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Induction of Autophagic Cell Death by Thymoquinone in Docetaxel Resistant Prostate Cancer Cells

Dosetaksel Dirençli Prostat Kanseri Hücrelerinde Timokinon Tarafından Otofajik Hücre Ölümünün İndüklenmesi

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ABSTRACT

Aim: Acquired docetaxel (DOC) resistance of prostate cancer (PCa) is still a clinical problem. In addition to failure in chemotherapy treatment, it causes tumor recurrence. Therefore, novel and more effective compounds are needed in DOC-resistant PCa treatment. This study aimed to investigate the possible cytotoxic and cell death-inducing activities of thymoquinone (TQ), one of the main active components of *Nigella sativa* L., on DOC-resistant prostate cancer cells.
Material and Methods: DOC-resistant PC3 cells (DOC-R/PC3) were developed by the continuous culture with increment concentrations of DOC (1-10 nM) until they improved their growth and division abilities. The cell viability was determined by MTT assay. The Muse™ Annexin V & Dead Cell kit was performed to detect apoptotic cell death. Autophagic vacuoles were observed by staining autophagic vacuoles. The levels of LC3I, LC3II and Beclin-1 proteins were investigated via western blot analysis.
Results: TQ inhibited the viability of DOC-R/PC3 cells in a dose- and time-dependent manner ($p=0.014$). The IC50 value of TQ for DOC-R/PC3 cells was calculated as 60 µM at 72 h. Treatment of TQ did not induce apoptotic cell death in DOC-resistant prostate cancer cells but induced the formation of autophagic vacuoles. Moreover, Beclin-1 and LC3-II protein levels were increased in TQ-treated DOC-R/PC3 cells, however, LC3-I levels were decreased in DOC-R/PC3 cells.
Conclusion: All these results show that TQ may become a new therapeutic target for DOC-resistant prostate cancer in the future.
Keywords: Autophagy; Beclin-1; LC3; prostate cancer; resistance; thymoquinone.

ÖZ

Amaç: Prostat kanserinde (prostate cancer, PCa) edinilen dosetaksel (docetaxel, DOC) direnci hala klinik bir sorundur. Kemoterapi tedavisinde başarısızlığa ek olarak tümör nüksüne neden olmaktadır. Bu nedenle, DOC'a dirençli PCa tedavisinde yeni ve daha etkili bileşiklere ihtiyaç duyulmaktadır. Bu çalışmanın amacı, *Nigella sativa* L. bitkisinin etken bileşenlerinden biri olan timokinon (thymoquinone, TQ)'un, DOC dirençli prostat kanseri hücreleri üzerindeki olası sitotoksik ve hücre ölümünü tetikleyici aktivitelerinin araştırılmasıdır.
Gereç ve Yöntemler: DOC dirençli PC3 hücreleri (DOC-R/PC3), büyüme ve bölünme yeteneklerini geliştirene kadar artan DOC (1-10 nM) konsantrasyonlarında devamlı kültürle çoğaltıldı. Hücre canlılığı, MTT yöntemi kullanılarak belirlendi. Muse™ Annexin V & Dead Cell kiti, apoptotik hücre ölümünün tespiti için kullanıldı. Otofajik vakuoller spesifik boya kullanılarak gösterildi. TQ mualemesi sonucu LC3-I, LC3-II ve Beclin-1 protein düzeylerindeki değişiklikler western blot analizi ile araştırıldı.
Bulgular: TQ muamelesi, DOC-R/PC3 hücrelerinin canlılığını doza ve zamana bağlı olarak inhibe etti ($p=0.014$). DOC-R/PC3 için TQ'nun IC50 değeri 72. saatte 60 µM olarak hesaplandı. TQ uygulaması, DOC dirençli prostat kanseri hücrelerinde apoptotik hücre ölümünü indüklemedi, ancak otofajik vakuol oluşumunu indükledi. Ayrıca TQ ile muamele edilen DOC-R/PC3 hücrelerinde Beclin-1 ve LC3-II protein seviyelerinin arttığı, ancak DOC-R/PC3 hücrelerinde LC3-I seviyelerinin azaldığı tespit edildi.
Sonuç: Tüm bu sonuçlar, TQ'nun gelecekte DOC dirençli prostat kanseri için yeni bir terapötik ajan olabileceğini göstermektedir.
Anahtar kelimeler: Otofaji; Beclin-1; LC3; prostat kanseri; direnç, timokinon.

