Asterisk-damaged crystae of mitochondria, Triangle-membrane blebbing.

4. Conclusions and discussion

Combination therapy is the focus of researchers due to its potential to reduce or completely eliminate multiple drug resistance in the treatment of many different types of cancer [12]. Today, combination therapy studies have become an important consideration in cancer treatments in order to strengthen therapeutic efficacy and overcome drug resistance and metastasis [13]. Autophagy is a self-digesting product that is evolutionarily protected and, however, is indicated as a

process in which proteins and other cytoplasmic materials are recycled to backup cell survival under stressful terms (i.e. cancer treatment). It is recommended as a resistance mechanism to treatments such as radiotherapy and chemotherapy [14]. If we talk about another way of death such as autophagy, apoptosis is one of the main mechanisms of cell death in response to cancer treatments [15]. Changes in susceptibility to apoptosis not only contribute to neoplastic development. In addition to this contribution, it can also increase resistance to traditional anticancer treatments that are still being applied, such as radiation to the patient and treatments with cytotoxic agents [16]. In this study, we used chloroquine to inhibit autophagy and B13 to involve apoptosis in MCF-7 cells. ATP viability test results and SRB results imply to the enhenced cytotoxicity of the agent used in combination (Figures 1 and 2). Our ATP finding indicated to the dose and time dependency of applied agent combination for application time of 24 and 48 hours. The obtained IC_{50} value of the combination for 72 hours was found to be higher then that for 48 hours that may mean resistance development by the MCF-7 cells to the used combination (Figure 2). Our viability and cytotoxicity test finding were discussed as potent inhibition of authophay and enhenced programmed cell death via potent arise in intracellular ceramide. Chloroquine is a lysosomal enzyme inhibitor and with it, it inhibits the late autophagy state by altering the pH of lysosomes and thus also affects the degradation of proteins enveloped in the autophagosome [17] and chloroquine, often used as an anti-malarial drug, it also has potential anti-cancer effects. It shows effects such as inhibition of cell growth in A549 (human lung cancer cells) cells and glioma cells. In addition, it has been shown to increase the inhibitory effects of other chemotherapeutic agents on tumors, thanks to its use in therapy [18]. Treatment methods in cancer are constantly improving, and recently there has been an increase in the work of classes of agents other than chemotherapy. Among these new agents being studied are targeted drugs, immunotherapies and hormonal agents, but a clearer understanding of cancer biology and pathogenesis paves the way for molecular targeted therapies [4]. Through research, it has been shown that the biological effect of chloroquine is concentration dependent. For example, at low concentrations, chloroquine has been found to inhibit the growth of the lung cancer cell line A549. At higher concentrations or longer durations, chloroquine has been reported to directly induce apoptosis and necrosis [19]. Based on findings from different studies, ceramidase inhibitors are considered to be a potential new therapeutic class of antiproliferative and cytostatic drugs. D-NMAPPD, also known as B13, is a new member of the ceramidase inhibitor group. B13 has been shown to induce apoptosis on different cancer cells in in vitro and in vivo studies, and there is also evidence that the associated ceramidase inhibitor B13 can suppress acid ceramidase activity [9]. Our study describes a systematic time and dose-dependent approach to evaluate one of the drug combinations that are effective in killing cancer cells due to changes in drug exposure and duration. In a study with chloroquine alone, this agent has been found to inhibit the proliferation of breast cancer cells. Our study shows close results with our study only if the chloroquine inhibitor is examined. In a conducted study, different breast cancer cell lines (4T1, MCF-7, MDA-MB-231, MDA-MB-435S, T47D and Bcap-37) were used to investigate the cell growth inhibitory properties of chloroquine. A comparison was made between the six cell lines examined in this study and showed that Bcap-37 cells were very susceptible to chloroquine treatment. IC_{50} values of MCF-7, MDAMB-231, MDA-MB-435S, T47D and Bcap-37 were shown as 63.98, 30.18, 35.84, 132.87, 21.68 µM after 48 hours of chloroquine treatment [20]. Paralelly with this findings our results showed that chloroquine+B13 combination resulted in lower IC_{50} values then that in the literature. Additionally the antiproliferative and cytotoxic activities of the used combination were detected highly in the exposed MCF-7 cell ultrastructure and morphology (Figures 3 and 4). Confocal microscopic findings of morphological changes of MCF-7 cells exposed to B13+Chloroquine combination were fragmentation of nuclei and cytoskeleton, chromatin condensation and hole formation. These findings were taken as morphological indicators of apoptotic cells death and interpreted to be caused by inhibition of ceramidase enzyme activity by B13 and autophagy by chloroquine (Figure 3). TEM findings of MCF-7 cells exposed to the used combination showed chromatin condensation, blebbing in membranes, collapse in the nucleus membrane, loss of crystae of mitochondria as ultrastructural changes (Figure 4). Mitochondrial dysfunction was implied by the broken mitochondrial inner sides especially its criste. This finding is taken as a clear apoptotic sign.

Herein, it has been determined that these compounds are potential anti-cancer agents but the combination was disscussed as the most relevant agent of the study to involve cell death with dual action, autophagy inhibition and apoptosis induction. The potential of the this combination as a treatment agent is thought to contribute to public health, and that is a good potent candidate for drug design and production after the further and deeper studies. In line with the study we have conducted, it is recommended to carry out studies to produce the B13+Chloroquine combined compound as an anti-cancer drug. It is also anticipated that it can be used in the pharmaceutical field for synthesizing targeted therapeutic agent after drug design. As a conclusion, B13+Chloroquine combination is recommended for use in designing and synthesizing an effective pharmaceutical agent for cancer treatment, after investigating its further effects on other cancer cell lines and *in vivo*.

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