ORIGINAL ARTICLE

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Investigation of the Strengthening Effect of Toyocamycin on Docetaxel in Human Ovarian Cancer Cells

İnsan Ovaryum Kanseri Hücrelerinde Toyokamisinin Dosetaksel Üzerindeki Güçlendirici Etkisinin Araştırılması

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ABSTRACT

Background: Docetaxel is an anti-microtubule chemotherapeutic agent classified in the taxane group and is widely used in the treatment of many types of cancer. However, systemic toxic effects can be observed in patients treated with docetaxel, and the development of *de novo* or acquired resistance limits its use and therapeutic efficacy. Thus, investigation of adjuvant treatment approaches has attracted interest. This study aimed to investigate the effects of toyocamycin-mediated inhibition of the Inositol-Requiring Enzyme 1 alpha (IRE1α)-X-box Binding Protein 1 (XBP-1) pathway, a physiological mechanism in mammalian cells, on the sensitivity of ovarian cancer cells to docetaxel.

Materials and Methods: Human ovarian adenocarcinoma cell lines SKOV3 and Caov-3 were used in the studies. The effects of toyocamycin and docetaxel on cell viability were investigated using the Water-Soluble Tetrazolium-1 assay. The inhibitory effect of toyocamycin on the IRE1α-XBP-1 pathway was confirmed by immunoblotting studies. The impact of combined treatment with toyocamycin and docetaxel on migration and invasion was evaluated using wound-healing and Matrigel-coated Boyden-chamber invasion assays. The expression levels of some pro-apoptotic and anti-apoptotic genes, such as B-cell lymphoma 2 (*BCL2*)-associated X apoptosis regulator, BH3-interacting domain death agonist, *BCL2* and *BCL2*-like 1, were analyzed by quantitative real-time polymerase chain reaction (qRT-PCR).

Results: Our results showed that toyocamycin-mediated inhibition of IRE1α-XBP-1 signaling significantly suppressed cell viability, migration, and invasion in ovarian cancer cells. In combined treatment with toyocamycin and docetaxel, it was determined that the viability, migration, and invasion were more effectively suppressed in a dose-dependent manner with increasing concentrations of toyocamycin than with either agent alone. Similar results were obtained from qRT-PCR studies. Combination treatments upregulated pro-apoptotic genes and downregulated anti-apoptotic genes in both cell lines.

Conclusion: The present data suggest that pharmacological targeting of IRE1α/XBP-1 signaling could significantly enhance the sensitivity of ovarian cancer cells to docetaxel.

Keywords: Docetaxel, ovarian cancer, toyocamycin, unfolded protein response

ÖZ

Amaç: Anti-mikrotübül ajanı olan dosetaksel taksan grubu sınıfındaki kemoterapötik ajanlardan birisidir ve çok sayıdaki kanser türünün tedavisinde yaygın olarak kullanılmaktadır. Ancak, dosetaksel'in kullanımına bağlı olarak hastalarda sistemik toksik etkiler gözlenebilmektedir. Bununla birlikte *de novo* veya edinilmiş direnci gelişimi dosetaksel'in kullanımını ve terapötik etkinliğini sınırlamaktadır. Bu nedenle adjuvan tedavi yaklaşımlarının araştırılması ilgi çekici hale gelmiştir. Bu çalışmada memeli hücrelerinde fizyolojik bir mekanizma olan İnozitol Gerektiren Enzim 1 alfa (IRE1α)-X-kutusu Bağlayıcı Protein 1 (XBP-1) yolunun *Streptomyces diastatochromogenes*'ten elde edilen bir adenozin analoğu olan toyokamisin aracılı inhibisyonunun ovaryum kanseri hücrelerinin dosetaksel'e olan duyarlılığı üzerine olan etkilerinin araştırılması amaçlanmıştır.

Gereç ve Yöntemler: Çalışmalarda insan ovaryum adenokarsinoma hücre hatları SKOV3 ve Caov-3 kullanılmıştır. Toyokamisin ve dosetakselin hücre canlılığı üzerine olan etkileri Suda Çözünebilen Tetrazolyum-1 hücre canlılık testi ile incelendi.

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ÖZ

Toyokamisinin IRE1α-XBP-1 yolu üzerindeki inhibe edici etkisi immünoblotlama çalışmaları ile doğrulandı. Toyokamisin ve dosetakselin kombinasyonel uygulamalarının migrasyon ve invazyon üzerine olan etkileri yara iyileşme ve matrijel-kaplı Boyden-chamber invazyon testleri ile değerlendirildi. Kantitatif gerçek zamanlı polimeraz zincir reaksiyonu (qRT-PCR) çalışmaları ile B-cell lymphoma 2 (*BCL2*)-associated X apoptosis regulator, BH3-interacting domain death agonist, *BCL2* ve *BCL2*-like 1 gibi bazı pro-apoptotik ve anti-apoptotik genlerin ifade düzeyleri analiz edildi.

Bulgular: Sonuçlarımız IRE1α-XBP-1 sinyalinin toyokamisin aracılı inhibisyonunun ovaryum kanseri hücrelerinin canlılığını, migrasyonunu ve invazyonunu önemli ölçüde baskıladığını gösterdi. Toyokamisin ve dosetakselin kombinasyonel uygulamalarında artan konsantrasyonlardaki toyokamisin uygulamasına bağlı olarak daha etkili hücrelerin canlılığını, migrasyonunu ve invazyonunun yalnız başına uygulamalara kıyasla daha etkili olarak baskılandığı belirlendi. Benzer sonuçlar qRT-PCR çalışmalarından da elde edildi. Her iki hücre hattında da kombinasyonel uygulamaların pro-apoptotik genleri yukarı regüle ettiğini, anti-apoptotik genleri ise aşağı regüle ettiğini gösterdi.

Sonuç: Mevcut araştırma verileri ovaryum kanseri hücrelerinin dosetaksel'e olan duyarlılığının geliştirilmesinde IRE1α/XBP-1 sinyalizasyonunun farmakolojik olarak hedeflenmesinin önemli bir potansiyele sahip olduğunu önermektedir.

