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RESEARCH ARTICLE

Novel combination of docetaxel and thymoquinone induces synergistic cytotoxicity and apoptosis in DU-145 human prostate cancer cells by modulating PI3K–AKT pathway

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Abstract
Background The treatment of castrate-resistant prostate cancer (CRPC) still remains as an important challenge of daily oncology practice. Docetaxel significantly prolongs overall survival in men with CRPC. Thymoquinone (TQ), one of the flavonoid compounds isolated from *Nigealla sativa*, has been shown to possess cytotoxic activity against a variety of cancer cell lines.
Materials and Methods The aim of the study was to investigate the possible synergistic cytotoxic/apoptotic effects of a novel combination, docetaxel and TQ in DU-145 hormone- and drug-refractory prostate cancer cells and their effects on PI3K and ERK signaling pathways.
Results We observed that the combination of docetaxel and TQ resulted in a significant synergistic cytotoxicy and apoptosis as compared to any single agent alone, in a dose-dependent manner. It was found that viability of the combination treated cells was not significantly changed in the presence of LY294002 as compared to inhibitor treated cells. However, in the presence of FR180204, viability of combination treated cells was significantly decreased as compared to inhibitor treated cells. In conclusion, cytotoxic effect of the docetaxel and TQ combination is correlated with the block of the PI3K/Akt signaling pathway in DU-145 cells.
Conclusion Therefore, this combination strategy may be an alternative approach for the challenging era of daily oncologic practice. Also, the combination of docetaxel and TQ might allow a reduction in docetaxel doses and diminish adverse effects of docetaxel while maintaining the therapeutic effect in patients with CRPC.

Keywords Thymoquinone · Docetaxel · Prostate cancer · Combination treatment · PI3K/Akt signaling pathways

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Introduction

Prostate cancer is the most common cancer and the second leading cause of death in men in the United States [1]. The clinical behavior of prostate cancer ranges from a microscopic, well-differentiated tumor to an aggressive, invasive cancer that finally results in metastases. Androgen deprivation therapy (ADT) is the standard initial approach for patients with metastatic prostate cancer. Patients being managed with ADT who have evidence of disease progression (increasing serum PSA, new clinical metastases, progression of existing metastases) are considered to have castrate-resistant prostate cancer (CRPC) [2, 3]. Docetaxel is the only chemotherapeutic agent that significantly prolong overall survival in clinical trials in men with CRPC [4, 5]. Because options for patients with CRPC are limited

following docetaxel failure, investigations are being directed at applying potential benefits of docetaxel-based combination therapy to the clinic, aiming to improve of clinical outcome [6].

A major benefit of combination therapies is that they reduce development of drug resistance, since tumor is less likely to have resistance to multiple drugs simultaneously. Thus, chemotherapeutics are more effective when given in combination (combination chemotherapy). When drugs with different effects are combined, each drug can be used at its optimal dose, without intolerable side effects. In phase II trials, combination treatment with docetaxel has shown increased efficacy compared with docetaxel alone. However, phase III trials of combination treatments with docetaxel in CRPC have failed to improve the efficacy of docetaxel [7–10].

Thymoquinone (TQ) is one of the major bioactive components (30–48 %) of *Nigella sativa* L. plant which is named as black seed or black cumin [11]. TQ has been used as antioxidant, anti-inflammatory and antineoplastic medicines for many years [12]. Many studies have revealed that TQ inhibits tumorigenesis through different molecular mechanisms [13]. Its cytotoxic and pro-apoptotic effects were predominantly demonstrated in prostate cancer cell lines such as PC-3, LNCaP and DU-145 [14, 15].

The family of serine–threonine protein kinases plays an important role in apoptosis. The PI3K/Akt and MAPK/ERK are important members of this family. The activation of the PI3K signaling pathway contributes to several aspects of tumorigenesis such as tumor development, progression, invasiveness, and metastasis [16]. Average 70 % of metastatic prostate cancers have genomic alterations in the PI3K signaling pathway members [17]. This high frequency of genomic alterations supports the rationale for investigating PI3K inhibitors in this tumor type [18]. ERK is not only cytoprotective but also directly promotes hypertrophy [19]. Previous investigations support pathogenetic relevance of the MEK/ERK pathway in prostate tumorigenesis and underscore the need for better understanding of the role of the pathway in prostate cancer cells [20].

