Original Research

Studies on molecular mechanisms of growth inhibitory effects of thymoquinone against prostate cancer cells: role of reactive oxygen species

Padma Sandeep Koka[1], Debasis Mondal[1], Michelle Schultz[1], Asim B Abdel-Mageed[2] and Krishna C Agrawal[1]

[1]Department of Pharmacology; [2]Department of Urology, Tulane University School of Medicine, New Orleans, LA 70112, USA
Corresponding author: Debasis Mondal, Department of Pharmacology, SL-83, Tulane University School of Medicine, New Orleans, LA 70112, USA. Email: dmondal@tulane.edu

Abstract

Thymoquinone (TQ), an active ingredient of black seed oil (*Nigella Sativa*), has been shown to possess antineoplastic activity against a variety of experimental tumors. However, the precise mechanism of action of TQ is not known. We investigated the mechanism of action of TQ in androgen receptor (AR)-independent (C4-2B) and AR naïve (PC-3) prostate cancer cells, as models of aggressive prostate cancers. Exposure (24–48 h) to TQ (25–150 μmol/L) inhibited the growth of both C4-2B and PC-3 cells, with IC_{50} values of approximately 50 and 80 μmol/L, respectively. Within one hour, TQ increased reactive oxygen species (ROS) levels (3-fold) and decreased glutathione (GSH) levels (60%) in both cell types. Pretreatment with *N*-acetylcysteine (NAC) inhibited both TQ-induced ROS generation and growth inhibition. TQ did not increase the activity of caspases and the caspase inhibitor, z-VAD-FMK did not decrease TQ-induced apoptosis. Furthermore, although TQ treatment resulted in the activation of Jun kinase (JNK), pretreatment with the JNK inhibitor, SP600125, did not protect cells from TQ. However, TQ significantly up-regulated the expressions of growth arrest and DNA damage inducible gene (GADD45α) and apoptosis-inducing factor-1 and down-regulated the expressions of several Bc12-related proteins, such as BAG-1, Bcl2, Bcl2A1, Bcl2L1 and BID. In C4-2B cells, TQ dose dependently inhibited both total and nuclear AR levels (4–5 fold) and AR-directed transcriptional activity (10–12 fold). Interestingly, this suppressive effect on AR was not prevented by NAC, which clearly suggested that TQ-induced cytotoxicity is not due to changes in AR regulation. These data suggest that TQ-induced cell death is primarily due to increased ROS generation and decreased GSH levels, and is independent of AR activity.

Keywords: thymoquinone, prostate cancer cells, reactive oxygen species, androgen receptor

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Introduction

With an estimated 186,320 new cases and 28,660 deaths in the United States during 2008 (NCI), prostate cancer is the second most common cancer in men. The mainstream treatment strategy for advanced prostate cancer is androgen ablation therapy (ADT).[1] Despite the initial success of ADT, most patients with advanced prostate cancer develop resistance to ADT and progress to hormone refractory disease, which is often lethal (mean survival 14–20 months) and resistant to chemotherapy.[2,3] Although many researchers have focused on inhibition of prostate cancer growth through the targeting of AR, alternative therapeutic approaches need to be developed to target those cells in which AR inhibition is ineffective.

Herbal medicines such as black seed oil, extracted from the seeds of *Nigella sativa*, have been used for more than 2000 years for the treatment of various diseases and the promotion of good health.[4] A major component of black seed oil is the lipid-soluble chemical, thymoquinone (TQ). TQ has been shown to exert anti-inflammatory, antioxidant and antineoplastic effects in both *in vitro* and *in vivo* conditions.[4] In a variety of cancer cell types, TQ has also been

Dr Krishna C Agrawal, Professor and Chairman of the Department of Pharmacology at Tulane Medical School, in New Orleans, LA, passed away Saturday, December 12, 2009. His exemplary life was inspired by his loving wife, his three children and seven grandchildren. Dr Agrawal's strong and revered leadership and his kind and gentle guidance will be missed by all.

shown to have growth inhibitory effects through the inhibition of DNA synthesis and the induction of cell cycle arrest.[5–7] It showed significant toxicity against pancreatic, lung, colon, uterine and leukemic carcinomas, but it had no significant effects on the growth of non-cancerous cell lines.[6,8] Previous reports demonstrated that TQ-induced apoptosis in neoplastic keratinocytes is via a p53-dependent mechanism, while in HL-60 cells apoptosis was induced by activation of caspase-8 and down-regulation of nuclear factor-kappa B activity.[7,9,10] In PC-3 prostate cancer cells, TQ has also been reported to suppress the activation of Akt and extracellular signal-regulated kinase signaling pathways.[11] Furthermore, in C4-2B prostate cancer cells, the growth inhibitory effect of TQ has been implicated to be the result of inhibition of AR and E2F-1 expression.[12] All these studies demonstrated that TQ induces growth inhibition through the suppression or activation of various signaling pathways. However, the precise mechanism by which TQ is able to alter these cellular pathways and induce growth inhibition has not been elucidated.

TQ belongs to a family of quinones that can undergo either enzymatic or non-enzymatic redox cycling with their respective semiquinone radicals to generate superoxide anion radicals.[13] Various quinones (e.g. furanonaphthoquinones, pyrrolo[2,1-c][1,4]benzodiazepine-anthraquinone, 2-acylamine-1,4-napthoquinone and 1,4-napthoquinone) induce apoptosis in cancer cells by generating reactive oxygen species (ROS).[14–17] In cancer cells, ROS has been shown to induce growth inhibition through various pathways including the depletion of glutathione (GSH) levels, activation of Jun kinase (JNK) and caspases, and over-expression of GADD45α.[18–22] We therefore hypothesized that TQ may generate ROS that is responsible for activation of cellular pathways that inhibit the growth of prostate cancer cells. A preliminary report from our laboratory has been published demonstrating the production of ROS and inhibition of Akt phosphorylation by TQ.[23] In this study, we investigated the role of TQ-induced ROS generation and GSH depletion, in inhibiting the growth of prostate cancer cells. We also investigated the role of caspases and several apoptosis-related proteins, in regulating TQ-mediated prostate cancer cell death.