Anahtar Kelimeler: Dosetaksel, ovaryum kanseri, toyokamisin, katlanmamış protein yanıtı sinyali

Introduction

Ovarian cancer is one of the leading causes of cancer-related deaths in women. According to the American Cancer Society, an estimated 20,890 women in the United States will be diagnosed with ovarian cancer in 2025, and approximately 12,730 of these women will die from ovarian cancer (1). Currently, many risk factors for ovarian cancer have been identified, including androgen levels, body weight, postmenopausal hormone therapy, and familial cancer syndromes (2). Local treatments, such as surgery and radiation therapy, and systemic treatment approaches, such as chemotherapy, hormone therapy, targeted drug therapy, and immunotherapy, are widely used in the treatment of ovarian cancer. However, the heterogeneity of cancer cells in tumor tissues, the development of resistance to systemic treatments, and recurrence after treatment may limit the effectiveness of treatment for ovarian cancer (3). Therefore, improving the utilization of existing agents with confirmed efficacy and enhancing the sensitivity of cancer cells to these agents continue to be important priorities. Adjuvant approaches remain important in this regard (4).

Docetaxel, an anti-microtubule agent belonging to the taxane family, is used as a first-line treatment for many cancers, including prostate and breast cancer. Docetaxel is a semi-synthetic, second-generation taxane with cytotoxic effects, derived from the needles of European yew trees (5). It blocks tubulin polymerization, leading to cell cycle arrest in ovarian cancer cells. However, patients develop *de novo* or acquired resistance to docetaxel following high-dose administration. This situation limits the use and therapeutic efficacy of docetaxel (6). We aimed to investigate the effects of combined application of docetaxel and toyocamycin, an adenosine analog obtained from *Streptomyces diastatochromogenes*, on the sensitivity of ovarian cancer cells to docetaxel.

Recent studies have reported that mechanisms involved in the control of endoplasmic reticulum (ER) stress, the unfolded protein response (UPR), and cellular proteostasis play a key role in tumor progression and the acquisition of drug resistance by cancer cells (7). UPR signaling is controlled by three main regulatory pathways: Inositol-Requiring Enzyme 1 alpha (IRE1α), PKR-like ER kinase, and activating transcription factor 6. Toyocamycin selectively inhibits the IRE1α–X-box Binding Protein 1 (XBP-1) signaling pathway (8). Under ER stress, IRE1α, localized in the ER membrane, oligomerizes, undergoes autophosphorylation of its cytosolic kinase domain, and mediates removal of a 26-bp intron from XBP-1 mRNA by alternative splicing through activation of its endoribonuclease domain. In this way, the expression of UPR target genes is selectively regulated by the formation of XBP-1s, the spliced form of XBP-1 (9,10). Studies have reported that alterations in the activity of the IRE1α-XBP1 signaling pathway significantly affect tumorigenic properties of cancer cells, including survival, drug resistance, invasion, metastasis, and epithelial-mesenchymal transition (9-12). Although the UPR signal serves as an adaptive mechanism in mammalian cells, reprogramming cells in response to stress, it can also activate programmed cell death when cellular stress is insurmountable (13). Given these regulatory roles, the IRE1α-XBP-1 signaling pathway has been suggested as an important target for the development of cancer therapeutics.

Toyocamycin was originally isolated from *Streptomyces* species and is a nucleoside antibiotic analogue of adenosine (14). It blocks RNA synthesis and ribosomal function, and programmed cell death. Toyocamycin also negatively

modulates the IRE1α-XBP1 pathway by affecting IRE1α autophosphorylation—a step required for IRE1α activation—thereby inhibiting the splicing of XBP-1 mRNA (14). Current *in vitro* and *in vivo* studies have revealed that it has potent anti-cancer activity against cancer cells (14-16). Considering this evidence, investigating the effect of toyocamycin as an adjuvant treatment is of particular interest.

Herein, we evaluated whether toyocamycin potentiates the anticancer activity of docetaxel in SKOV3 and Caov-3 human ovarian adenocarcinoma cell lines. For this purpose, their effects on cell viability and on the migratory and invasive abilities of ovarian cancer cells were assessed using several methods. Moreover, the effects on the expression levels of some pro-apoptotic and anti-apoptotic genes were examined by quantitative real-time polymerase chain reaction (qRT-PCR). Our results showed that combined treatment with docetaxel and toyocamycin has the potential to significantly enhance docetaxel's anticancer effects in ovarian cancer cells. Taken together, these findings suggest that simultaneous pharmacological targeting of the IRE1α-XBP1 arm of the UPR signaling may significantly improve the sensitivity of ovarian cancer cells to docetaxel.

Materials and Methods

Materials

All cell culture-compatible plastic materials were supplied by Sarsdeth. The culture medium, fetal bovine serum (FBS), trypsin (0.25% and 0.05%), and other supplements were purchased from Lonza Bioscience. Dimethyl sulfoxide (#20385.01), of suitable quality for cell culture studies, was purchased from SERVA. Rabbit polyclonal antibody XBP-1s (#24868-1-AP) (1:2500), one of the primary antibodies used in immunoblotting studies, was supplied by Proteintech. Mouse monoclonal beta-actin antibody (#A5316) (1:10.000) was purchased from Sigma-Aldrich. Horseradish peroxidase (HRP)-conjugated anti-mouse (#31430) (1:500) or anti-rabbit (#31460) (1:500) immunoglobulin G (H+L) secondary antibodies were obtained from Thermo Fisher Scientific. Toyocamycin (#sc-362812) and docetaxel (#sc-201436) were obtained from Santa Cruz Biotechnology. Thapsigargin (#T9033) was provided by Sigma-Aldrich.

Methods

Cell Culture

SKOV3 (HTB-77™) and Caov-3 (HTB-75™) human epithelial ovarian adenocarcinoma cell lines were obtained from the American Type Culture Collection. SKOV3 cells were cultured in McCoy's 5A Medium supplemented with 10% FBS and Caov-3 cells were cultured in Dulbecco's Modified Eagle's Medium containing 10% FBS and 2 mM L-glutamine under conventional cell culture conditions (37 °C and 5% CO_2). Mycoplasma contamination was routinely monitored using the easy PCR™ Mycoplasma Detection Kit (#20-700-20) (Biological Industries). Cell lines used in the studies were passaged between 5 and 10 times.

