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Investigation of the effects of bicalutamide and TRAIL signaling mechanism on docetaxel resistant prostate cancer cells

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ABSTRACT: Prostate cancer is the most common cancer among men, and developed metastasis spread to parts of the body. Clinically, prostate cancer can develop resistance to chemotherapy drugs, and generally, the initial treatment for metastatic prostate cancer is androgen deprivation therapy in combination with chemotherapy. This study aimed to further investigate the signaling mechanisms of Bicalutamide (BCLT) with TRAIL combination in resistance to Docetaxel (DX) on the prostate cancer cell. The human prostate cancer cell lines PC3 were generated by initially treating with DX for docetaxel resistance prostate cancer cells (PC3/DX). PC3/DX cells were transfected pAR plasmid for modeling androgen-dependent docetaxel resistance prostate cancer cells (PC3/DX/AR+) and were incubated with BCLT (2.5 µM) and/or TRAIL (2.5 ng/mL). After the treatment, cell proliferation was determined with an MTT assay. Apoptotic cells were investigated with AO/EB staining by fluorescence microscope. Levels of Caspase-3, Caspase-8, AR, TNF-α protein and gene expression were evaluated. We found apoptosis increased with TRAIL (5 ng/mL) in PC3/DX cells, whereas with the combination of 2.5 ng/mL TRAIL and BCLT (2.5 µM) in PC3/DX/AR+ cells. TNF-α protein and gene expression levels increase with TRAIL in PC3/DX and PC3/DX/AR+ cells. AR protein and gene expression levels increased with BCLT and TRAIL PC3/DX/AR+ cells. Our results indicate that BCLT and TRAIL combination has potential agents for inhibiting docetaxel resistance prostate cancer growth and metastasis.

KEYWORDS: Prostate cancer; docetaxel resistance; bicalutamide; TRAIL; apoptosis

1. INTRODUCTION

Prostate cells are androgen-dependent cells to increase growth, function, and proliferation [1]. Androgen receptors mediate the activation of testosterone and dihydrotestosterone (DHT); it is also a phosphoprotein that acts as a transcription factor [2]. Androgens (AR) receptors are stimulated by DHT or testosterone and bind to androgen response regions, target sites on DNA, thereby activating the transcription of androgen-associated target genes. These genes are related to events such as proliferation and differentiation. AR is active in the cytoplasm, and when stimulated, they show activity by binding to both genomic and cytoplasmic targets. Androgen deprivation therapy (ADT) is used in metastatic prostate cancer. Although these treatments keep the disease under control for a few years, the development of the disease continues and becomes resistant to castration [3-5]. In prostate cancers resistant to castration, cancer cells metastasize to distant organs such as the liver, lungs, brain, and bone [6]. Cancer metastasis is a complex process in which transformation, proliferation, local invasion, and destruction of parenchymal cells occur in cells. The migration of cells forms the primary tumor. It then spreads in the blood and lymphatic circulation and eventually proliferates to form a secondary tumor by settling in various organs and inducing neurovascularization [7,8]. BCLT, an antiandrogen, is a drug used to treat androgen-dependent prostate cancer. It blocks AR. It is used in ADT studies and stabilizes prostate tumors. Although it slows down prostate cancer and increases the survival time, it is not curative. Therefore, it is suitable to be used in cancer treatment, not alone, but in combination with drugs [9]. In the treatment of prostate cancer, taxane group drugs have attracted attention in recent years. The taxane group drugs bind to microtubules in cells, preventing the division of cancer cells, stopping both

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the spread and growth of cancer [10]. Studies have shown that docetaxel, paclitaxel, and cabazitaxel, among the taxane group drugs, have different effects on prostate cancer cells [11]. While the primary function of docetaxel's therapeutic effect is to suppress microtubule dynamics, its other function is to provide phosphorylation of Bcl-2 [12,13]. However, it is reported that long-term use of docetaxel causes resistance mechanisms in cancer [14,15]. The docetaxel-resistant prostate cancer cells (PC3/DX) cell line was developed in previous studies to understand docetaxel-induced drug resistance mechanisms better. It has been reported that the resistance mechanism in PC3/DX cells develops via the mTORC2 signaling pathway and causes the development of castration-resistant prostate cancer [16].