Materials and methods

Cell culture

The prostate cancer cell line PC-3, isolated from the vertebral metastases of a prostate cancer patient, was obtained from American Type Tulture Collection (ATCC; Manassas, VA, USA; CRL-1435). These cells were grown in F12K medium supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin. C4-2B cells were provided by Dr L Chung (Emory University School of Medicine, Atlanta, GA, USA) and subsequently grown in RPMI supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin.

Reagents

TQ and *N*-acetylcysteine (NAC) were purchased from Sigma (St Louis, MO, USA). Monochlorobimane (Fluka, Milwaukee, WI, USA) and dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) (Invitrogen, Carlsbad, CA, USA) were purchased as indicated. Z-VAD-FMK, a caspase inhibitor, was obtained from BD Pharmingen (Franklin Lakes, NJ, USA). The WST-8 assay kit was purchased from Dojindo Molecular Technologies, Inc (Salt Lake City, UT, USA). The caspase assay kit was obtained from Biovision Inc (Mountain View, CA, USA). Antibodies towards GADD45α and phospho-c-Jun were purchased from Santa Cruz Biotechnologies (San Diego, CA, USA) and Biovision, respectively. Bcl2, Bcl2A1, BCL2L1, BID, apoptosis-inducing factor (AIF), and β-actin were all purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies towards AR and TATA binding protein were purchased from Abcam (Cambridge, MA, USA) and BAG-1, p-JNK and pan-JNK were purchased from BD Biosciences (Franklin Lakes, NJ, USA).

Cell survival assay

Colorimetric 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfo-phenyl)-2H-tetrazolium, monosodium salt (WST-8) assay was used to detect viable cells. When WST-8 is reduced by dehydrogenases in live cells, it produces a yellow-colored product called formazan that is soluble in the tissue culture medium. Prostate cancer cells were seeded at a density of 5000 cells per well in 96-well plates and treated with 25–150 μmol/L TQ for 24 and 48 h, and 10 μL of CCK-8 solution was added and after 1 h incubation at 37°C, absorbance was measured at 450 nm.

ROS Assay

Intracellular ROS levels were measured in prostate cancer cells using the fluorescent dye, CM-H_2DCFDA (Invitrogen). CM-H_2DCFDA is a cell-permeable indicator for ROS that fluoresces when acetate groups are removed by intracellular esterases and oxidation occurs within the cell. Cells were cultured in 24-well Krystal black plates (Labnet International, Woodbridge, NJ, USA) and washed once with warm phosphate-buffered saline (PBS) before being incubated with 10 μmol/L CM-H_2DCFDA in serum-free media for 20 min at 37°C. Cells were then washed with warm PBS and treated with various concentrations of TQ for three hours. After treatment, ROS levels were monitored every 30 min for two hours, at excitation and emission wavelengths of 485 and 520 nm, respectively, using a *FL_x800* fluorimeter (Bio-Tek Instruments, Winooski, VT, USA). No dye controls were used to determine background fluorescence and were subtracted from experimental samples. Results are expressed as percent change in relative fluorescence units (RFUs) as compared with the vehicle controls.

GSH assay

Monochlorobimane (Sigma) was used to measure the intracellular reduced GSH levels. Monochlorobimane binds to reduced GSH and fluoresces at 420 nm when excited at 380 nm. For these studies, cells were grown in Krystal 24-well opaque black plates (Labnet, NJ, USA) and treated for 30 min with 25–150 μmol/L TQ. After the 30-min TQ

exposure, cells were washed with PBS and incubated with 50 μmol/L monochlorobimane for 20 min. After incubation, cells were washed with PBS and fluorescence was measured for up to one hour at 10-min intervals.

Western immunodetection

After TQ treatment, cells were lysed (Cell Signaling Technology) and 50 μg of protein was separated on 4–20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels (Bio-Rad, Hercules, CA, USA) at 90 V. Protein was then transferred to a nitrocellulose membrane and blocked with 5% non-fat dry milk prepared in Tris-buffered saline containing 0.1% Tween-20. Primary antibody was incubated overnight at 4°C. Protein levels were determined by densitometry with Quantity One (Ver. 4.6.1). Densitometric values were compared with corresponding $\beta-$ actin controls and fold change from control was reported.

Caspase assay

The caspase assay is based on the colorimetric detection of chromophore *p*-nitroanilide (pNA) after cleavage from a labeled substrate (caspase-3 – DEVD-pNA; caspase-6 – VEID-pNA; caspase-8 – IETD-pNA; and caspase-9 – LEHD-pNA). PC-3 cells were treated with TQ (50 and 100 μmol/L) and lysed with cell lysis buffer. Cell lysate was centrifuged and quantified by the BCA method (Pierce). Approximately, 100 μg of protein from each sample was loaded onto a 96-well plate and diluted to 50 μL in 2× reaction buffer containing 10 mmol/L dithiothreitol, and 5 μL of substrate was added and absorbance was measured at 405 nm.

RNA isolation and reverse transcriptase-polymerase chain reaction

The trizol reagent (Invitrogen) was used to isolate RNA from TQ-treated cells and semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using an assay kit (Promega). Approximately 2.0–3.0 μg of total RNA was used in each RT reaction mixture (60 μL) containing MMLV-RT (0.5 U), RNase inhibitor (0.1 U), oligo-dT (1 μg) and deoxynucleotides (dNTPs, 1 mmol/L each), and incubated for 60 min at 42°C. Each 50 μL PCR reaction mix contained cDNA template, 2× REDTaq Ready mix, forward primer (0.2 μmol/L) and reverse primer (0.2 μmol/L). The primer sequences and amplification protocols for GADD45α and GAPDH were according to a previous publication.[21] The PCR products were electrophoresed on a 2.0% agarose gel containing ethidium bromide, and the intensity of each product was evaluated using a UV gel Documentation system (Bio-Rad). GAPDH was used as the internal loading control and densitometric values for GADD45α expression were calculated as compared with GAPDH levels in the respective samples.