Water-Soluble Tetrazolium-1 (WST-1)-Based Cell Viability Assay

Cells were seeded into 96-well cell culture dishes at a density of 10,000 cells/well; after 24 hours, toyocamycin, docetaxel or combinations of the two agents were applied to the cells for 48 hours. An equal volume of the solvent was applied as a control. The master stocks of all agents were prepared at a 2000-fold concentration. 48 hours later, cell viability was analyzed using WST-1 reagent (#MK400) (Takara, Japan), following the manufacturer's recommended protocol. 20 µL of WST-1 was added to each well, and the culture dish was incubated for 2 hours at 37 °C. Absorbance was measured at 450 nm using a microplate reader (BioTek Epoch 2). Experimental studies were performed in 3 technical and 3 biological replicates. Results are presented graphically as % viability ± standard deviation (SD). Inhibitory concentration 50 (IC_{50}) values for the agents were calculated using GraphPad Prism 8.0.

Quantitative Real-Time PCR

Total RNA was isolated using the Monoarch® Miniprep Total RNA Isolation Kit (#T2010S) (New England Biolabs, USA), following the manufacturer's recommended protocol. The concentrations and purity of the obtained RNA samples were determined by A260/A280 absorbance measurements using a microspectrophotometer (Allsheng Nano 400A). 1 µg of RNA sample was used to synthesize complementary DNA (cDNA) using the iScript™ cDNA Synthesis kit (#1708890) (Bio-Rad). Real-time PCR analyses were performed on the CFX Connect Real-Time PCR system using iTaq Universal SYBR® Green Master Mix (#1725120; Bio-Rad). mRNA expression levels of BCL2-associated X apoptosis regulator (*BAX*), *BCL2*, *BCL-xL*, and BH3-interacting domain death agonist (*BID*) were analyzed. Expression levels of ribosomal protein lateral stalk subunit P0 were evaluated for use as a housekeeping gene. Relative gene expression changes were calculated using the Livak method. Results are presented as fold change ± SD in a bar graph. qRT-PCR studies were performed with three biological replicates; each cDNA sample was analyzed in three technical replicates. Melting curve analysis was performed at the end of each qRT-PCR run to evaluate the specificity of the PCR.

Western Blotting

Immunoblotting studies were conducted as previously described in Erzurumlu et al. (17). Cell pellets were lysed in radioimmunoprecipitation assay buffer containing 1% mammalian protease inhibitor (SERVA #39102.01) for 30 min. The samples were centrifuged at 14,000 r.p.m. for 20 minutes at 4 °C; the pellet was removed, and the supernatant was stored for use in subsequent steps. Total protein content was determined using bicinchoninic acid (#23225) (Thermo Scientific, MA). Protein samples were denatured in 4x Laemmli protein loading buffer at 70 °C for 15 minutes. Protein samples were loaded onto hand-cast sodium dodecyl sulfate–polyacrylamide gels at approximately 25 µg per lane and were subjected to electrophoresis for 2 h. Protein samples separated on the gel were transferred onto an Immun-Blot® polyvinylidene difluoride (PVDF) membrane (Bio-Rad; #1620177). The PVDF membrane was subjected to blocking, washing, primary antibody treatment, washing, HRP-conjugated secondary antibody treatment, and washing; chemiluminescence imaging was then performed using Clarity™ Western ECL substrate (#1705061) (Bio-Rad). Chemiluminescence imaging was performed on the Fusion Pulse (Vilber Lourmat) system.

Wound-Healing Assay

Cells were seeded into 12-well cell culture dishes at a concentration of 3.5 x 10^5 cells per well. 24 hours later, wound areas were created using a sterile 200-µl micropipette tip. After washing the cells with 1x DPBS, fresh complete medium was added to the cells, and the cells were treated with agents for 72 hours. Wound areas were photographed at 0 and 72 hours using a phase-contrast microscope equipped with a camera system (Sunny SopTop microscope). Wound closure rates (%) were analyzed with ImageJ software (http://imagej.nih.gov/ij/). Each group was studied in three technical and three biological replicates, and the results were presented as % wound-closure area (mean ± SD) in a bar graph.

Matrigel-coated Boyden-Chamber Invasion Assay

Invasion assays were performed as previously described by Erzurumlu et al. (17). Matrigel (BD Biosciences) and serum-free medium were combined in a tube at a ratio of 1:8. 45 µl of the mixture was applied to the surface of a transwell (Sarstedt) with a pore size of 8 µm and incubated at 37 °C for 1 hour. The upper surface of the transwell was filled with 100 µl of serum-free medium and incubated for 30 minutes. 10,000 cells were seeded into each transwell. A culture medium containing 20% FBS was added to the culture dish holding the lower part of the transwell, and then the cells were treated with the agents. After 72 hours, cells that migrated to the lower surface of the membrane filter were fixed, stained with crystal violet, and counted. Each sample was analyzed in three biological and two technical replicates. Results are presented as percent invasion in a bar graph (mean ± SD).

Statistical Analysis

Statistical significance of differences between groups was determined by two-tailed Student's t-test (assuming equal variances) or one-way analysis of variance, with a confidence level of at least 95%, using GraphPad Prism 8.0. Statistical significance was accepted at $p < 0.05$. Results are presented as mean ± SD.

Results

Evaluation of the Effects of Toyocamycin and Docetaxel on Viability in Ovarian Cancer Cells

The effects of toyocamycin and docetaxel on cell viability in SKOV3 and Caov-3 human ovarian adenocarcinoma cells were investigated using the WST-1 cell viability assay. For this purpose, cells were treated with 10, 12.5, 25, 32.5, 40, 50, 62.5, 75, and 100 nM toyocamycin, and with 0.1, 0.125, 0.25, 0.325, 0.45, 0.5, 0.625, 0.75, and 1 nM docetaxel for 48 hours, after which WST-1 viability analysis was performed. Our results show that docetaxel and toyocamycin significantly suppressed cell viability in SKOV3 and Caov-3 cells in a concentration-dependent manner (Figures 1A and 1B). The IC_{50} concentrations of toyocamycin were determined to be 55.08 and 51.33 nM for SKOV3 and Caov-3 cells, respectively, and for docetaxel, they were calculated to be 0.585 and 0.563 nM.