TRAIL, Tumor Necrosis Factor (TNF) Related Apoptosis-Inducing Ligand, is a TNF ligand family member cytokine known as Apo2L/TNFSF10/CD253. TRAIL protein is 32 kDa in weight and 281 amino acids long. TRAIL is regulated by growth factors, cytokines, chromatin modification, viral infection, and several transcription factors such as Stat1, Sp1, NF-κB, IRF-1/3/7, and Egr-2/3Membrane-dependent and soluble forms of TRAIL can selectively induce apoptosis in many tumor and cancer cells, while normal cells are generally known to be resistant to the cytotoxic effects of TRAIL [17]. TRAIL can bind to five different receptors [18] that act as death receptors are TRAIL-R1 and TRAIL-R2. In order for TRAIL to induce apoptotic cell death, it must bind to these receptors. TRAIL-R3, TRAIL-R4, and osteoprotegerin receptors are called false receptors. It is defined as a false receptor because although TRAIL can bind to these receptors, it cannot induce apoptotic cell pathways [19]. This feature of TRAIL promises promise for cancer treatment and paves the way for combination therapies. In this study, bicalutamide and TRAIL combination therapy on docetaxel-resistant prostate cancer cells was investigated.

2. RESULTS

It has been shown that TRAIL administration in PC3/DX cells, depending on the dose, stops the growth of the cells after 24 hours of incubation (Figure 1A). When the dose-dependent TRAIL treatment, the cell growth was significantly reduced compared to the control cells. In particular, TRAIL doses of 2.5 ng / mL ($p<0.01$) and 5ng / mL ($p<0.001$) were found to reduce the growth of cells (Figure 1B). In PC3/DX /AR$^+$ cells, BCLT (2.5 µM) or TRAIL (2.5 ng/mL) alone or in combination reduced the cell proliferation (Figure 1C-1D). TRAIL alone appears to was reduced cell viability by 50% in PC3/DX /AR$^+$ cells. The combination of BCLT and TRAIL has been found to reduce cell viability close to TRAIL alone.

AO/EB staining is a method used to identify apoptotic cells in microscopy. While AO dyeing in green color, EB dyeing in red. The status of the ratio of green dye to red staining and the viability of the cells is evaluated. After treatment of different concentrations of TRAIL to PC3/DX cell and incubating for 24 hours, AO/EB staining was performed (Figure 2A). Green/red ratio was significantly decreased in PC3/DX cells at 2.5ng / mL and 5 ng/mL compared to the control group (Figure 2B). Images of PC3/DX/AR$^+$ and BCLT (2.5 µM), TRAIL (2.5 ng / mL), and AO/EB staining made due to the combination of these two compounds are shown in Figure 2C. While green staining was observed to be intense in the control group, it is seen that some red staining started in BCLT. As seen in the Figure 2C, the intensity of red staining increased in the cells treated with TRAIL, while the red staining increased in the cells treated with TRAIL. As shown in the figure, the green/red ratio is seen in the lowest combination. It was determined that PC3/DX/AR$^+$ green/red staining ratio decreased in BCLT, but it was not statistically significant. While the rate decreased statistically with TRAIL application compared to the control group ($p<0.01$), similarly, it was observed that the green/red staining ratio was significantly decreased in the BCLT-TRAIL combination compared to the control ($p<0.001$). At the same time, when the BCLT-TRAIL combination was compared to the BCLT group, it was found that the green/red staining ratio decreased ($p<0.01$). Accordingly, cell death has occurred the same in the TRAIL and BCLT-TRAIL combination in PC3/DX/AR$^+$ cells (Figure 2D).