In this study, we aimed to investigate the possible synergistic cytotoxic/apoptotic effects of a novel combination, docetaxel and TQ in DU-145 hormone- and drug-refractory prostate cancer cells and their effects on PI3K and ERK signaling pathways.

Materials and methods

Cell lines and reagents

Human prostate cancer cells (DU-145) were obtained from ICLC (Genova, Italy). The cells were grown as monolayer adherent cell lines and were routinely cultured in RPMI 1640 supplemented with 10 % heat-inactivated fetal bovine serum (FBS), 1 % L-glutamine, 1 % penicillin–streptomycin in 75 cm^2 polystyrene flasks (Corning Life Sciences, UK) and maintained at 37 °C in a humidified atmosphere with 5 % CO_2. Growth and morphology were monitored and cells were passaged when they had reached 90 % confluence. Cell culture supplies were obtained from Biological Industries (Israel). TQ and docetaxel were obtained from Sigma Chemical Co (USA). Stock solution of docetaxel (10 nM) and TQ (10 μM) were prepared in dimethyl sulphoxide (DMSO). Final dilutions were made immediately before use, and new stock solutions were prepared for each experiment. DMSO concentration in the assay was <0.1 %, which had no cytotoxic effect on the tumor cells. All other chemicals, unless mentioned, were purchased from Sigma Chemical Co. PI3-K and ERK inhibitors were obtained from Tocris Bioscience.

XTT viability assay

For the viability assay, after verifying cell viability using the trypan blue dye-exclusion test in a Cellometer automatic cell counter (Nexcelom Inc., Lawrence, MA, USA), cells were seeded at 10^4/well in 200 μl into 96-well flat-bottom microtitre plates. After an overnight incubation, cells were treated with drugs alone or in combination. Moreover, to test the effect of PI3K and ERK inhibitors (LY294002 and FR 180204), cells were treated with the inhibitors for 1 h, and then treated with docetaxel in combination with TQ. Plates were incubated at 37 °C in a 5 % CO_2 incubator for the indicated periods. At the end of incubation, 100 μl of XTT (2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide) (Roche Applied Science, Mannheim, Germany) was added to each well, and plates were incubated at 37 °C for a further 4 h. Absorbance was measured at 450 μM against a reference wavelength at 650 μM using a microplate reader (DTX 880 Multimode Reader, Beckman Coulter, Fullerton, CA, USA). The mean of triplicate experiments for each dose was used to calculate the 50 % inhibitory concentration (IC_{50}) and the combination index (CI) values.

Evaluation of apoptosis by DNA fragmentation analysis

Apoptosis was measured with a Cell Death Detection ELISA Plus Kit (Roche Applied Science, Germany) according to the manufacturers' instructions. The relative amounts of mono- and oligo-nucleosomes generated from the apoptotic cells were quantified using monoclonal antibodies directed against DNA and histones by ELISA. Briefly, the cytoplasmic fraction of the untreated control,

TQ-, Docetaxel- and combination treated cells were transferred onto a streptavidin-coated plate and incubated for 2 h at room temperature with a mixture of peroxidase conjugated anti-DNA and biotin labeled anti-histone. The plate was washed thoroughly, incubated with 2,29-azino-di-[3-ethylbenzthiainesulphonate] diammonium salt, and the absorbance was measured at 405 nm with a reference wavelength at 490 nm (DTX 880 Multimode Reader, Beckman Coulter, Fullerton, CA, USA).

Statistical analysis

All experiments were conducted in triplicate and the results expressed as the mean ± SD, with differences assessed statistically p values determined by Students' t test. The median dose–effect analysis was used to assess the interaction between agents. Determination of the synergistic vs additive vs antagonistic cytotoxic effects of the combined treatment of cells with TQ and Docetaxel were assessed by Biosoft CalcuSyn software (Ferguson, MO, USA). CI was used to express synergism ($CI < 1$), additive effect ($CI = 1$), or antagonism ($CI > 1$) (Chou and Talalay, 1984).