Investigation of the Effects of Co-Administration of Toyocamycin with Docetaxel on the Viability of Ovarian Cancer Cells

To evaluate whether toyocamycin enhances the suppressive effect of docetaxel on the viability of SKOV3 and Caov-3 ovarian cancer cells, toyocamycin at ¼x, ½x, and 1x IC_{50} and docetaxel at 1x IC_{50} were applied simultaneously to the cells, and cell viability was analyzed. We found that combined application of toyocamycin and docetaxel suppressed cell viability more strongly than either agent alone (Figure 2). Our findings revealed that co-administration of toyocamycin and docetaxel resulted in enhanced anticancer responses in ovarian cancer cells.

Confirmation of the Inhibitory Effect of Toyocamycin on XBP-1s Production by Immunoblotting

Toyocamycin, an adenosine analog obtained from *S. diastatochromogenes*, prevents the cleavage of XBP-1

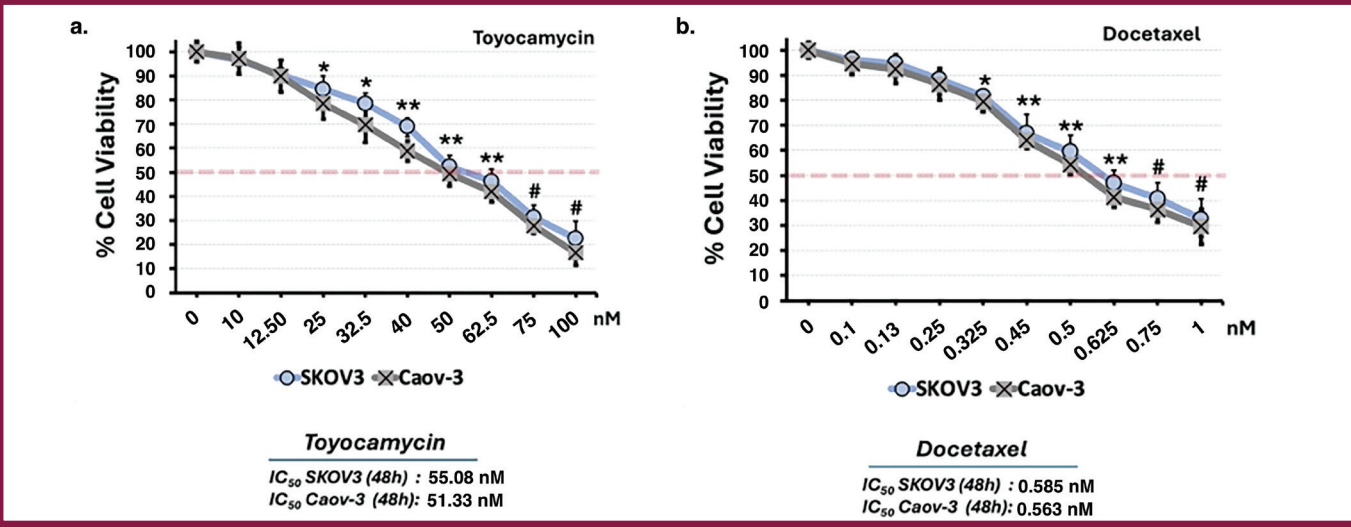

Figure 1. Investigation of the effects of toyocamycin and docetaxel on the viability in SKOV3 and Caov-3 human ovarian adenocarcinoma cells and the determination of IC_{50} concentrations. SKOV3 and Caov-3 cells were treated for 48 hours with (a) 10, 12.5, 25, 32.5, 40, 50, 62.5, 75, and 100 nM toyocamycin and (b) 0.1, 0.125, 0.25, 0.325, 0.45, 0.5, 0.625, 0.75, and 1 nM docetaxel . Cell viability was assessed using the WST-1 assay. Each experiment was performed with three independent biological replicates and three technical replicates. IC_{50} concentrations of the agents were calculated using GraphPad Prism 8.0. Comparisons with control were indicated as: *p < 0.05, **p < 0.005, #p < 0.001. IC_{50}, inhibitory concentration 50; WST-1, Water-Soluble Tetrazolium-1.

mRNA and blocks the formation of XBP-1s, which function as active transcription factors in UPR signaling. (14). Immunoblotting studies were performed to confirm XBP-1 inhibition at the IC_{50} concentrations determined in cell viability assays. In these studies, thapsigargin, which can activate UPR signaling by inducing ER stress, was used as a positive control. SKOV3 and Caov-3 cells were treated with 5 nM thapsigargin for 1 hour to induce ER stress (18). As expected, thapsigargin administration resulted in increased XBP-1s levels in SKOV3 and Caov-3 cells compared to the control group. Thapsigargin-induced increases in XBP-1s levels were suppressed by toyocamycin in a dose-dependent manner (Figure 3). These results confirm that toyocamycin inhibits XBP-1s signaling at the applied concentrations in SKOV3 and Caov-3 ovarian cancer cells.

Investigation of the Effects of Combined Applications of Toyocamycin and Docetaxel on the mRNA Expression Levels of Apoptotic Genes in Ovarian Cancer Cells

To examine the effects of toyocamycin and docetaxel treatments on the expression of pro-apoptotic (*BAX, BID*) and anti-apoptotic (*BCL2, BCL-xL*) genes in SKOV3 and Caov-3 cells, mRNA levels were evaluated by qRT-PCR. For this purpose, cells were treated with docetaxel at 1x IC_{50} and with toyocamycin at ¼x, ½x, and 1x IC_{50} or with combinations of the two agents, for 24 h. Our data revealed that toyocamycin and docetaxel alone significantly up-regulated the expression levels of the pro-apoptotic proteins *BAX* and *BID*, while the expression levels of the anti-apoptotic proteins *BCL2* and *BCL-xL* were down-regulated compared to the control group (Figures 4A and B). Combined treatment with increasing concentrations of toyocamycin and docetaxel strongly upregulated the expression of the pro-apoptotic proteins *BAX* and *BID* and downregulated the expression of the anti-apoptotic proteins *BCL2* and *BCL-xL*, compared with treatment with toyocamycin or docetaxel alone (Figures 4A and B). These results indicate that co-treatment with toyocamycin and docetaxel induces apoptotic responses more effectively than either agent alone in SKOV3 and Caov-3 cells.