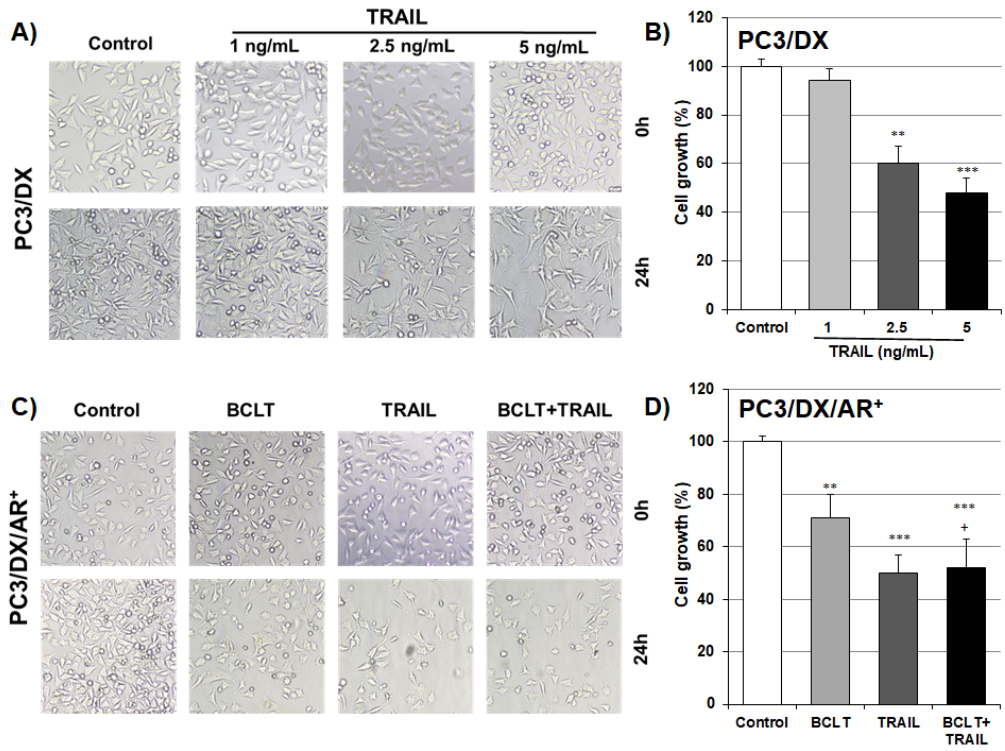

Figure 1. Effect of TRAIL treatment on cell growth **A)** Dose dependent TRAIL treatment microscope images in PC3/DX cells (10X) **B)** Dose dependent TRAIL treatment cell growth percentages in PC3/DX cells **C)** BCLT and TRAIL treatment in PC3/DX/AR+ cells microscope images (10X) **D)** BCLT and TRAIL treatment cell growth percentages in PC3/DX/AR+ cells (** p<0.01, *** p<0.001 compared to control, + p<0.05 compared to BCLT)

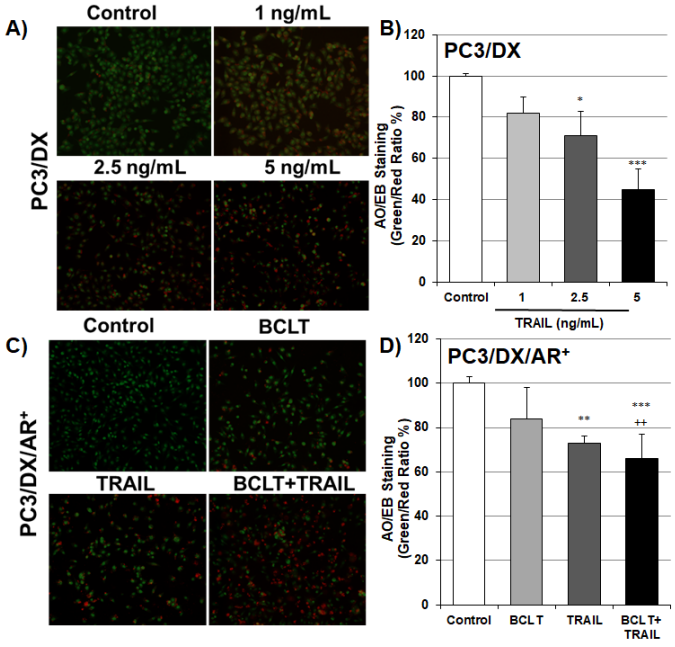

Figure 2. A) AO/EB staining images of TRAIL on PC3/DX cells (20X) **B)** Green/Red ratio of PC3/DX cells **C)** AO/EB staining images of BCLT and TRAIL on PC3/DX/AR+ cells **D)** Green/Red ratio of PC3/DX/AR+ cells (* p<0.05, ** p<0.01, *** p<0.001 compare to control, ++ p<0.01 compare to BCLT)

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TNF-α, cas-3, cas-8 protein expression differences in cells after incubation of TRAIL treatment on PC3/DX cells are shown in Figure 3. TNF-α protein expression in PC3/DX cells (Figure 3A) increased slightly when 0.5ng/mL TRAIL treatment compared to control, but no significant change was detected. When 1ng/mL TRAIL was treatment, it was observed that TNF-α increased ($p<0.05$). At the same time, a significant increase ($p<0.001$) was detected when 2.5 ng/mL TRAIL was treated (Figure 3B). Cas-3 and cas-8 protein expressions (Figure 3C) in PC3/DX cells were found to be significantly increased ($p<0.001$) especially at 2.5 ng/mL TRAIL when compared to control (Figure 3D). In PC3/DX cells, no significant change was observed in cas-8 protein expression in dose-dependent TRAIL administration (Figure 3E).