INTRODUCTION

Castrate-resistant prostate cancer (PCa) is one of the most prevalent cancers in men worldwide, after lung cancer (1). In most cases, PCa is detected as advanced, depending on the diagnosed stage and prostate-specific antigen levels. Although PCa is diagnosed as much as possible in the early stages, metastases may develop in patients after various local treatments (2).

In recent years, drugs and drug combinations used in the treatment of PCa have been an important development for PCa patients. Among them, docetaxel (DOC), which averts the depolarization of microtubules and mitotic division, is a standard chemotherapeutic treatment method for PCa patients (3,4) Although DOC is an effective treatment in PCA, it can cause various undesirable side effects such as fatigue, pneumonia, and infusion reactions. Moreover, DOC resistance resulting from an exaggeration of ABC transport proteins, which confines the cellular amounts of the drug, is an important problem in PCa treatment. Therefore, novel and more effective compounds are needed in DOC-resistant PCa treatment (5).

Thymoquinone (TQ), known as black cumin, is one of the main active components of *Nigella sativa* L. essential oil (6). Black seed is utilized both as a spice and in the treatment of diseases in many societies, especially in Arab countries. Many studies have been conducted to identify the cytotoxic effects of TQ on prostate, colon, breast, liver, lung, colon, brain cancer cells *in vitro* (7). It has been discovered that many cancer-related mechanisms such as angiogenesis, invasion, metastasis, cell death, and tumor growth are impaired by TQ (8,9). Besides its anticancer properties, TQ fortifies the immune system, protects normal cells from the effects of oxidative stress, and averts various side effects (10). TQ can make cancer cells sensitive to traditional treatments such as chemotherapy and radiotherapy by regulating resistance mechanisms (11,12), therefore it is recommended that TQ be used as an adjuvant in combination with radiotherapy and chemotherapy (7).

The two primary types of programmed cell death (PCD) are apoptosis and autophagy, which can be recognized by their morphological characteristics. Apoptosis, or type I PCD is characterized by cell shrinkage, nuclear condensation and fragmentation, dynamic membrane blebbing, and phosphatidylserine externalization (13). Autophagy or type II PCD is a catabolic process characterized by the formation of autophagosomes, double membrane-bound structures surrounding cytoplasmic macromolecules and organelles (14). Beclin-1 and microtubule-associated light chain 3 (LC3) are two autophagy-related (Atg) genes/proteins that play key functions and are commonly considered as potential markers of autophagy. Beclin-1 is involved in the nucleation phase (early stages) of autophagosome formation and is an important molecule for the initiation of autophagy. LC3 is a key autophagy-related protein that exists in two forms, LC3-I and LC3-II. When autophagy is triggered, the cytosolic form of LC3 (LC3-I) is transformed to LC3-II, a lipid molecule known as phosphatidyl ethanolamine (PE), which is then incorporated into the membrane of autophagosomes (15). It suggests that reduced autophagy contributes to the progression of prostate cancer (16).

This study aimed to investigate the possible cytotoxic and cell death-inducing activities of TQ on DOC-resistant prostate cancer cells.

MATERIAL AND METHODS

Cell Culture Conditions and Chemicals

DOC and TQ were provided from Sigma (USA). The DOC (10 mM) and TQ (10 mM) stock solutions were formulated in dimethyl sulphoxide (DMSO) and stored at -20 °C. We arranged new stock solutions before each experiment and formulated the dilutions just before application.

Human PC3 prostate cancer cell lines were purchased from Interlab Cell Line Collection (ICLC). The cells were maintained in RPMI 1640 with heat-inactivated fetal bovine serum (10%), penicillin-streptomycin (1%), and L-glutamine (1%) supplementation. Cells were cultivated at 37 °C and 5% CO_2- containing incubators. The growth and morphology of the cells were monitored daily under an inverted light microscope. All cell culture procurations were purchased from Sigma.

DOC-resistant derivatives of PC3 (DOC-R/PC3) were produced by the continuous culture at increasing doses of DOC until they improved their growth and division abilities. PC3 cells were cultured with the increasing doses of DOC (1-10 nM) for 6 months before they were able to survive and differentiate in the presence of 10 nM DOC (a >10-fold rise in the IC_{50} value for DOC). MDR1 protein levels were measured using western blot analysis at each stage. When the PC3 cells began to divide in 10 nM of DOC medium and increased MDR1 activity, they were named DOC-R/PC3 and these cells were used for the next experimental steps. Sigma provided all of the other chemicals not listed above.