Luciferase assay

Approximately 5 million C4-2B cells were co-transfected with pPSA-LUC and pCMV-β-gal plasmids using lipofectamine plus (Promega), according to the manufacturer's instructions. At 24 h post-transfection, cells were treated with 0–50 μmol/L TQ for 48 h, growth medium was removed, cells were washed with PBS and 30 μL of lysis buffer (Promega) was added to each well. For the luciferase assay, cell lysate was pelleted and 20 μL of the supernatant was added to 100 μL of luciferase assay reagent (Promega) for each reaction. Similarly, the β-galactosidase assay was performed in 20 μL of cell extracts according to the manufacturer's instructions. The luciferase luminescence values (RLU) were normalized to the β-galactosidase activity in respective samples.

Statistical analysis

All experiments were performed at least three times, with values obtained from an average of 3–4 replicates per treatment condition. In Western hybridizations and PCR experiments, the data were normalized to the internal controls. All statistical analyses were performed with the GraphPad Prism version 4.00 Software (San Diego, CA, USA). Results are expressed as $\pm$ standard error of means. The significance of changes from control was determined by a two-tailed Student's *t*-test and comparison of three or more groups was done by one-way analysis of variance with Dunnett's post-test.

Results

Concentration-dependent effect of TQ on prostate cancer cell viability

The benign prostate hyperplasia (BPH), LNCaP, PC-3 and C4-2B cells were treated with TQ (25–150 μmol/L) for 24 or 48 h and their percent survival was monitored using a WST-8 assay (Figure 1). Exposure to TQ showed a dose- and time-dependent suppression in cell survival. As compared with the BPH cells, TQ-mediated toxicity was significantly higher in the tumorigenic lines LNCaP, C4-2B and PC-3. At 24 h, the IC_{50} values of TQ were 92 μmol/L in LNCaP, 86 μmol/L in PC-3 cells and 100 μmol/L in C4-2B cells. At 48 h, the IC_{50} values of TQ were 49, 74 and 87 μmol/L, in PC-3, LNCaP and C4-2B cells, respectively. At these lower concentrations (25–100 μmol/L) no significant growth inhibition was observed in the BPH cells and close to 50% inhibition in survival was seen only at the highest concentration of 150 μmol/L TQ. This clearly suggested that TQ exposure may have a more cytotoxic effect in the aggressive prostate cancers.

Exposure to TQ acutely increases ROS generation and depletes GSH levels

Quinones generate ROS through the formation of semiquinone radicals.[16] Therefore, we determined the effect of TQ on ROS production in prostate cancer cells. PC-3 and C4-2B cells were treated with 25–100 μmol/L TQ for 1 h before ROS levels were measured using CM-H_2DCFDA. The values were obtained at 30 min intervals up to 2 h and RFU values were normalized to protein content in cells. In both PC-3 and C4-2B cells, TQ treatment acutely (1 h) increased ROS expression and the peak was determined at the 1-h time point, as shown in the bar

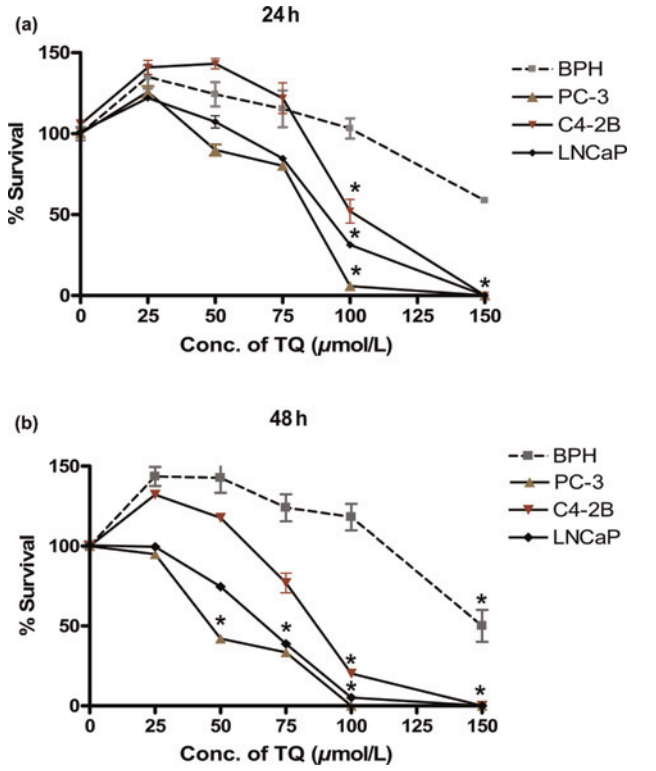

Figure 1 TQ-induced growth inhibition of prostate cancer cells. PC-3, LNCaP, C4-2B and BPH cells were treated with 25–150 μmol/L TQ for 24 h (a) and 48 h (b) in a 96-well plate. Growth inhibition of prostate cancer cells was measured by OD readings using the WST-8 dye. Change in survival with increasing concentrations of TQ, as compared with untreated controls (100%) is shown in the line graphs. Error bars represent the standard error of means (SEM) generated from four independent experiments ($n = 4$) carried out in triplicates, and significant changes represent $^{*}P < 0.05$. TQ, thymoquinone; BPH, benign prostate hyperplasia (A color version of this figure is available in the online journal)