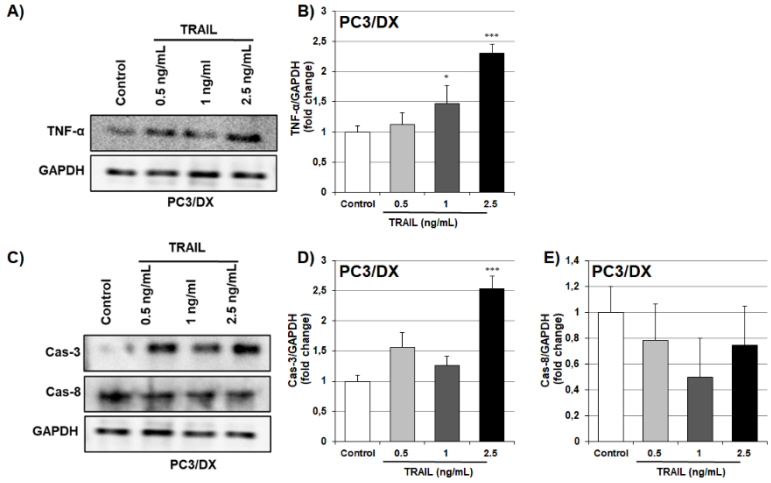

Figure 3. A) TNF-α western blotting bands of TRAIL on PC3/DX cells **B)** Densitometric analysis of TNF-α/GAPDH on PC3/DX cells **C)** Cas-3 and cas-8 western blotting bands of TRAIL on PC3/DX cells **D)** Densitometric analysis of Cas-3/GAPDH and Cas-8/GAPDH on PC3/DX cells (* $p<0.05$, *** $p<0.001$ compare to control)

AR and TNF-α protein expression changes in PC3/DX/AR$^+$ cells are shown in Figure 4A. When AR protein expression was compared to control, a statistically significant increase was found in BCLT ($p<0.05$). While there was no significant change in AR protein expression when TRAIL was administered, a significant increase in AR protein expression was also detected in PC3/DX/AR$^+$ cells treated with BCLT-TRAIL combination compared to control ($p<0.01$). At the same time, a statistically significant change was found in the AR protein expression in PC3/DX/AR$^+$ cells applied with BCLT-TRAIL combination compared to BCLT ($p<0.05$, Figure 4B). When TNF-α protein expression in PC3/DX/AR$^+$ cells were compared to control, a statistically significant decrease was found in BCLT ($p<0.05$). While there was no significant change in TNF-α protein expression when TRAIL was administered, similarly, no significant change was found in TNF-α protein expression in PC3/DX/AR$^+$ cells treated with BCLT-TRAIL combination (Figure 4C). Figure 4D and Figure 4E showed no significant change in cas-3 protein expression in PC3/DX/AR$^+$ cells. When BCLT was applied in PC3/DX/AR$^+$ cells, no significant change in cas-8 protein expression was detected. Similarly, when TRAIL was administered, there was no significant change in cas-8 protein expression, but when the combination of BCLT-TRAIL was applied, a statistically significant decrease was found in cas-8 protein expression compared to the control group ($p<0.01$ Figure 4F).

As seen in Figure 5A, no significant change was detected in the AR gene expression level in the PC3/DX cell TRAIL dose treatment. Although a slight increase was observed in the TNF-α gene expression level at 0.5 ng/mL in PC3/DX cells, no significant change was detected. When compared to the control, a significant increase was found at 1ng/mL ($p<0.01$) and 2.5 ng/mL ($p<0.001$) (Figure 5B). Although some change was observed in the level of cas-3 gene expression in 0.5 ng/mL TRAIL in PC3/DX cells, no significant change was detected. A significant increase in cas-3 gene expression level was detected in 1 ng/mL TRAIL treatment ($p<0.05$) and 2.5 ng/mL TRAIL application ($p<0.001$) compared to the control. Accordingly, the highest increase was seen in the 2.5 ng/mL TRAIL treatment (Figure 5C). There was no significant change in AR gene expression level with TRAIL treatment in PC3/DX/AR$^+$ cell.