Results

Effects of docetaxel and TQ alone on the viability of DU-145 cells

To evaluate the effects of docetaxel and TQ on the viability of human prostate cancer cells, DU-145 cells were exposed to increasing concentrations of docetaxel (from 0.01 to 1,000 nM) and TQ (1 to 120 μM) for 24, 48 and 72 h. Both docetaxel and TQ decreased cell proliferation in a time- and dose-dependent manner (data not shown). As shown in Fig. 1, there were 8, 15, and 60 % decreases in cell proliferation in 0.1, 1 and 10 nM docetaxel applied DU-145 cells, as comparing to untreated controls at 72 h (Fig. 1, $p < 0.05$). Highest cytotoxicity was observed at 72 h and IC50 value of docetaxel in DU-145 cells was calculated from cell proliferation plots and was found to be 10 nM. We also examined the effect of TQ on DU-145 cells. There were 6, 36, and 72 % decreases in cell viability of DU-145 cells exposed to 10, 40, and 80 μM of TQ, respectively, when compared to untreated controls at 72 h (Fig. 2, $p < 0.05$). IC_{50} value of TQ was 130 μM for DU-145 cells (Fig. 2).

Synergistic effects of docetaxel and TQ in DU-145 prostate cancer cells

To study the possible synergistic/additive effects of docetaxel and TQ combination, DU-145 cells were exposed to

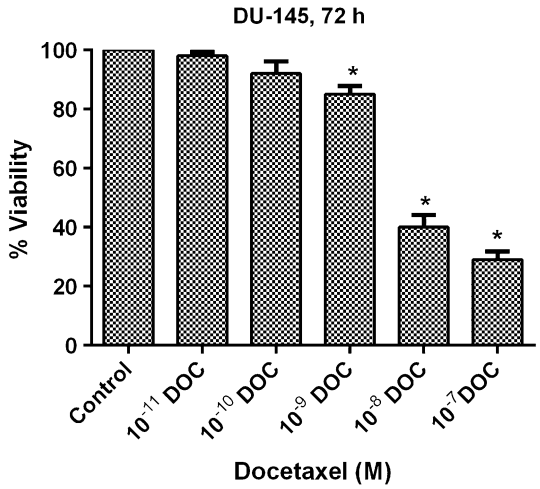

Fig. 1 Effects of docetaxel on the viability of DU-145 cells. The XTT assay was performed using triplicate samples in at least two independent experiments. The *error bars* represent the standard deviations, and when not seen, they are smaller than the thickness of the lines on the graphs. $p < 0.05$ was considered significant

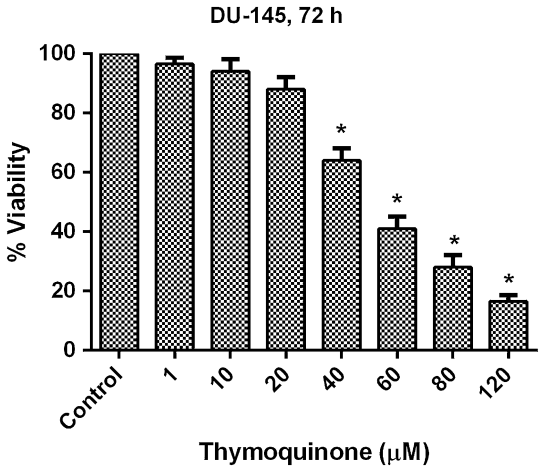

Fig. 2 Effects of TQ on the viability of DU-145 cells. The XTT assays were performed using triplicate samples in at least two independent experiments. The *error bars* represent the standard deviations, and when not seen, they are smaller than the thickness of the lines on the graphs. $p < 0.05$ was considered significant

different concentrations of docetaxel or TQ alone, and in combination of both for 24, 48 and 72 h. Combination of different concentrations of docetaxel and TQ were evaluated at different time points (data not shown). Results showed significant synergistic toxicity on DU-145 prostate carcinoma cells at 72 h, as compared to any agent alone as shown in Table 1. The results revealed that while 10 nM docetaxel and 60 μM TQ resulted in 43.5 and 15 % decrease in proliferation of DU-145 cells, respectively, the combination of both drugs at the same doses caused 68.5 % decrease in cell proliferation as compared to untreated

Table 1 Combination index values of docetaxel in combination with TQ on the viability of DU-145 cells

Concentration of drugs	CI value
DOC (0.1 nM) + TQ 60 (μM)	0.964 Synergism
DOC (10 nM) + TQ 60 (μM)	0.445 Strong synergism

Combination index (CI) values were calculated from repeated XTT cell viability assays

controls (Fig. 3). CI values were represented in Table 1 for each combination (Table 1).