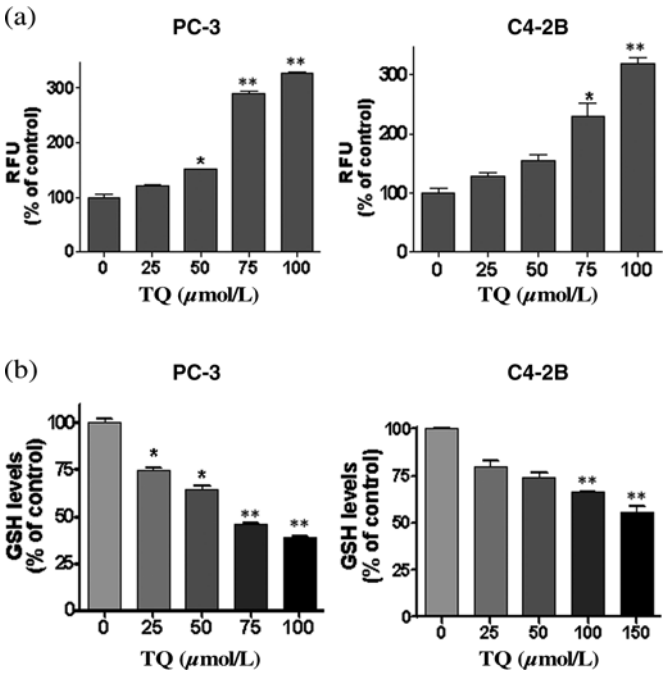

Figure 2 TQ-induced ROS generation and depletion of GSH in PC-3 and C4-2B cells. (a) PC-3 and C4-2B cells were treated with increasing concentrations of TQ (25–100 μmol/L) for one hour and ROS levels were monitored. Data are represented as percent change in relative fluorescent units (RFUs) as compared with control. (b) PC-3 and C4-2B cells were treated with TQ for 30 min and the levels of GSH were determined with a monochlorobimane dye. Data are expressed as percent change in GSH activity from control. Error bars represent the SEM generated from three independent experiments ($n = 3$) carried out in triplicates, and significant changes represent $^{*}P < 0.05$ and $^{**}P < 0.01$. GSH, glutathione; TQ, thymoquinone; ROS, reactive oxygen species; RFU, relative fluorescent unit; SEM, standard error of the mean

graphs (Figure 2a). A dose-dependent increase in ROS generation was clearly evident at this time point. Almost a 3.25-fold increase in ROS levels were observed with 75 and 100 μmol/L of TQ in both PC-3 and C4-2B cells. These results suggest that the ROS generated by TQ may play a role in growth inhibition in PC-3 and C4-2B cells.

As the increases in ROS and conjugation of quinones with GSH are known to result in the depletion of GSH levels, we investigated the role of TQ in depleting GSH in prostate cancer cells.[13,18] PC-3 and C4-2B cells were treated with 25–150 μmol/L TQ and levels of reduced GSH were measured using monochlorobimane. Data generated at 15-min intervals were normalized to protein contents in respective samples and GSH levels at the 30-min interval, when the largest decrease was observed, are shown in the bar graphs (Figure 2b). Acute treatment with TQ clearly resulted in a concentration-dependent decrease in GSH levels in both PC-3 and C4-2B cells. As compared with controls, GSH levels were significantly decreased by 50 and 100 μmol/L TQ, showing 35% and 65% reductions in GSH levels in PC-3 cells and 25% and 45% reductions in C4-2B cells, respectively. These results demonstrated that treatment with TQ lead to GSH depletion, which preceded the peak ROS generation in PC-3 and C4-2B cells.

Pretreatment with NAC protected PC-3 and C4-2B cells against TQ-induced ROS generation and growth inhibition

To confirm the role of ROS in TQ-induced growth inhibition, ROS levels were monitored in PC-3 and C4-2B cells that were pretreated with NAC (4 mmol/L) for 1 h and then with TQ (100 μmol/L) for 1 h, washed and stained with CM-H_2DCFDA. We clearly observed that NAC pretreatment significantly inhibited TQ-induced ROS production in both PC-3 and C4-2B cells (Figure 3a). The NAC control group did not show any significant changes in growth inhibition compared with untreated controls. Interestingly, in parallel experiments where NAC was removed before the TQ exposure, we observed no increase in TQ-induced ROS generation (data not shown), clearly indicating the persistence of the effects of NAC on TQ. In order to monitor the effects of NAC and TQ-induced growth inhibition, PC-3 and C4-2B cells were pretreated with NAC for 1 h and then exposed to 100 μmol/L TQ for an additional 24 h, following which cell viability was determined. Pretreatment with NAC significantly protected both PC-3 and C4-2B cells against the growth inhibitory effects of TQ (Figure 3b). This suggests that increased ROS generation and concomitant depletion of GSH are essential in TQ-mediated cell death.

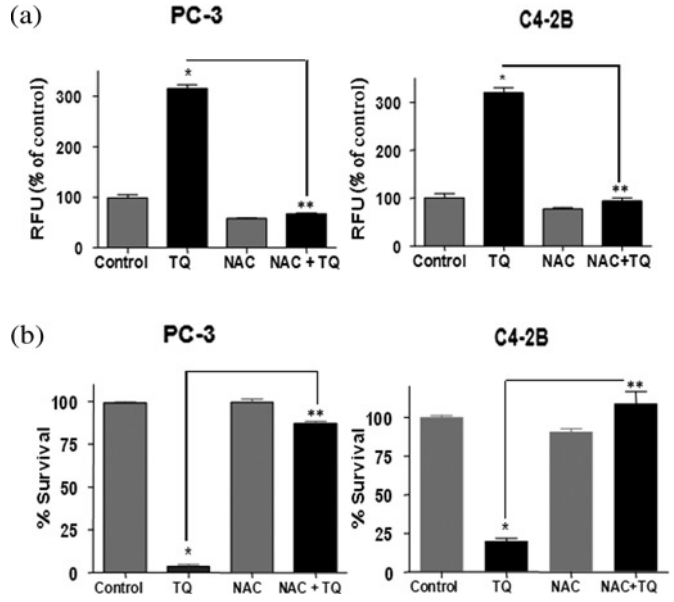

Figure 3 Pretreatment with NAC inhibits TQ-induced ROS generation and growth inhibition. (a) PC-3 and C4-2B cells were pretreated with NAC (4.0 mmol/L) for 1 h then incubated with TQ (100 μmol/L) for 1 h, and ROS levels were monitored. (b) PC-3 and C4-2B cells were pretreated with NAC (4.0 mmol/L) for 1 h followed by treatment with TQ for 24 h and cell survival was monitored by WST-8 assay. Error bars represent the SEM generated from three independent experiments ($n = 3$) and significant changes represent $^{*}P < 0.001$ and $^{**}P < 0.005$. NAC, *N*-acetylcysteine; TQ, thymoquinone; ROS, reactive oxygen species; RFU, relative fluorescent unit; SEM, standard error of the mean

TQ-induced JNK stress kinase activation is not linked to TQ-mediated PC-3 cell death

By carrying out DNA fragmentation analysis, we documented that the TQ-induced cell death in both PC-3 and C4-2B cells is primarily due to apoptosis (data not shown). Hence, in the following studies we wanted to delineate which apoptosis pathway is up-regulated following TQ exposure. The c-JNK, a stress kinase often activated under conditions of increased oxidative stress,[24] was first monitored in PC-3 cells treated with 100 μmol/L of TQ for 24 h. Western blot analysis was carried out to determine the levels of total-JNK, phosphorylated-JNK and phosphorylated c-jun levels (Figure 4a). Fold change in expressions were normalized to the β-actin levels. As early as 15 min after treatment, TQ increased JNK phosphorylation by 2–3-fold and JNK activation was maintained for two hours. However, TQ treatment did not change the levels of total JNK in cells. Interestingly, a 6–7-fold increase ($P < 0.01$) in c-Jun phosphorylation was observed within 15 min post-TQ exposure, but this activation did not persist for more than 30 min. Interestingly however, pretreatment with the JNK inhibitor, SP-600125 (10 μmol/L) did not protect PC-3 cells against TQ-induced growth inhibition, suggesting that JNK activation may not have a significant role in TQ-induced apoptosis in prostate cancer cells (Figure 4b).