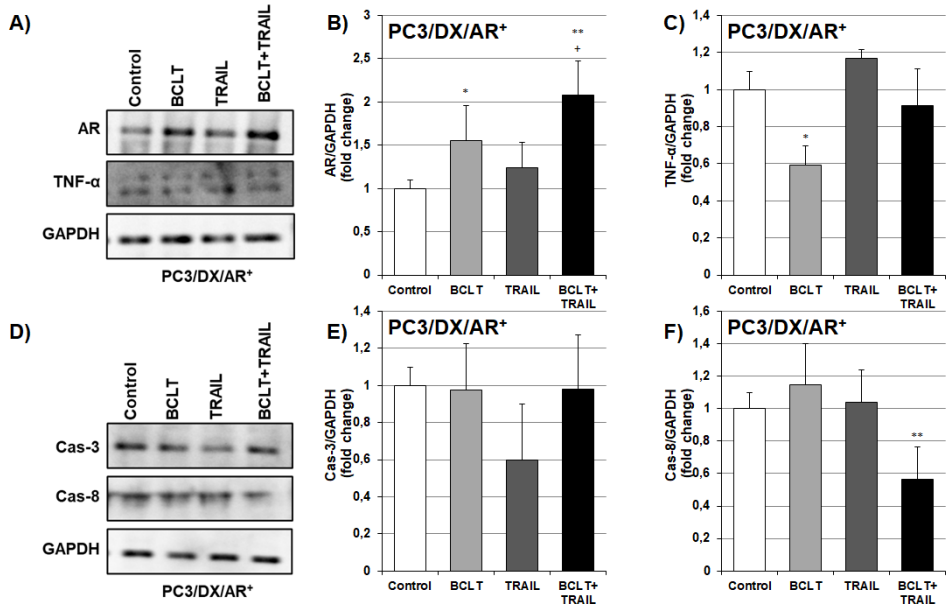

Figure 4. **A)** AR and TNF-α western blotting bands of TRAIL on PC3/DX/AR+ cells **B)** Densitometric analysis of AR/GAPDH on PC3/DX/AR+ cells **C)** Densitometric analysis of TNF-α/GAPDH on PC3/DX/AR+ cells **D)** Cas-3 and Cas-8 western blotting bands of TRAIL on PC3/DX/AR+ cells **E)** Densitometric analysis of Cas-3/GAPDH on PC3/DX/AR+ cells and **F)** Densitometric analysis of Cas-8/GAPDH on PC3/DX/AR+ cells (* p<0.05, *** p<0.001 compared to control, + p<0.05 compare to BCLT)

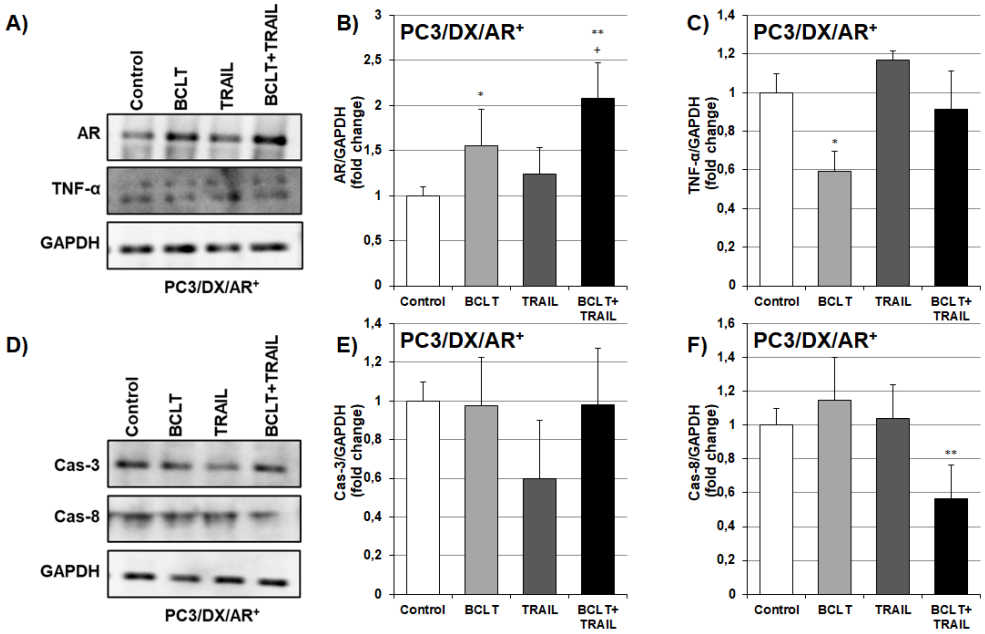

Figure 5. A) AR gene expression levels on PC3/DX cells **B)** TNF-α gene expression levels on PC3/DX cells **C)** Cas-3 gene expression levels on PC3/DX cells**. D)** AR gene expression levels on PC3/DX/AR+ cells **E)** TNF-α gene expression levels on PC3/DX/AR+ cells **F)** Cas-3 gene expression levels on PC3/DX/AR+ cells**.** Gene expression levels of BCLT and/or TRAIL treatment on PC3/DX/AR+ cells **D)** Cas-3 and Cas-8 western blotting bands of TRAIL on PC3/DX/AR+ cells **E)** Densitometric analysis of Cas-3/GAPDH on PC3/DX/AR+ cells and **F)** Densitometric analysis of Cas-8/GAPDH on PC3/DX/AR+ cells (*p<0.05, **p<0.01 , ***p<0.001 compared to control, +p<0.05, +++p<0.001 compare to BCLT)

When compared to control, it was found that AR gene expression increased significantly with BCLT treatment (p<0.01). Compared to the control group, a significant increase in AR gene expression level was

detected with BCLT-TRAIL combination treatment ($p<0.001$); it was found that AR gene expression increased significantly with BCLT-TRAIL combination treatment compared to BCLT ($p<0.05$, Figure 5D). There was no significant change in TNF-α gene expression level with BCLT treatment. It was found that it increased significantly with TRAIL treatment compared to control ($p<0.01$). Similarly, a significant increase in TNF-α gene expression level was detected in BCLT + TRAIL combination treatment ($p<0.01$). At the same time, when the combination treatment was evaluated according to BCLT, it was found that it increased significantly ($p<0.001$, Figure 5D). There was no significant change in Cas-3 gene expression level with either BCLT or TRAIL treatment. While it increased in BCLT-TRAIL combination treatment compared to control ($p<0.001$), a significant increase ($p<0.001$) was found when compared to the BCLT group (Figure 5E).