Evaluation of the Effects of Co-treatment of Toyocamycin and Docetaxel on the Migration and Invasion Ability of Ovarian Cancer Cells

To evaluate the effects of co-treatments with toyocamycin and docetaxel on the migratory and invasive abilities of ovarian cancer cells, SKOV3 and Caov-3 cells were treated with 1x IC_{50} docetaxel and ¼x, ½x, and 1x IC_{50} toyocamycin, or combinations of both agents, for 48 hours in migration assays and 72 hours in invasion assays. Our results showed that treatment with either toyocamycin or docetaxel alone significantly inhibited the migration and invasion of SKOV3 and Caov-3 cells compared with the control group (Figures 5A and 5B). In our trials in which combinations of

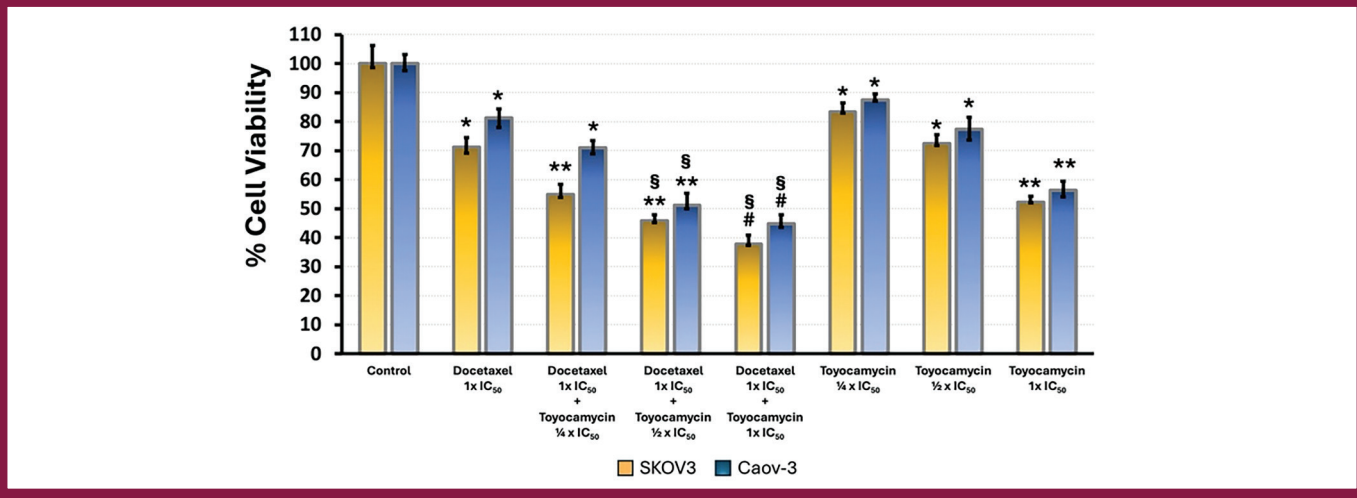

Figure 2. Investigation of the effects of combined application of toyocamycin and docetaxel to SKOV3 and Caov-3 human ovarian adenocarcinoma cells on cell viability. SKOV3 and Caov-3 cells were treated with toyocamycin at ¼x, ½x, and 1x IC_{50}, with docetaxel at 1x IC_{50}, or with combinations thereof for 48 hours. Cell viability was examined by WST-1 assay. Results are presented in the graph as % viability relative to the control group. Each experiment was performed with three independent biological replicates and three technical replicates. Toyocamycin concentrations corresponding to ¼x, ½x, and 1x IC_{50} are 13.77, 27.54, and 55.08 nM for SKOV3 and 12.83, 25.67, and 51.33 nM for Caov-3, respectively. The 1x IC_{50} docetaxel concentrations were 0.585 nM and 0.563 nM for SKOV3 and Caov-3, respectively (comparisons with control: $^{*}p < 0.05$; $^{**}p < 0.005$; $^{\#}p < 0.001$, comparisons with docetaxel: $^{\S}p < 0.05$; $^{\S}p < 0.01$). IC_{50}, inhibitory concentration 50; WST-1, Water-Soluble Tetrazolium-1.

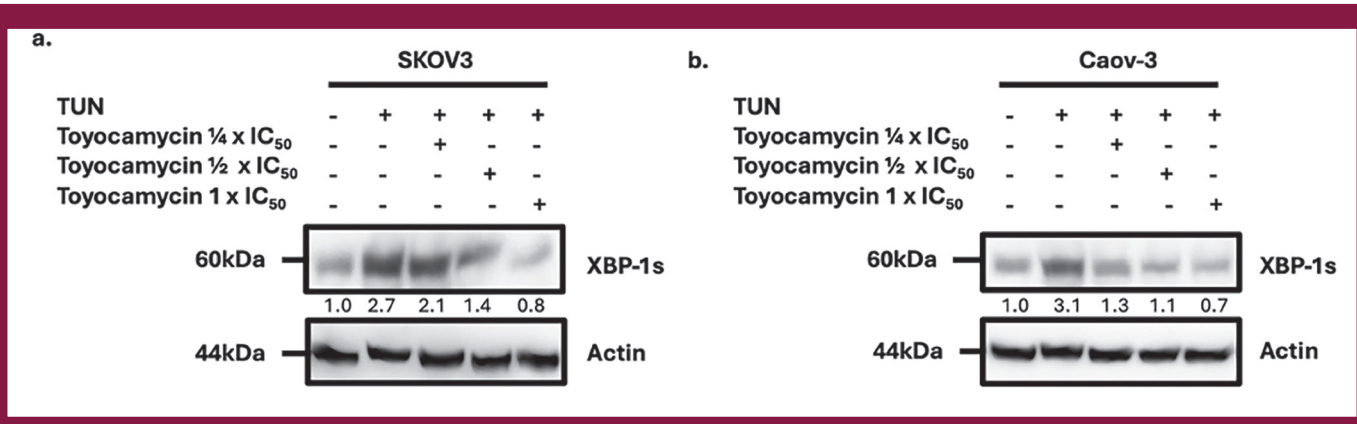

Figure 3. Confirmation of the inhibitory effect of toyocamycin on XBP-1 by immunoblotting studies. (a) SKOV3 and (b) Caov-3 cells were treated with combinations of 5 nM thapsigargin with toyocamycin at ¼x, ½x, and 1x IC_{50} for 24 hours. Total protein was subsequently isolated from the cells, and XBP-1s levels were examined by immunoblotting. Beta-actin was used as a loading control in these studies. Thapsigargin was used as a positive control. Densitometry results of XBP-1s levels, normalized to beta-actin bands, are presented below the blot images. ¼x, ½x and 1x IC_{50} toyocamycin concentrations are 13.77, 27.54 and 55.08 nM for SKOV3 and 12.83, 25.67 and 51.33 nM for Caov-3, respectively. IC_{50}, inhibitory concentration 50; XBP-1, X-box Binding Protein 1.