Treatment with TQ did not alter the activation of caspase-3, -6, -8 and -9, in PC-3 cells

Activation of caspases is one of the mechanisms through which oxidative stress can induce growth inhibition.[20] TQ inhibition of cell growth in HL-60 cells had been shown to be caspase-dependent.[9] Hence, in order to determine the role of caspases in TQ-mediated effects, PC-3 cells were treated with increasing concentrations of TQ (25–150 μmol/L) for six hours and the cleavage of caspases-3 and -9 were analyzed by Western immunodetection. Although treatment with TQ down-regulated the protein expression of both caspase-3 and caspase-9, no cleaved products indicating the activation of caspases were observed (Figure 4c). Furthermore, in cells exposed to the pan-caspase inhibitor, Z-VAD-FMK (10 μmol/L) we did not observe any significant protection in TQ-mediated suppression of cell survival (Figure 4d). To confirm these results, we carried out further caspase activity assays for caspase-3, -6, -8 and -9, and saw no significant changes in caspase activation in TQ-treated samples (Figure 4d). These findings suggested that TQ-mediated effects on PC-3 cell death is caspase-independent, as well.

Expression of other apoptosis-related proteins in TQ-treated PC-3 cells

After determining that the activation of JNK or caspases may not be involved in TQ-induced cell death, we measured mRNA (Figure 5a) and protein levels (Figure 5b) of two apoptosis-inducing proteins, GADD45α and AIF, as well as a number of Bcl2 family proteins, Bcl2, BAG-1, Bcl2A1, Bcl2L1 and BID, by Western immunodetection (Figure 5c). Within 12 h following treatment with 100 μmol/L TQ, both GADD45α and AIF protein levels increased by 2.1- and 3-fold, respectively. However, treatment with TQ down-regulated the expression all of the antiapoptotic proteins tested, by as much as 50–60%. This clearly indicated that decrease in Bcl2 family proteins and increase in GADD45α and AIF is responsible for the TQ-induced apoptosis in PC-3 cells.

TQ-mediated growth inhibition of C4-2B cells is AR-independent

Previous studies by Kaseb *et al.*[12] had shown that TQ exposure suppresses AR levels in both LNCaP and C4-2B cells and had suggested a role for this inhibitory effect in TQ-mediated growth inhibition in AR+ cells. In C4-2B cell lysates, we determined the effects of TQ (25–100 μmol/L) on total AR expression by Western blot analysis (Figure 6a). TQ treatment had a concentration-dependent effect in decreasing total AR levels. We further examined the effect of TQ on dihydrotestosterone (DHT) (0.1 and 1.0 nmol/L)-induced nuclear translocation of AR in C4-2B cells (Figure 6b). Within 30 min following treatment of C42B cells, a 6- and 10-fold increase in AR nuclear translocation was observed with 0.1 and 1.0 nmol/L of DHT. Pretreatment of cells with increasing concentrations of TQ (25 and 50 μmol/L) inhibited this nuclear localization of