3. DISCUSSION

In cancer resistance mechanisms, factors such as drug intake, increased excretion of the drug, failure of drug binding to the target, drug metabolism, repairing DNA damage, essential mediators in the cell cycle, stimulation of mediators to control the apoptotic pathway are critical [20,21]. Chemotherapeutic agents are highly effective, especially in the initial stages of cancer treatment, and cause cell death primarily but develop significant resistance. In taxane group drugs, their efficiency is high, but their resistance development potential is high in the treatment of prostate cancer resistant to castration [22]. It has been demonstrated in clinical trials with docetaxel, but it is still not understood how its effects and combinations with different drugs ocur [23,24]. Docetaxel stabilizes microtubules by binding to β-tubulin and stops the proliferation of cancer cells in the mitosis step [25]. There are various opinions that docetaxel through MDR (multidrug resistance protein) or apoptotic proteins [26,27]. However, it has been suggested that docetaxel effectively affects inflammatory markers in castration-resistant cancers and changes the microenvironment in which the tumor is located [28]. Our study modeled a new cell line by treating PC3 cells with docetaxel for 3-5 weeks to develop docetaxel resistance. The cell line we have obtained is an in vitro modeling of a typical clinical situation that is not affected when it is docetaxel in the PC3/DX environment and has gained resistance to the apoptotic effects docetaxel. It is also suggested that taxane group drugs are effective on TNF-α and TNF-α mediated receptors. It has been shown that it exerts apoptotic effects in cells through this receptor and its family [29]. In the study, paclitaxel and docetaxel were placed on MCF-7 breast cancer and A2780 ovarian cancer cells, depending on the dose, and TNF-α secretion was measured.

At the same time, the colony formation and growth capacities of docetaxel relationship TNFR1 and TNFR2 receptors were examined. It has been suggested that docetaxel directs apoptosis in cells by its TNF-receptors. Docetaxel has been shown in studies to increase caspase-3 in prostate cancer. In androgen-independent DU145 cells, when the expression levels of TNF-α in the cell were increased, it was determined that Bcl-2 expression was suppressed, and the caspase-3 levels increased, causing cell death. At the same time, it was observed that cell death occurred in LNCAP androgen-dependent cells with the administration of docetaxel, cisplatin, and TNF-α. Therefore, there is an opinion in the study that using molecules such as TNF-α and chemotherapeutic agents together may be more beneficial in cancer treatment [30]. In another study, it was determined that 0.3 μM docetaxel activates caspases in PC3, DU145, and RWPE-2 prostate cancer cells and at the same time activates the lysosomal pathway and causes cell death [31]. In another study conducted with the melanoma cells IgR3 and MM200 cell lines, it was shown that docetaxel caused an increase in caspase-3 and active caspase-3 at a concentration of 20 nM and could also control mitochondrial apoptosis [32]. Our study observed that TNF-α levels and caspase-3 levels increased when we applied TRAIL treatment in prostate cancer cells with and without androgen receptors that resisted docetaxel. While BCLT slows down the growth of cells as an anti-androgen, it contributes to activating the death mechanisms of the cells together with TRAIL in resistant prostate cancer cells. Clinical studies suggest that BCLT and docetaxel administration in prostate cancer patients can be used in dose-dependent alternative combinations to prevent the development of resistance mechanisms [33,24]. Our study may be preliminary data for these recommendations that BCLT application with TRAIL can be practical in cells that develop resistance. Thus, even in the cell that develops resistance, TRAIL's effect on pro-apoptotic genes in the cell and leading the cells to apoptosis may be necessary for the treatment. On the other hand, when we examine TRAIL and prostate cancer studies, although apoptotic effects are observed, its effect on resistant cells has not been determined yet. TRAIL is a TNF-associated apoptosis-inducing ligand, and it has been reported that it may develop resistance in long-term use in cancer