increasing concentrations of toyocamycin and docetaxel were applied, co-treatment with toyocamycin and docetaxel more strongly inhibited the migratory and invasive abilities of SKOV3 and Caov-3 cells than either agent alone. Our results showed that the combination of toyocamycin and docetaxel produced more potent anti-migratory and anti-invasive effects.

Discussion

Gynecological cancers continue to be a significant health problem; they are commonly diagnosed and are a frequent cause of death (19). In many patients, resistance to conventional therapies or distinct genomic and proteomic profiles of tumor cells limit treatment success (20). Therefore, research on adjuvant approaches has gained importance.

Taxane-class agents are an important group of chemotherapeutic agents used in cancer therapy (21). Responses to treatments with taxane-class drugs may vary depending on whether patients have received previous therapy. Patients may develop resistance to these agents (22). In the present study, the combined effect of the taxane-class drug docetaxel and toyocamycin on improving its anticancer activity was evaluated in an *in vitro* ovarian cancer model using several methodologies.

Toyocamycin, also known as vengicide, is an adenosine analog. Isolated from *S. diastatochromogenes*, toyocamycin exhibits activity against multiple targets and functions as an antibiotic with antitumor activity (14,23). Studies have shown that toyocamycin inhibits the IRE1α-XBP-1 signaling pathway, a physiological signaling mechanism in mammalian cells (14).

IRE1α signaling regulates numerous pathways in mammalian cells, including lipid biogenesis, chaperone synthesis, protein degradation, control of quality-control protein levels, regulation of autophagic responses, and increased expression of pro-survival genes (24,25). Recent studies have revealed that changes in the activity of the IRE1α-XBP-1 signaling pathway promote carcinogenesis (9-12). Thus, it is suggested as one of the important mechanisms to be targeted in cancer therapy. Toyocamycin is a small-molecule agent that inhibits the IRE1α-XBP-1 signaling pathway by blocking the kinase activity of IRE1α (14). Its selective effect on IRE1α-XBP-1 signaling makes it an important agent for investigating its efficacy in anticancer therapies.

Our studies investigating the possible enhancing roles of toyocamycin on the anticancer activity of docetaxel