Effects of the sequential treatment

We examined the effect of sequential treatment with either docetaxel or TQ and subsequent treatment with the second agent in DU-145 cells. Pretreatment of tumor cells with docetaxel for 36 h, washing, and then treatment for an additional 36 h with TQ resulted in synergistic cytotoxicity in DU-145 cells. Also, pretreatment of tumor cells with TQ for 36 h, washing, and then treatment for an additional 36 h with docetaxel resulted in synergistic cytotoxicity in DU-145 cells (data not shown). Thus, significant synergistic cytotoxicity of the drugs was observed regardless of which agent was applied first.

Effects of docetaxel and TQ combination on DNA fragmentation in DU-145 prostate cancer cells

To examine the possible synergistic effects of combination of docetaxel and TQ, as compared to any agent alone, on induction of DNA fragmentation as a marker of cell death, we quantified the levels of mono-oligo nucleosome fragments using Cell Death Detection Plus Elisa Kit (Roche Applied Science, Mannheim, Germany). We treated DU-145 cells in different concentrations of docetaxel or TQ and the combination of both for 72 h before analysing DNA fragmentations (Fig. 4). The results showed that when DU-145 cells exposed to 10 nM docetaxel and 60 μM TQ, there were 8.2 and 3.3-fold increase observed in DNA fragmentation as the combination of both induced DNA fragmentation 20 fold more as compared to untreated controls (Fig. 4).

Effects of caspase 3/7 activity in combination treated DU-145 cells

Next, we investigated the molecular mechanisms underlying apoptosis induced by combination treatment in prostate cancer cells. For this aim, we evaluated the possible role of the executioner DEVD caspases 3/7. The combination of docetaxel with TQ did not affect the activation of caspase 3/7 activity in DU-145 cells (data not shown) ($p > 0.05$).

Effects of docetaxel and TQ combination on PI3K/AKT and MAP/ERK signal pathways

Further, to elucidate the underlying mechanisms of synergistic effects of the combination treatment, we have investigated whether combination-induced cytotoxicity was related to inhibition of PI3K/Akt and MAPK/ERK signaling pathways. For this aim, effects of specific PI3K

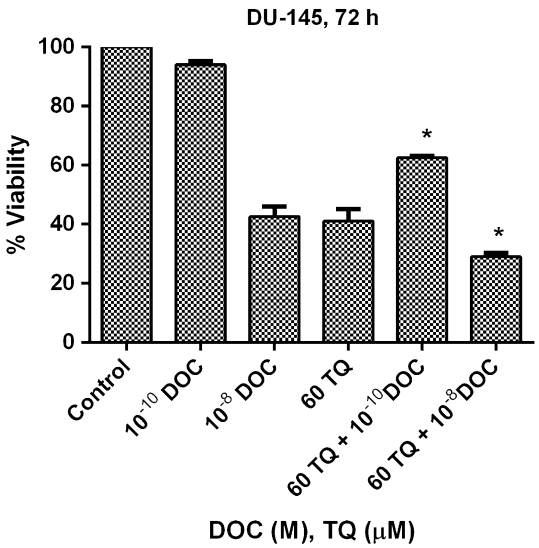

Fig. 3 Effects of docetaxel and TQ combination on the viability of DU-145 Cells. The results are expressed as the mean of three different experiments. The *error bars* represent the standard deviations, and when not seen, they are smaller than the thickness of the lines on the graphs. $p < 0.05$ was considered significant

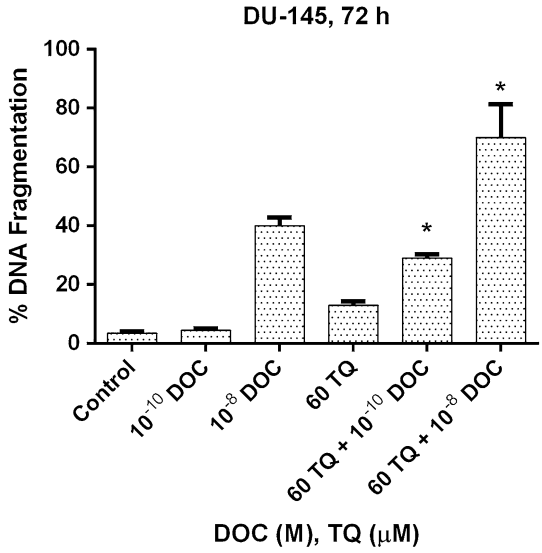

Fig. 4 Apoptotic effects of docetaxel and TQ alone or in combination on DU-145 cells through DNA fragmentation analyses. The results are the means of two independent experiments. The *error bars* represent the standard deviations, and when not seen, they are smaller than the thickness of the lines on the graphs. $p < 0.05$ was considered significant