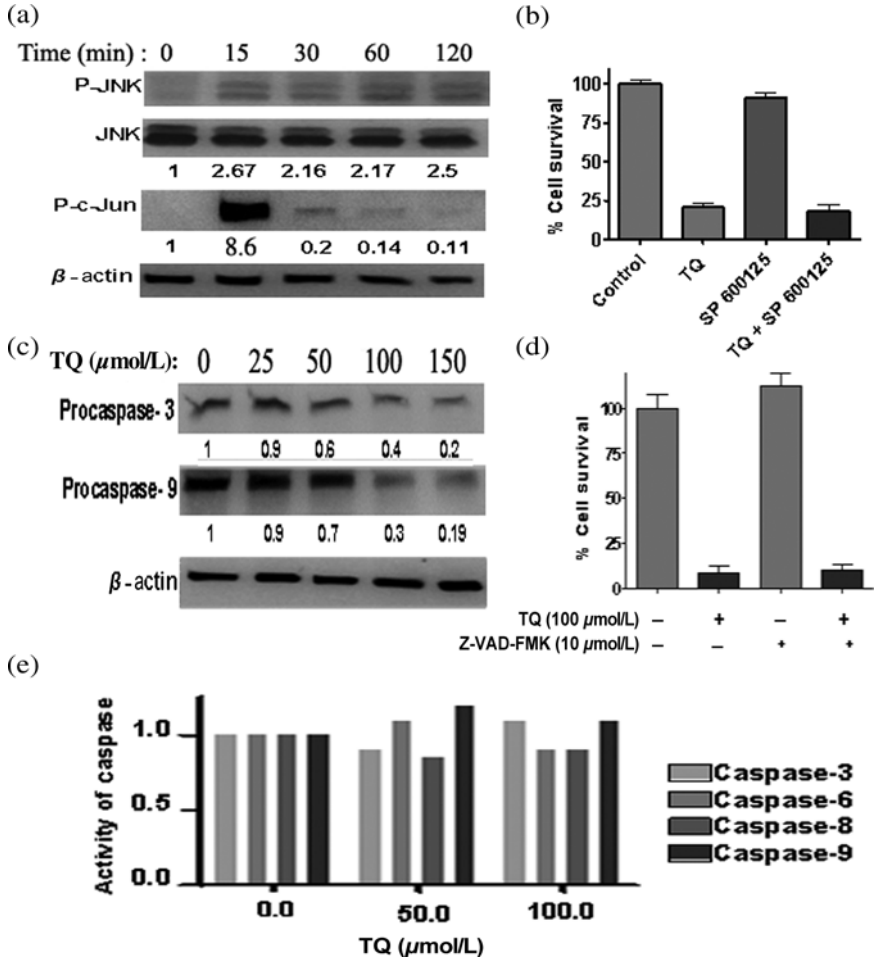

Figure 4 Effect of TQ on the activation of JNK and caspases in PC-3 cells. (a) PC-3 cells were treated with TQ (100 μmol/L) from 15–120 min and whole-cell lysates were analyzed by Western blot for total JNK and phosphorylated JNK and c-Jun. β-actin was used as a loading control and densitometric values normalized to β-actin levels are shown in the bottom of each panel. (b) PC-3 cells were treated with TQ (100 μmol/L) for 24 h, in the presence or absence of SP-600125 (10 μmol/L) and percent cell survival was monitored. (c) PC-3 cells were treated with increasing TQ concentrations (25–100 μmol/L) for six hours and the cleavage of caspase-3 and -9 was determined by Western blot analysis. Densitometric values for each pro-caspase were normalized to β-actin levels and are shown in the bottom of each panel. (d) The effect of TQ (10 μmol/L) on survival of PC-3 cells was monitored in the presence or absence of the caspase inhibitor Z-VAD-FMK (10 μmol/L). Error bars represent the SEM generated from two independent experiments ($n = 2$). (e) The effect of TQ on the activation of caspase-3, -6, -8 and -9 was monitored in PC-3 cells using functional assays in which each sample was incubated with a specific caspase substrate. Bar graphs represent the activity of each caspases in the presence or absence of TQ exposure. TQ, thymoquinone; JNK, Jun kinase; SEM, standard error of the mean

AR. In order to determine the effect of TQ on AR-directed transcription, we transfected C4-2B cells with pPSA-Luc and monitored the effect of TQ on reporter activity. Exposure to TQ (25 and 50 μmol/L) significantly inhibited luciferase activity by 30% and 90% (Figure 6c). Similar to previous observations,[12] our studies indicated that TQ suppresses AR expression and function in C4-2B cells. In order to see whether the decrease in AR levels is linked to TQ-induced ROS generation, we monitored both total (Figure 7a) and nuclear (Figure 7b) AR levels, in cells pretreated with NAC (4.0 mmol/L) and then exposed to TQ treatment. Interestingly, NAC was not able to decrease the effects of TQ on either basal AR or DHT-induced AR levels. Treatment with TQ inhibited AR expression by 50% and 65% of controls; however, TQ was still able to reduce AR levels in NAC-pretreated cells. Since NAC had completely protected C4-2B cells against the cytotoxic effects of TQ (Figure 3), our data suggest that TQ-induced growth inhibition of C4-2B cells is independent of the effects of TQ on the AR signaling pathway.

Discussion

TQ belongs to a family of quinones that can undergo enzymatic or non-enzymatic redox cycling with their corresponding semiquinone radicals to generate superoxide anion radicals.[13] TQ has been shown to inhibit growth in various cancer cell lines, including prostate cancer cells, but the mechanism of TQ-induced growth inhibition has not been delineated. Our study, in concert with previous publications, showed that treatment with TQ induced growth inhibition in the prostate cancer cell lines, PC-3, C4-2B and LNCaP, but not in the non-tumorigenic prostate cell line, BPH.[12] Our data showed that treatment with 100 μmol/L of TQ resulted in significant up-regulation of ROS expression in both PC-3 and C4-2B

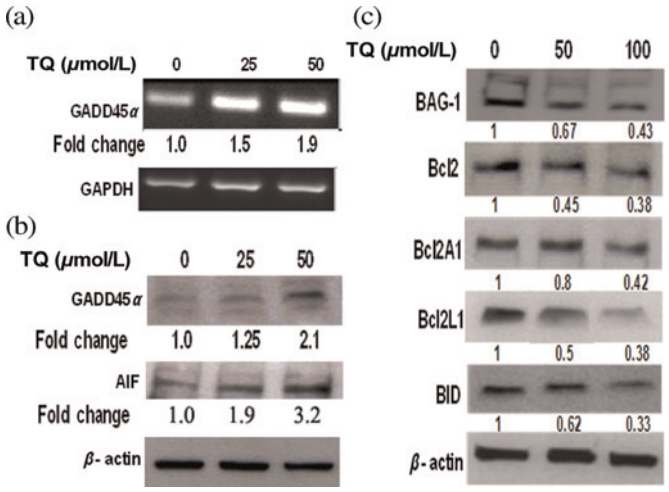

Figure 5 Effect of TQ on expression of GADD45α, AIF, BAG-1, Bcl2, Bcl2A1, Bcl2L1 and BID. PC-3 cells were treated with 25 and 50 μmol/L of TQ for six hours and (a) RT-PCR was performed for GADD45α gene expression. Changes in GADD45α mRNA expression are represented as fold change and all values were normalized to GAPDH levels. (b) Total GADD45α and AIF protein levels were measured by western blot analysis of cells treated with TQ for six hours. Changes in GADD45α and AIF protein levels are represented as fold change following normalization to β-actin levels. (c) PC-3 cells were treated with 50 and 100 μmol/L of TQ for six hours and the changes in total expression of BAG-1, Bcl2, Bcl2A1, Bcl2L1 and BID were monitored by Western blot analysis. Changes in protein levels are represented as fold change following normalization to β-actin levels. One representative picture from three independent experiments ($n = 3$) are shown. TQ, thymoquinone; AIF, apoptosis-inducing factor