treatment [34]. Especially in recent years, research on recombinant TRAIL and TRAIL-targeted monoclonal antibodies is in phase1 and phase2 [35]. TRAIL binds to DR5 and DR4 death receptors on cells, causing increased caspases [36]. The triple homodimer of TRAIL allows it to bind to these receptors, and the receptor trimerization takes place, and DISC (Death-Inducing Signaling Complex) occurs in the cytoplasmic part [37]. With the binding of TRAIL, DR4 and DR5 activate caspases that initiate apoptosis through the adapter molecule Fas-associated Death Domain-containing adapter molecule (FADD) containing the death unit. The activation of caspases is activated by breaking the Bid molecule, a member of the proapoptotic Bcl-2 family. By interacting with Bax and Bak molecules, the apoptotic process continues step by step with the release of cytochrome c from the mitochondria. At this stage, if apoptosis protein inhibitor binds to Smac/Diablo, caspase 3 'activation may stop, but apoptosis cannot be reversed after caspase-3 is activated [38]. TRAIL has also been found to trigger proliferation, growth, and survival in cells through activation of NF-κB, MAP kinases, and Akt, in addition to apoptosis [39]. It has been determined that TRAIL can trigger the survival/proliferation of tumor cells with its anti-apoptotic effect in some tumor types, and it is also reported that it can develop a resistance mechanism [40]. In some cancer cell lines, it is known that TRAIL-mediated increased caspase 3 triggers the induction of the internal apoptotic pathway [41-43]. TRAIL's response in prostate cancer may differ. For example, while its apoptotic effect is dominant in androgen-independent prostate cancer cells PC3 and DU145, TRAIL treatment is observed to develop resistance in androgen-dependent cells such as LNCAP [36]. It has been shown that TRAIL increases the activation and expression of androgen in LNCAP cells [44]. For this reason, TRAIL is thought to develop resistance [45,46]. On the other hand, it is suggested that the androgen induces TRAIL and develops resistance in the cell [47,48]. In addition to the resistance development of TRAIL, it has been reported that it is also effective in its anti-tumoral effect in *in vivo* studies. In *in vivo* studies with TRAIL, when 500 µg of recombinant TRAIL protein was administered intravenously, tumor shrinkage was found in SCID mice formed with MDA-MB-231 breast cancer cells. It was not observed significant toxicity in tissue integrity, blood count, and mouse lifetime. In addition, it has been shown that TRAIL-induced apoptotic death in tumor-bearing regions approximately 9-12 hours after the application [49]. Another study reported that repeated systemic application of TRAIL in monkeys did not cause a detectable change in clinical and histopathological tests [50]. When the studies of BCLT and its combinations in cancer cells developing AR-related resistance are examined, very different studies are seen.
Our study observed that when we expressed AR in PC3/DX cells, the expression in the group treated with TRAIL significantly changed compared to the control group. Accordingly, TRAIL plays a role in AR expression. In our study, we applied TRAIL using BCLT, which is an anti-androgen, in order to block the effect of AR. Although BCLT is known as an anti-androgen, it has been shown in recent studies to increase apoptosis in prostate cancer cells (PWR-1E, PC-3 and DU-145) independent of androgen [51]. It has been shown in proteomic studies that the administration of bicalutamide in LNCaP cells also activates fodrin-related apoptotic signaling pathways [52]. It has been reported that when bicalutamide and curcumin are applied to MUC-1 cells, changes in genes in ERK and MAPK signaling pathways are observed in cells [53]. Studies on the use of bicalutamide and molecules with known apoptotic activity are constantly increasing to increase treatment options and prevent the development of resistance. In this case, significant advances can be made in the treatment of prostate cancer, and it would be beneficial to try various combinations, as in this study.

4. CONCLUSION

In our study, we observed that TRAIL significantly increased AR expression at both gene and protein levels. However, we found that in BCLT and TRAIL treatment, apoptosis increased in cells with resistance developed and AR expression, and that cell death occurred via the intrinsic pathway, especially not by caspase-8 but by caspase-3. Our findings showed that it might be an effective treatment for androgen-dependent and independent prostate cancer cells that develop a resistance mechanism against a chemotherapeutic agent.
Studies show that the development of resistance in cancer and elucidation of the molecular mechanisms of the response of the agents used in treatment will contribute to the development of different combined therapies in the future.

5. MATERIALS AND METHODS

5.1. Docetaxel resistance cell line and AR transfection

PC3 cells (ATCC CRL-1435) were inoculated in 25cm² flasks. RPMI-1640 medium containing 10% FBS, Penicillin-Streptomycin (100 units), L-Glutamine, and $NaHCO_3$ was used as cell culture medium. The cells were incubated at 37°C, 95% humidity, and 5% CO_2 and multiplied. PC3 cells were treated with a dose of Docetaxel (DX) of 1µM. After two days, the cell medium was renewed, and 0.5 µM DX was added to the renewed medium. By applying 1 µM DX to the cell passaged on the 4th day, PC3/DX cells of the cell were formed in this way for about 20 days. The B-cell lymphoma 2 (Bcl-2) that the cell gains resistance to DX has been detected. Then enough resistant cells were grown and used in studies. Transfection was performed using pAR plasmid (Addgene #28235). Liposome based DNA transfection reagent (LipoD293, Signagen) was used as the transfection reagent, and transfection was checked after 24 hours with 1 µg of DNA for a 6-well plate. PC3/DX/AR$^+$ cells were created by expressing androgen in cells with docetaxel resistance.