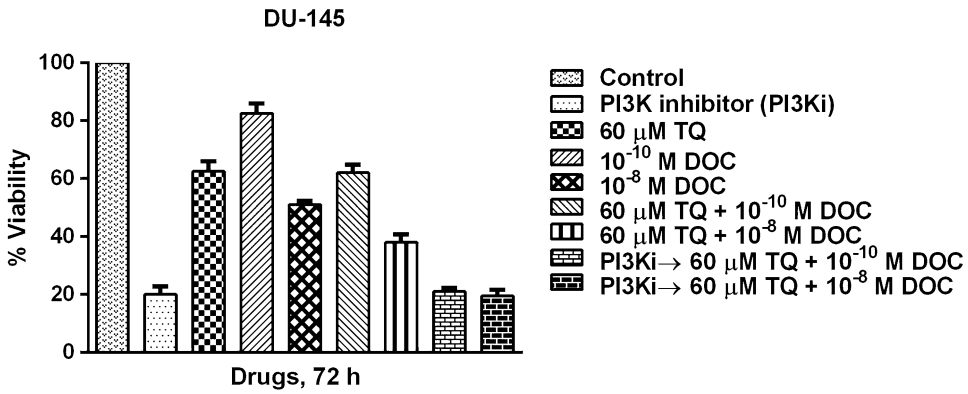

Fig. 5 Effects of LY294002, a specific PI3K inhibitor on the viability of docetaxel and TQ combination treated DU-145 cells. The results are the means of two independent experiments. The *error bars* represent the standard deviations, and when not seen, they are smaller than the thickness of the lines on the graphs. $p > 0.05$ was considered not significant

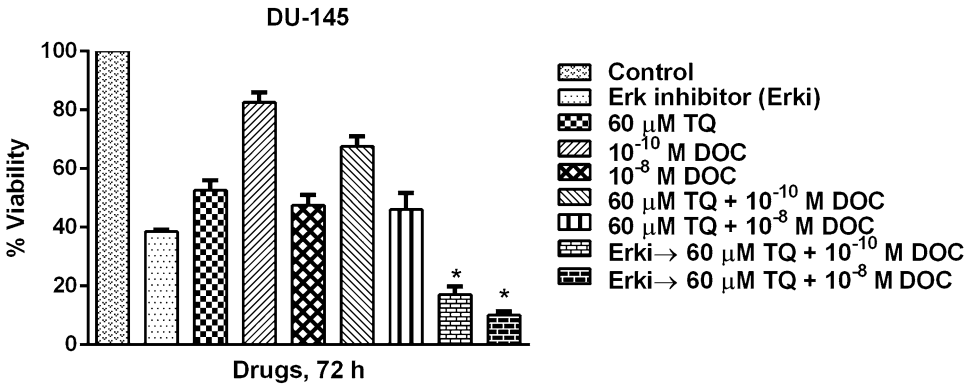

Fig. 6 Effects of FR180204, a specific ERK inhibitor on the viability of docetaxel and TQ combination treated DU-145 cells. The results are the means of two independent experiments. The *error bars* represent the standard deviations, and when not seen, they are smaller than the thickness of the lines on the graphs. $p < 0.05$ was considered significant

(LY294002) and ERK (FR180204) inhibitors were investigated on the viability of DU-145 cells by XTT viability assay. It was found that viability of the combination treated cells was not significantly changed in the presence of LY294002 (50 μM) as compared to inhibitor treated cells (Fig. 5) ($p > 0.05$). However, in the presence of FR180204 (50 μM), viability of combination treated cells were significantly decreased as compared to inhibitor treated cells (Fig. 6) ($p < 0.05$).