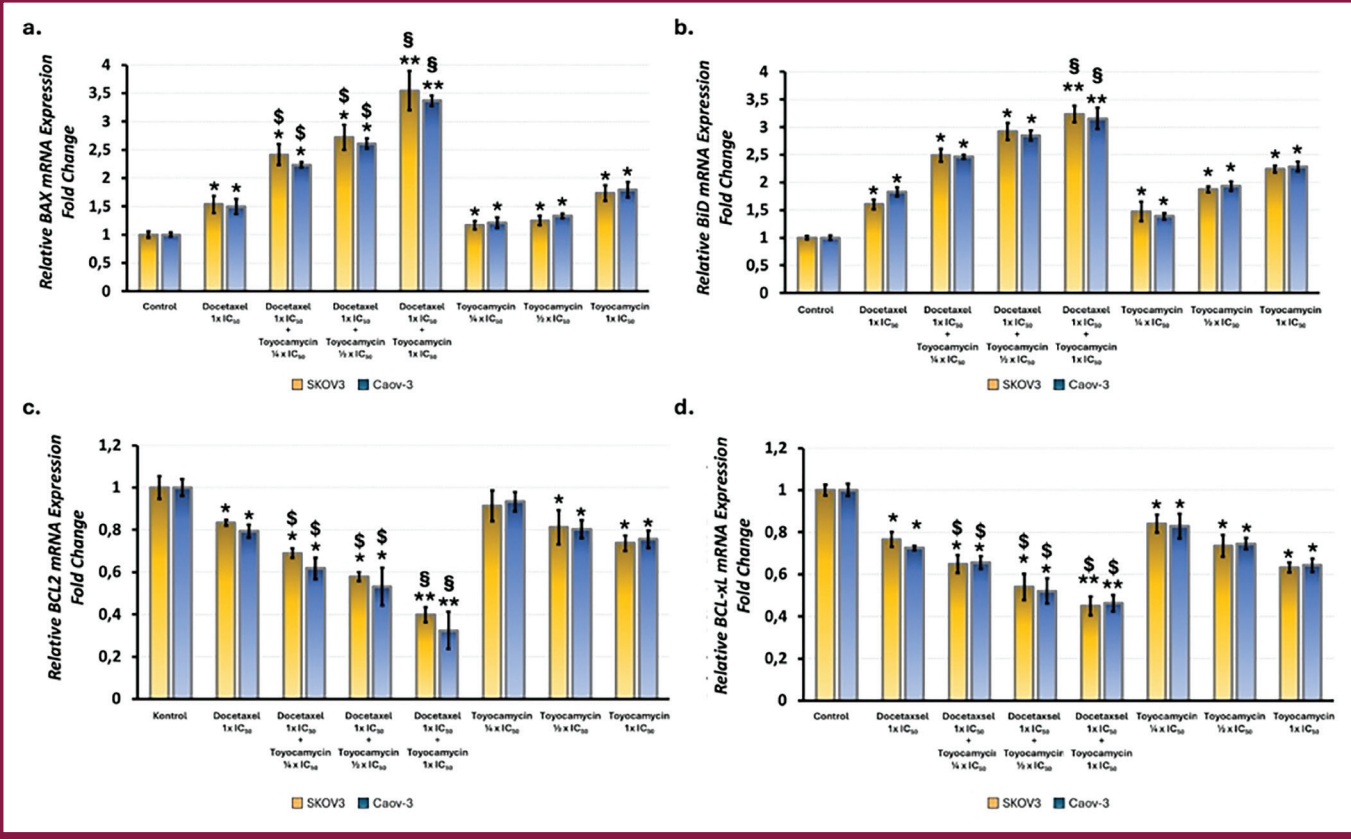

Figure 4. Investigation of mRNA expression levels of pro-apoptotic and apoptotic genes in SKOV3 and Caov-3 cells by qRT-PCR. Cells were treated for 24 hours with toyocamycin at ¼x, ½x, or 1x IC_{50}; with docetaxel at 1x IC_{50}; or with combinations of both agents. mRNA expression levels of the genes (a) *BAX*, (b) *BID*, (c) *BCL2*, and (d) *BCL-xL* were analyzed by qRT-PCR. RPLP0 was used as the housekeeping gene. Results are presented as fold changes with SDs (±) in a bar graph. The control was set to 1. Each experiment was performed in three independent biological and three technical replicates. ¼x, ½x and 1x IC_{50} toyocamycin concentrations are 13.77, 27.54 and 55.08 nM for SKOV3 and 12.83, 25.67 and 51.33 nM for Caov-3, respectively. 1x IC_{50} docetaxel concentration is 0.585 nM and 0.563 nM for SKOV3 and Caov-3, respectively (comparisons with control; *p < 0.05; **p < 0.005; #p < 0.001, comparisons with docetaxel; $p < 0.05; §p < 0.01). *BAX*, BCL2-associated X apoptosis regulator; *BCL-xL*, BCL2-like 1; *BCL2*, B-cell lymphoma 2; *BID*, BH3-interacting domain death agonist; RPLP0, ribosomal protein lateral stalk subunit P0; IC_{50}, inhibitory concentration 50; qRT-PCR, quantitative real-time polymerase chain reaction; SD, standard deviation.

determined that the combination of docetaxel and toyocamycin, across increasing concentrations, suppressed the viability of ovarian cancer cells to a significantly greater extent than docetaxel alone (Figure 1B). These findings suggest that targeting the IRE1α-XBP-1 pathway with toyocamycin, in combination with docetaxel as an adjuvant therapy, may represent an important approach to achieve more effective anticancer responses. Previous reports have shown that UPR signaling is an important mechanism of drug resistance in cancer cells (26). From this perspective, toyocamycin may offer an important approach to preventing resistance to docetaxel. However, our findings, which are limited to an *in vitro* experimental model of simultaneous combination therapy, should be validated in *in vivo* studies of long-term docetaxel treatment to confirm the effect of toyocamycin on docetaxel therapy.

Few studies have examined the combined effects of toyocamycin. Ri et al. (14) demonstrated that toyocamycin exerts therapeutic effects in multiple myeloma (MM) cells by inhibiting IRE1α-XBP-1. These studies also demonstrated that combinations of toyocamycin with bortezomib, a proteasome inhibitor, had greater antitumor activity in an *in vivo* MM model (14). Park et al. (15) reported that toyocamycin induces apoptosis through crosstalk between oxidative stress and the MAPK signaling pathway in human prostate cancer cells. Additionally, a Phase I study was conducted of toyocamycin