MTT Viability Assay

The MTT assay was employed to evaluate the cytotoxic effect of TQ on DOC-R/PC3 cells. In 100 µL of culture media, DOC-R/PC3 cells were seeded at 10^4 cells per well in a 96-well plate. TQ formulated by dilution in increasing concentrations (25, 50, 75, 100, 150 µM) was applied to the cells in well-plate for 24, 48, 72 hours. A control without TQ treatment was also incorporated. After incubation term, TQ treated and untreated cells were exposed to 20 µL MTT solution and held at 37 °C for 4 h. Then, all the media was abolished and DMSO was applied to the cells to dissolve formazan crystals. Then, the optic densities of the wells were measured using a spectrophotometer (Tecan Infinite 200 PRO) at 570 nm wavelength. For DOC-R/PC3 cells, the IC_{50} value, indicating the TQ concentration displaying 50% cell viability, was determined using Biosoft CalcuSyn version 2.0 software (USA).

Apoptosis Assay

The Muse™ Annexin V & Dead Cell kit (Millipore) was used to detect the presence of apoptosis in DOC-R/PC3 cells in response to treatment with TQ. The DOC-R/PC3 cells were plated at $4x10^5$ cells and were subjected to pre-determined IC_{50} values of TQ (60µM) or TQ free media (control). The cells were then held at 37 °C for 72 h in a CO_2 incubator. Centrifugation (1000 rpm, 10 min) was used to extract full cells, which were then washed in PBS. The cells were resuspended in RPMI-medium combined with Muse™ Annexin V & Dead Cell reagent and

maintained for 20 min at RT. Finally, apoptosis analysis was assessed with the Muse™ Cell Analyzer (Merck Millipore, Billerica, MA, USA) (17).

Detection of the Autophagic Activity of TQ

In DOC-R/PC3, the Autophagy Assay Kit (ab139484) was used to determine which type of cell death triggered in response to TQ therapy. The cells were subjected to TQ (60 μM) for 72h. Cells were harvested and washed in PBS after being treated. Finally, cells were stained and maintained for 30 minutes at 37 °C in a dark environment concerning the manufacturer's guide. Wide-field fluorescence microscopy was used to monitor stained cells.

Western Blot

First, cells were exposed to 60 μM TQ for 72 h. Cell pellets were prewashed in phosphate-buffered saline (PBS) and then M-PER Mammalian Protein Extraction Reagent (Thermo Scientific) was utilized to make cell lysates. After centrifugation at 14 000 g for 15 min, total protein contents were evaluated by using the Bradford method. Polyacrylamide gel electrophoresis was used to isolate equal quantities of protein loaded onto SDS polyacrylamide. For PAGE, the gels were run at 120 V for 2 hours. Proteins were separated and then moved to nitrocellulose membranes (Bio-Rad Laboratories) under 115 V for 75 minutes. The membranes were blocked for 1 h with a blocking buffer with 5% nonfat dry milk prepared in TBS with 0.1 percent Tween 20. After blocking the membranes, they were incubated with primary antibodies against LC3 (1:750; Cell Signaling) and Beclin-l (1:2000; abcam). After overnight incubation with the primary antibodies, membranes then treated with secondary antibodies (1:2000) for 1 h. Membranes were washed three times with TBS containing Tween 20 for 10 min. The Kodak Gel Logic 1500 Imaging System was utilized to monitor protein bands. Abcam provided all the antibodies (Cambridge, UK). β-Actin was the loading control, and an orbital shaker was used throughout the incubation process (18). Western blot bands were analyzed via Image J software.

Statistical Analysis

Statistical analysis was conducted via one-way analysis of variance (ANOVA) followed by a Dunnett's t-test for multiple comparisons (Normality analysis was done via Shapiro-Wilk test). Statistical analysis and graphs were done via Graph Pad Prism 5 (Graphpad Software). Statistical significance was attributed to values with a $p<0.05$.