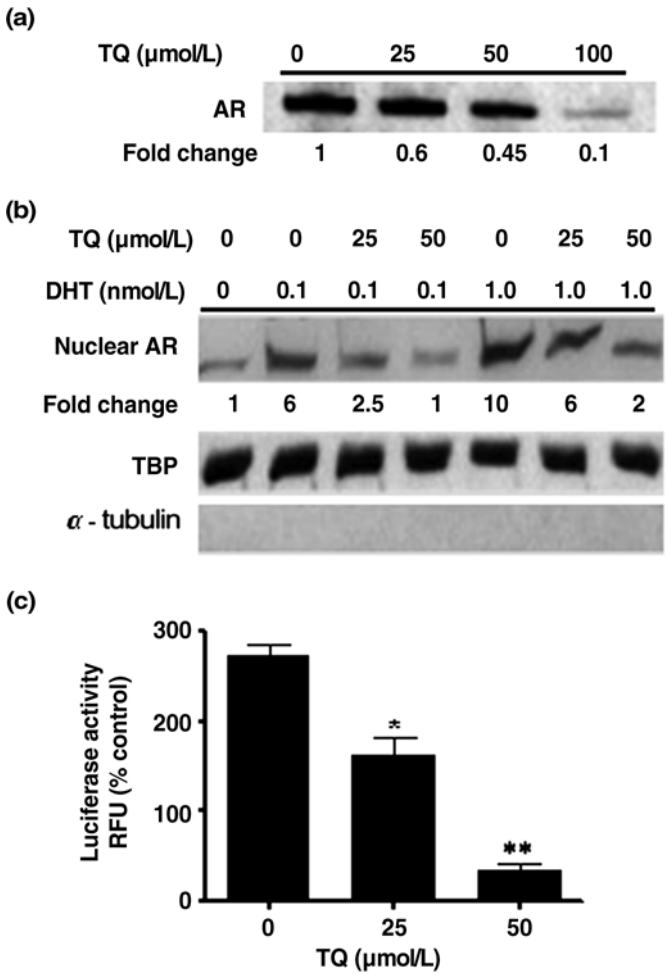

Figure 6 Effect of TQ on AR expression and AR-directed transcriptional activity. (a) C4-2B cells were treated with TQ for 24 h and total protein was isolated and immuno-blotted for AR expression. (b) Following treatment with TQ for 12 h, the C4-2B cells were stimulated with DHT (0.1 and 1.0 nmol/L) for 30 min and nuclear proteins were isolated for AR levels. TBP and α-tubulin were used as loading controls for nuclear and cytoplasmic proteins, respectively. (c) Cells were co-transfected with pPSA-Luc and pβ-gal plasmids, treated with TQ for 24 h, then luciferase and galactosidase activity were determined. Luciferase values were normalized to the respective β-gal values. Error bars represent the SEM generated from two independent experiments ($n = 2$) and significant changes are represented as $^{*}P < 0.05$ and $^{**}P < 0.01$. TQ, thymoquinone; AR, androgen receptor; DHT, dihydrotestosterone; TBP, TATA binding protein; RFU, relative fluorescent unit

cells. Free radicals can oxidize cysteine residues in proteins (altering protein structure and function), catalyze oxidation of lipids and lipid hydroperoxides, and all of these mechanisms of ROS may lead to growth inhibition.[25,26] After showing TQ-induced ROS generation in PC-3 and C4-2B cells, we monitored the effects of TQ on GSH levels in these cells. Oxidative stress is usually accompanied by GSH depletion.[18,27] Indeed, we saw that exposure of PC-3 and C4-2B cells to 100 μmol/L of TQ resulted in 65% and 35% GSH depletion, respectively. To confirm the role of ROS generation in TQ-induced cell death, we also used NAC to scavenge the ROS.[28] Pretreatment of PC-3 and C4-2B cells with NAC not only abrogates the induction of ROS by TQ, but it also significantly protected these cells against TQ-induced growth inhibition. These findings clearly corroborated our hypothesis that TQ induces growth inhibition in prostate cancer cells via the generation of ROS and modification of molecular pathways that affect cancer cell growth.

A number of previous publications suggest that preincubation with NAC is required to inhibit the cytotoxicity of quinones because NAC can form adducts with quinones and reduce their toxicity.[29,30] This may be a mechanism for the potent effects of NAC in suppressing the growth inhibitory effects of TQ. Indeed, in our experiments, the protective effects of NAC persisted even after its removal from the media, suggesting that adduct formation and inactivation of TQ by NAC may indeed be a mechanism of its action. The blockade of TQ-induced ROS up-regulation and growth inhibition by NAC pretreatment confirmed the role of ROS in TQ-induced growth inhibition. Although TQ is a known antioxidant at lower concentrations, a number of recent publications support our current report on the pro-oxidant effects of TQ at higher concentrations.[4,11,23,31–34] The shift between pro-oxidant and anti-oxidant activity of TQ can be attributed to variations in semiquinone (single electron transfer) and hydroquinone (two electron transfer) formation in a given cell type.[13]

Increases in ROS levels have been shown to induce growth inhibition in malignant cell types by activating both caspases and stress kinases.[19,20] Activation of caspases is a very common mechanism via which eukaryotic cells undergo apoptosis.[20] Caspases exist as zymogens containing a pro-domain and a protease domain. However, our data indicated that the activation of caspases is not involved in TQ-induced cytotoxicity. Both caspase-3 and caspase-9

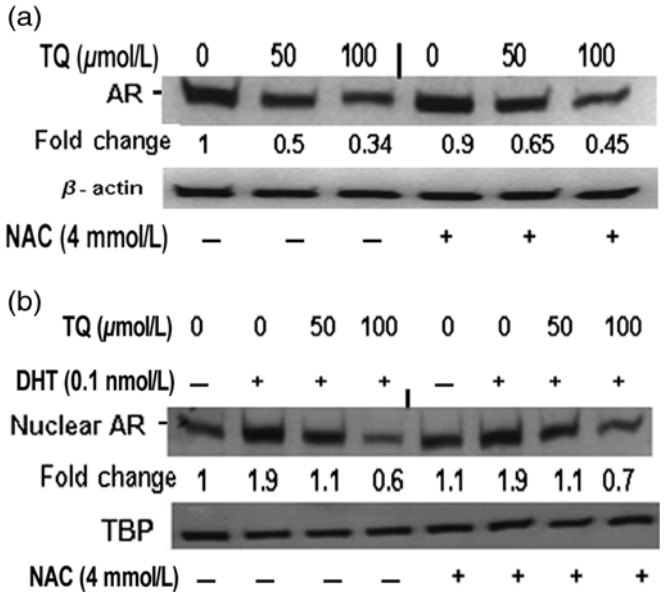

Figure 7 Effect of NAC on TQ-mediated inhibition of AR expression and nuclear translocation. (a) C4-2B cells pretreated with NAC for one hour were incubated with TQ (50 and 100 μmol/L) for 12 h and total AR levels were determined by Western immunoblot analysis. Changes in AR protein levels are represented as fold change following normalization to β-actin levels. (b) C4-2B cells treated with TQ (50 and 100 μmol/L) and DHT (0.1 nmol/L) in the presence of NAC (4.0 mmol/L) were analyzed for nuclear AR protein levels. Changes in nuclear AR levels are represented as fold change following normalization to TBP levels. TQ, thymoquinone; AR, androgen receptor; NAC, *N*-acetylcysteine; DHT, dihydrotestosterone; TBP, TATA binding protein