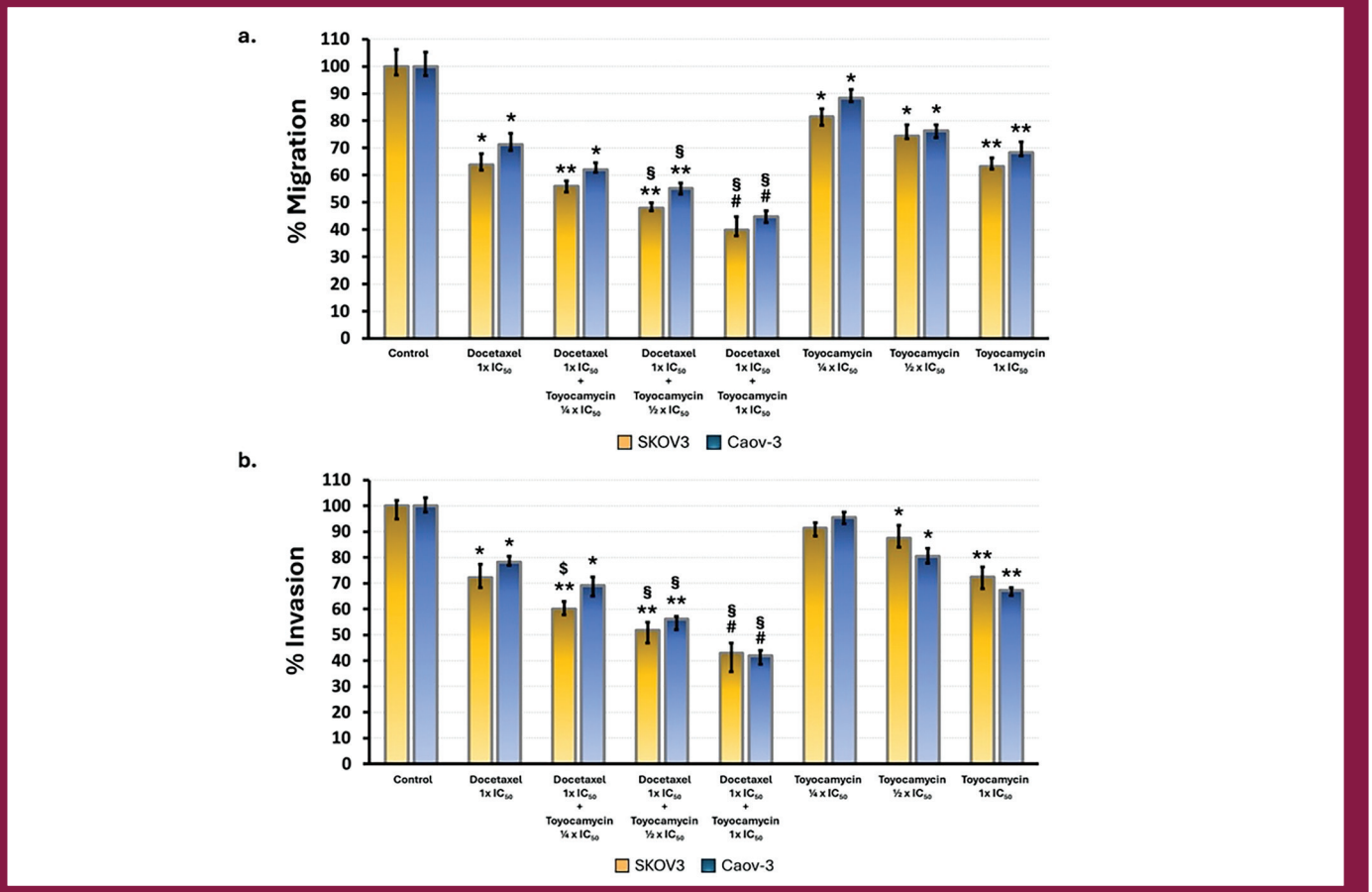

Figure 5. Evaluation of the effects of toyocamycin and docetaxel applications on migration and invasion ability in SKOV3 and Caov-3 cells. (a) SKOV3 and Caov-3 cells were treated with toyocamycin at ¼x, ½x, and 1x IC_{50}, with docetaxel at 1x IC_{50}, or combinations of both agents. Wound areas were monitored for 72 hours during migration experiments. % changes in wound closure were calculated from closure areas measured at 0 and 72 hours using ImageJ software. Each experiment was performed in three independent biological and three technical replicates. (b) In invasion assays, SKOV3 and Caov-3 cells were treated with toyocamycin at ¼x, ½x, and 1x IC_{50}, with docetaxel at 1x IC_{50}, or with combinations of both agents, for 72 hours. Cells exhibiting invasion were stained with crystal violet and counted. Results are presented as fold changes ± SDs in bar graphs. Each experiment was performed in three independent biological and three technical replicates. ¼x, ½x and 1x IC_{50} toyocamycin concentrations are 13.77, 27.54 and 55.08 nM for SKOV3 and 12.83, 25.67 and 51.33 nM for Caov-3, respectively. 1x IC_{50} docetaxel concentration is 0.585 nM and 0.563 nM for SKOV3 and Caov-3, respectively (comparisons with control; *$p < 0.05$; **$p < 0.005$; #$p < 0.001$, comparisons with docetaxel; $^{\$}p < 0.05$; $^{\$}p < 0.01$). IC_{50}, inhibitory concentration 50; SD, standard deviation.

in patients with advanced solid tumors. However, the study results were not carried forward to subsequent stages, in which patients did not demonstrate a significant clinical response to toyocamycin treatment. One important finding of this study was that no systemic side effects, such as organ dysfunction or cytopenia, were observed in association with toyocamycin treatment. Local foci of necrosis were observed only at the infusion sites in tissues treated with toyocamycin. This suggests that the side effects of toyocamycin infusion via central venous catheters would be manageable. This issue paves the way for its clinical use (27). Taken together, these findings suggest that toyocamycin is a promising agent and that further studies are required to examine its effects in different cancer groups and to determine its possible additive effects with different treatments.

Radiotherapy and systemic or targeted cytotoxic agents, commonly used in cancer therapy, stimulate the apoptotic cell-death pathway. Therefore, evasion of apoptosis by cancer cells is one of the main reasons for treatment failure (28). In our studies, we examined the expression levels of the pro-apoptotic genes *BAX* and *BID* and the anti-apoptotic genes *BCL2* and *BCL-xL*. Our findings showed that toyocamycin and docetaxel reprogrammed the cells by upregulating pro-apoptotic gene expression and downregulating anti-apoptotic gene expression in SKOV3 and Caov-3 cells. Moreover, combination therapy induced the expression of apoptotic genes more effectively than either therapy alone (Figures 4A and B). These findings suggest that toyocamycin enhances docetaxel-mediated induction of apoptosis in ovarian cancer cells additively, thereby providing more effective anticancer responses.

Study Limitations

One of the major limitations to successful cancer treatment is that cancer cells become more aggressive, exhibiting increased migration and invasion. This allows cancer cells to spread more rapidly. Furthermore, drug resistance contributes to the failure of currently applied therapies (29,30). In this respect, the simultaneous use of combinatorial approaches alongside traditional treatments is important for improving therapeutic efficacy. Our findings showed that treatment of ovarian cancer cells with increasing concentrations of toyocamycin combined with docetaxel significantly reduced the migratory and invasive abilities of SKOV3 and Caov-3 cells compared with treatment with docetaxel or toyocamycin alone (Figures 5A and B). Previous studies have reported that pharmacological targeting of UPR signaling suppresses the motility-related properties of cancer cells (31). Consistent with this, present data suggest that toyocamycin treatment in combination with docetaxel, which has an inhibitory effect on cell division, results in more effective responses than either agent alone, due to the suppression of tumorigenic properties of ovarian cancer cells and to toyocamycin-mediated disruption of the UPR signal, which functions as an adaptive mechanism. Although our findings are limited to *in vitro* results, studies in the literature emphasize the importance of targeting the IRE1α-XBP-1 arm of the UPR signaling pathway for new therapeutic approaches. The importance of UPR-targeted approaches has been highlighted in numerous *in vitro* and *in vivo* studies, particularly in prostate, breast, pancreatic, ovarian, glioblastoma, and hematological cancers (11,12,32-38). Collectively, our research findings suggest that targeting IRE1α-XBP-1 signaling to improve cellular sensitivity to docetaxel in ovarian cancer may contribute to the development of new treatment protocols.

Conclusion

In conclusion, our *in vitro* findings suggest that the XBP-1 inhibitor toyocamycin, which targets the IRE1α arm of the UPR, has significant potential to sensitize ovarian cancer cells to docetaxel. Our data, limited to *in vitro* experimental findings, support further studies examining the potential use of toyocamycin in cancer cells. Although obtaining data from *in vitro* systems is a significant limitation of our study, the present findings suggest that the potential effects of toyocamycin may guide the design of further *in vivo* experimental models.

Ethics

Ethics Committee Approval: This study does not require any ethical permission.

Informed Consent: Not required.

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Footnotes

Authorship Contributions

Concept: Y.E., Design: Y.E., Data Collection or Processing: Y.E., Y.D., Analysis or Interpretation: Y.E., Literature Search: Y.E., Y.D., Writing: Y.E.

Conflict of Interest: No conflict of interest was declared by the author(s).

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