5.2. Cytotoxicity Assay

MTT method was used to determine cell viability, and measurements were made following the kit procedure. Bicalutamide (BCLT) 0.2 µM, 0.5 µM, 1 µM, 2.5 µM, 5 µM, 10 µM, 25 µM; The doses of TRAIL of 1 ng / mL, 2.5 ng / mL, 5 ng / mL, 10 ng / mL, 25 ng / mL, 50 ng / mL, 100 ng / mL were also administered in combinations. Twenty-four hours after the application, 10 µL of 12 mM MTT solution was added and left to incubate for 4 hours at 37˚C in an oven containing 5% CO_2. DMSO was added to dissolve the purple-colored formazan crystals formed at the end of 4 hours and left to incubation at 37˚C in an oven containing 5% CO_2. The absorbance of the purple color formed after 4 hours was measured at 570 nm with an ELISA plate reader. At the end of the experiment, 2.5 µM of BCLT and 2.5 ng/mL of TRAIL were determined for the combination. Since TRAIL reduces cell viability at high doses, it was used only in cytotoxicity experiments in the study, data not shown.

5.3. Acridine Orange / Ethidium Bromide (AO/EB) Staining

AO/EB staining was done under a microscope to detect living and dead cells. After PC3/DX and PC3/DX/AR$^+$ cells were seeded in a 24-well plate, 2.5 µM doses of BCLT and 2.5 ng / mL of TRAIL were administered separately and in combination. Twenty-four hours after the application, the cell medium was discarded, and AO and EB stock (ODC0015B) in PBS was diluted 1/1000 and applied to the wells. After incubating for 15 minutes in the dark, images were taken under a fluorescent microscope under 20X objective in blue and green light. Live cells were stained green with AO, while dead cells were stained red with EB. Calculations were made in the cells by taking the Green/Red ratio.

5.4. Gene expression with qPCR

Total RNA isolation from the cells was performed using the PureLink RNA Mini Kit by the kit procedure. According to the commercial kit procedure, the cDNA recovery was performed according to the procedure with the Applied Biosystems kit. Gene expression experiments with qRT-PCR were performed using the Applied Biosystems SYBR Green Master Mix kit following the procedure (Applied Biosystems Step One Plus). Primers was used sequences of Caspase-3 (forward 5 'CAGTGGAGGCCGACTTCTG 3', reverse 5 'TGGCACAAAGCGACTGAT 3'); AR (forward 5'AAGACGCTTCTACCAGCTCACCA3 ', reverse 5'TCCCAGAAAGGATCTTTGGGCACT3'); TNF-α (forward 5'-GGAGAAGGG GACCGACTCA-3 ', reverse 5'-CTGCCCAGACCTCGGCAA-3'); GAPDH (forward 5'-AGGGCTGCTTTTAACTCTGGT-3 ', reverse 5'-CCCCACTTGATTTTGGAGGGA-3 '). GAPDH was used as a reference gene in the calculations with $2^{-\Delta\Delta Ct}$ methods.

5.5. Protein expression with western blotting

Cells were harvested with RIPA lysis buffer and lysed with sonicator at 4 °C. Total protein determination in samples was made using a BCA kit. Samples were mixed with sample loading buffer at a ratio of 1: 1 and denatured at 95 °C for 5 minutes. 4-12% SDS-PAGE gels were prepared, and samples containing 25 µg / mL protein were loaded into each well. In electrophoresis, samples were first run at 100V for 1 hour. A semi-dry system (Biorad, Semi-Dry Transfer Cell) was used to transfer the proteins in the gel to the membrane after electrophoresis. Proteins separated in the gel were transferred to the PVDF membrane by blotting at 300 mA for 1 hour. PVDF membrane was blocked with 3% BSA in the room for 2 hours. Primary antibody (1: 500 dilution; GAPDH sc-25778, TNF-α sc-1351, Caspase-8 sc-7890, Caspase-3 sc-81651, AR sc-816) was incubated on the orbital shaker overnight at 4^0 C. After incubation; it was washed three times with TBST for 10 minutes. Then, the membrane was incubated with HRP conjugated secondary antibody (1: 1000 dilution; sc-2030, sc-2020) at room temperature for 2 hours. Next, it was incubated with the solution containing the chemiluminescence substrate (Santa Cruz sc-2048) in the dark for 1 minute. The bands obtained with the imaging system (Syngene G: Box) were evaluated by densitometric analysis. GAPDH was used as a reference protein.

5.6. Statistical analyses

Statistical analysis was made using the SPSS 15.00 analysis program. To compare groups, One Way Anova and Tukey test as post-hoc tests were used. The significance level was accepted as $p < 0.05$.

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