RESULTS

Cytotoxic Effect of TQ on the DOC-R/PC3 Cells

The effect of TQ on the viability of DOC-R/PC3 was evaluated by the MTT assay. The increasing concentrations of TQ (25-150 μM) were applied to DOC-R/PC3 cells for 24, 48 and 72 h. MTT assay results showed that cell viability decreased depending on time and increasing TQ concentration. There were 12%, 15%, 25%, 38%, 57% reductions in the cell viability of DOC-R/PC3 cells exposed to 25, 50, 75, 100, 150 μM of TQ, respectively, at 24 h ($p=0.028$). There were 12%, 15%, 33%, 58%, 76% reductions in the cell viability of DOC-R/PC3 cells exposed to 25, 50, 75, 100, 150 μM of TQ, respectively, at 48 h ($p=0.020$). Cell viability after 72 hours of TQ exposure was significantly reduced in TQ-exposed cells relative to non-exposed cells (control). There were 20%, 38%, 60%, 75%, 82% reductions in the cell viability of DOC-R/PC3 cells exposed to 25, 50, 75, 100, 150 μM of TQ, respectively, at 72 h ($p=0.014$). The IC_{50} of TQ for DOC-R/PC3 was measured as 60 μM at 72 hours, when the highest cytotoxic effect was observed.

Evaluation of Apoptosis in TQ Treated DOC-R/PC3 Cells

To determine the proportion of apoptotic cells after TQ treatment, AV/PI staining was conducted, and flow cytometric analysis was done. Since AV-FITCH has a good affinity for phosphatidylserine, AV/PI staining cells indicates early apoptotic cells. Furthermore, while AV-/PI- cells indicate viable cells, AV+/PI+ cells indicate late apoptotic cells. Based on a comparison of TQ-exposed and non-exposed cells, dot plots of flow cytometric apoptosis analysis revealed the proportion of early apoptosis and late apoptosis (Figure 1A, 1B). There was no statistically significant difference in the viability between TQ-treated (%8.2 apoptotic cells) and TQ-untreated cells (%0.1 apoptotic cells) ($p=0.088$), as shown in Figures 1A and 1B. This showed that TQ had no apoptotic effect on human prostate cancer cells resistant to docetaxel (DOC-R/PC3).

Induction of Autophagy in TQ Treated DOC-R/PC3 Cells

The induction of autophagy was examined in DOC-R/PC3 via Autophagy Assay Kit (ab139484) coupled with fluorescence microscopy. Fluorescent microscopy analysis showed the autophagic vacuoles in DOC-R/PC3 cells exposed to TQ for 72 h compared to untreated DOC-R/PC3 cells (Figure 1C, 1D).

LC3-I, LC3-II, and Beclin-1 Protein Expression Levels in DOC-R/PC3 Cells

Western blot analysis was used to determine the levels of LC3-I, LC3-II, and Beclin-1 protein expression in DOC-R/PC3 cells as markers of autophagy. Levels of Beclin-1 protein were induced by 3.2±0.2 fold in TQ-treated DOC-R/PC3 cells (Figure 2). Levels of another autophagy related protein LC3-II were increased by 2.8±0.4 fold in TQ-treated DOC-R/PC3 cells, but LC3-I levels were decreased by 2.4±0.6 fold in DOC-R/PC3 cells compared to untreated control cells, suggesting enhanced autophagic activity in DOC-R/PC3 cells (Figure 2).

DISCUSSION

In patients with metastatic prostate cancer, DOC-based therapy is the preferred first-line treatment. Previous research has suggested that when used as a high-dose monotherapy for prostate cancer, DOC, has major adverse effects and results in resistance. The current study aimed to identify a novel drug with low or no cytotoxicity that could inhibit viability and induce cell death in DOC-resistant human PCa cells. By exposing PC3 cells to gradually the concentrations of DOC, DOC-R/PC3 were created. The resistance to DOC of the subclones was validated by evaluating cell viability and MDR1 activity.

In the literature, it was proven that TQ inhibits cell viability of PCa cells in a dose- and time-dependently (19,20). However, there is no study investigating the possible cytotoxic effect of TQ on DOC-resistant PCa cells. The findings of this study showed that treating the DOC-resistant PCa cell line DOC-R/PC3 with TQ, a new and more powerful drug, resulted in substantial cytotoxic activity.