Discussion

Data presented here provide the evidence that treatment of hormone- and drug-resistant prostate cancer cell line, DU-145 with a novel combination, docetaxel and TQ, results in a significant synergistic cytotoxic activity and apoptosis compared to any single agent alone. This effect was observed in a dose and time-dependent manner. However, it is found that the combination treatment of docetaxel and TQ did not induce caspase 3/7 in DU-145 cells. We have looked for the possible underlying mechanisms of synergy achieved by combination of docetaxel and TQ and we have shown that combination significantly correlated with the block of the PI3K/AKT signal pathways in DU-145 cells. However, the novel combination had no effect on MAPK/ERK pathway.

In DU-145 cells exposed to docetaxel and TQ combination, we demonstrated drug concentration dependent increases in DNA fragmentation, while there was no caspase 3/7 activation. The reason of apoptosis in our DU-145 cell lines may be due to the ROS dependent apoptotic mechanisms of TQ as there was no role of caspase activation like in the studies of Zubair et al. and Koka et al. [14, 21].

The PI3K/Akt and MAPK/ERK plays an important in regulating cell proliferation, growth and apoptosis. [19, 22, 23]. Given its important role in cancer, there is great interest in the development of inhibitors able to target on the signaling pathways in preclinical trials. Metastatic CRPC is one such tumor type being investigated using this strategy. The primary negative regulator of the PI3K pathway is Pten. Loss of Pten expression in prostate cancer is one of the most frequent molecular aberrations and 65 % of metastatic cases have some form of alteration in the PI3K pathway [24, 25]. The best-characterized result of Pten loss is increased survival signaling through PI3K/Akt [26]. This high frequency of alterations supports the rationale for investigating PI3K inhibitors in prostate cancer. Previous studies demonstrate a novel role of the MAPK/ERK pathway in regulating androgen receptor expression in androgen-dependent prostate cancer cells [27, 28]. Docetaxel has been shown to effect prostate cancer cell lines by modulating both of these pathways [29, 30].

Targeting multiple signaling molecules is essential to induce enhanced apoptosis rather than single molecular target in cancer therapy. Therefore, in this study, we employed combination therapy to aim multiple molecular targets in inducing apoptosis, besides reducing the therapeutic doses of Docetaxel. Therefore, a dietary phytochemical TQ the main active ingredient of the volatile oil of *Nigella sativa*, employed to overcome docetaxel evinced serious side effects and assist in decreased resistance on docetaxel administration.

Previous studies have shown that TQ exhibits inhibitory effects on cell proliferation of many cancer cell types including prostate cancer cells [31]. Anticancer effects of TQ depend on the inhibition of proliferation, angiogenesis, invasion and metastasis [13]. In the present study, we have identified that ERK inhibitor FR180204 enhanced the combination treatment of docetaxel and TQ effects on inhibition of cell viability (Fig. 6). Moreover, combination treatment of docetaxel and TQ-induced cytotoxicity was attenuated after the inhibition of PI3K/AKT pathway by LY294002 (Fig. 5).

The neuroendocrine (NE) cells represent in the normal prostate. Important in tumorigenesis of NE cells has been showed in basic trails [32, 33]. In prostate carcinomas, under androgen-ablation therapy, the NE cell population is increased by several folds [34]. Those tumors with an increased NE cell population are often more aggressive and become androgen-independent [35]. Upregulated ERK1/2 activity is detected in NE cells in PCa as well as in NE-differentiating PCa cell lines while various stimulations that activate the Raf/MEK/ERK pathway can induce NE differentiation of PCa cells in in vitro and in vivo environments [36]. In a study, Hong et al. [20] showed that the Raf/MEK/ERK pathway-mediated growth inhibition and NE differentiation could be mediated via mechanisms dependent or independent of AR downregulation. The results of same the study demonstrate that the Raf/MEK/ERK pathway has a novel role in regulating AR levels in a subset of LNCaP prostat cancer cells. We observed that TQ and docetaxel combination treatment do not have effect over MAPK/ERK pathway. But during the studies that will be done over this pathway, androgen-ependent cells lines should be used also the androgen receptor status and the NE differentiation status of the cells should be known. Our study is limited in this way.

Conclusion

Inhibition of PI3K/AKT pathway by the combination treatment of docetaxel and TQ might be one of the critical routes underlying the synergistic cytotoxic and apoptotic effect in hormone-and drug-refractory prostate cancer cell line, DU-145. These data suggest that the novel combination of these drugs might allow reduction in docetaxel doses and diminish docetaxel related adverse effects while maintaining the therapeutic effect in patients with CRPC.

Conflict of interest The authors declare no conflict of interest.

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