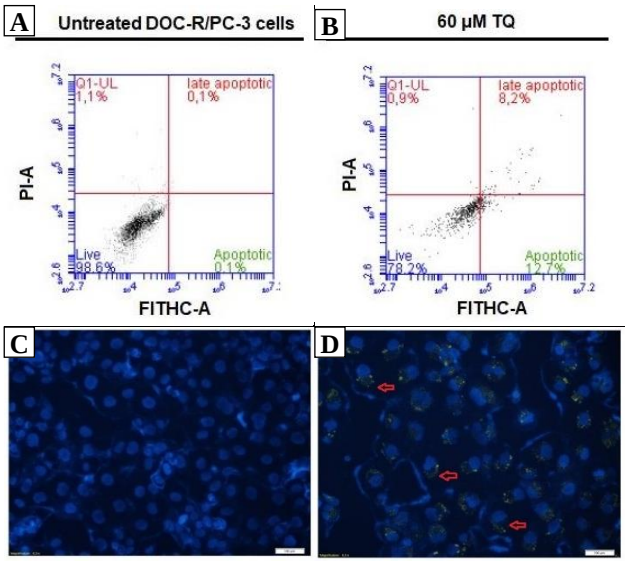

Figure 1. Flow cytometric analysis of **(A)** untreated DOC-R/PC3 cells and **(B)** 60 µM TQ treated DOC-R/PC3 cells via the AV/PI staining. Fluorescent microscopy analysis of **(C)** untreated and **(D)** 60 µM TQ treated DOC-R/PC3 cells

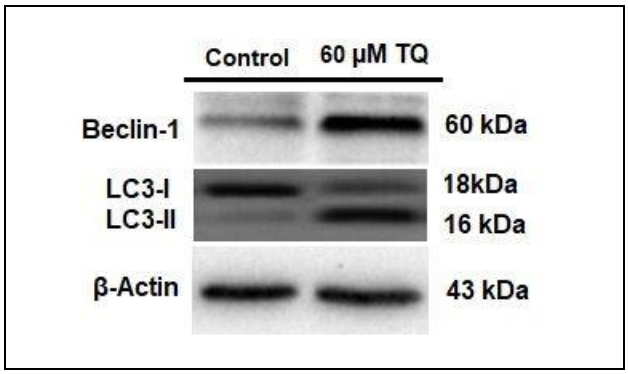

Figure 2. Western blot analysis of LC3-I, LC3-II and Beclin-1 protein levels after 60 µM TQ treatment in DOC-R/PC3 cells

This effect was observed depending on time and concentration manner. It was also reported in previous studies that TQ triggers apoptosis in various human cancer cells including prostate cancer cells (5,21-24). Thus, to investigate the possible apoptotic feature of TQ on DOC-R/PC3 cells, Annexin V-FITC and propidium iodide (PI) levels were evaluated. Annexin V-FITC detects phosphatidylserine externalization in apoptotic cells, while PI attaches to DNA and recognizes necrotic cells. Apoptosis was not induced in TQ-treated DOC-R/PC3 cells, according to flow cytometry.

Another main mechanism underlying effective anti-cancer chemotherapy treatments is autophagy. To enlighten the underlying cytotoxic mechanism of TQ on DOC-R/PC3 cells, the autophagic effect was investigated via staining autophagic vacuoles. The most prominent morphological change in autophagy is the vesicles formed in the cytoplasm surrounded by a membrane of two or more layers, containing parts of the cytoplasm and/or organelles. Lysosomal enzymes break down the cargo of these vesicles as they fuse with lysosomes (25).

To verify the autophagy-inducing effect of TQ in DOC-R/PC3 cells, LC3-I, LC3-II and Beclin-1 protein levels were evaluated. Autophagy is activated in response to similar stress stimuli, coordinated through some regulatory proteins. Various enzyme-substrate relationships (for example, caspase8 both cutting autophagy proteins and being the target of autophagy), protein-protein relationships (such as Atg5-FADD), and protein-protein competition (between autophagy proteins and pro-apoptotic proteins race) play a role. All these data show that there may be several molecular mechanisms underlying TQ inducing autophagy in DOC-R/PC3 cells. After the cellular stress and cell death-related kinases, DAPk or JNK1/2 phosphorylate Beclin1 and Bcl-2, respectively, Beclin1 can escape from the suppressive effect of Bcl-XL protein and activate autophagy (26,27). Moreover, a ubiquitin-like mechanism converts LC3-I into LC3B-II (28).

CONCLUSION

Our data showed that TQ has a significant cell death-inducing effect on PCa cells that have developed resistance to docetaxel, a traditional chemotherapeutic agent. Although studies are showing that TQ is a powerful anticancer agent, our findings also confirm this, however, more thorough research is required to determine the molecular mechanism of action. As a result, this report shed light on autophagy and TQ-induced cytotoxicity in human PCa cells. Based on these findings, TQ can be a new therapeutic option for DOC-resistant prostate cancer in the future.

Ethics Committee Approval: Since our study was not an experimental study including human or animal subject, ethics committee approval was not required.

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