are well-known proapoptotic caspases. It has been shown that cytochrome-C released from mitochondria forms a complex with caspase-9 and an adaptor molecule, apoptotic protease-activating factor 1. Active caspase-9 then processes and activates caspase-3. By using peptides resembling the recognition sites of caspases to block caspase activities, we have shown that caspases are required for most, if not all, types of apoptosis. However, although in our studies both procaspase-3 and procaspase-9 were down-regulated in TQ-exposed cells, we did not observe caspase activation. Furthermore, the pan-inhibitor of caspases, z-VAD-FMK, was also unable to suppress the TQ-induced cell death, clearly implicating that caspases are not involved. Activation of JNK can either promote or inhibit apoptosis and can modulate several cell growth mechanisms.[35] Although treatment with TQ resulted in the functional activation of JNK, which was indicated by increases in both JNK and c-JUN phosphorylation in TQ-treated cells, interestingly, however, pretreatment with a JNK inhibitor (SP600125) did not protect the cells against TQ-induced cytotoxicity, which suggested that this stress kinase pathway is also not invovled in TQ-induced cytotoxicity. Indeed, a recent publication by El-Najjar *et al.*[33] suggested that TQ-induced activation of JNK signaling may have a protective effect on cells in the presence of ROS generation.

In order to delineate the mechanism by which TQ induces apoptosis, we first conducted an apoptotic array to monitor changes in various apoptotic and antiapoptotic genes in PC-3 cells treated with TQ (data not shown). In these arrays, GADD45α levels were up-regulated and Bcl2 proteins were down-regulated, indicating their possible role in TQ-induced cell death. We confirmed these results by RT-PCR and Western blot and saw significant increases in both GADD45α mRNA and protein levels. GADD45α is a growth arrest and DNA damage-inducible gene that has been shown to be up-regulated by both genotoxic and non-genotoxic stresses in mammalian cells.[21,22,36] Recent studies showed that GADD45α associates with Cdc2 and CyclinB1, resulting in G2/M arrest.[37] Genes that were significantly down-regulated in the Western blots included BAG-1, Bcl2 and Bcl2A1. A number of previous studies have shown that down-regulation of BAG-1, Bcl2, Bcl2L1, Bcl2A1 and BID can increase cell death. BAG-1 is a multifunctional protein that interacts with a wide range of target molecules to regulate apoptosis, proliferation, transcription, motility and metastasis.[38] BAG-1 can associate with Bcl-2 to promote cell survival.[39] Bcl2 is an antiapoptotic mediator found in many cancer cells.[40] Its overexpression prevents cell death and confers hormone resistance in prostate cancer.[41,42] Previous studies have shown that both Bcl2 and BAG-1 are down-regulated in response to rapid increases in oxidative stress.[43] Treatment with TQ resulted in the down-regulation of Bcl-2-related proteins and the proapoptotic protein BID. BID functions to promote cell cycle progression to the S phase and may be involved in maintenance of genomic stability through regulation of a mitosis check point.[44] This suggests that the survival functions of BID might have been inhibited by TQ, resulting in cell death. Changes in intracellular GSH levels have also been shown to affect expression of Bcl2 proteins and induce growth inhibition.[27] Our findings indicate that the depletion of GSH levels by TQ in PC-3 cells may relate to this increased mechanism of apoptosis. Antiapoptotic members of the Bcl2 family inhibit the release of apoptogenic factors such as AIF. Increases in the cytosolic levels of AIF after TQ treatment in PC-3 cells correlated with decreases in the levels of antiapoptotic proteins of the Bcl-2 family.[45,46] Overexpression of AIF has been shown to induce externalization of phosphotidylserine on the cell surface, loss of mitochondrial function and inhibition of eukaryotic translation initiation factor 3 subunit p44 (eIF3g), which can induce apoptosis.[47,48] Moreover, significant up-regulation of ROS levels has been shown to induce AIF-dependent cell death that is caspase independent.[49] Our results corroborate with this mechanism suggesting that ROS-induced GADD45α and AIF and decrease in numerous Bcl2 family proteins may be directly involved in TQ-induced apoptosis in the AR naïve PC-3 cells.

In the AR-positive C4-2B cells, the presence of the AR signaling pathway is an important target for the development of chemotherapeutic agents against prostate cancer.[50] A recent report by Kaseb *et al.*[12] suggested that TQ-induced growth inhibition of prostate cancer cells may be due to inhibition of AR expression. In accordance with this published finding, we observed a decrease in AR expression in C4-2B cells exposed to TQ. Treatment with TQ significantly inhibited both basal levels and DHT-stimulated nuclear translocation of AR in these cells. Furthermore, exposure to TQ (50 μmol/L) significantly inhibited AR-mediated activation of the

PSA promoter activity in C4-2B cells by almost 90%. Indeed, a publication by Husbeck *et al.*[51] had demonstrated that superoxide radicals can inhibit AR expression and activity in prostate cancer cells. Although the decrease in GSH levels in TQ-exposed cells corroborate with the increase in cell death and the protective effects of NAC, it is possible that NAC may form adducts with TQ to potently suppress its cytotoxic effects.[29] Since our studies implicated the role of ROS in TQ-induced growth inhibition, we investigated the effects of TQ-induced ROS generation on AR expression. Interestingly, pre-exposure to NAC did not prevent TQ-mediated inhibition of AR expression and its DHT-mediated nuclear localization, clearly suggesting that TQ-induced inhibition of AR is independent of the ROS-induced cell death.

This manuscript catalogs the effects of TQ in two different prostate cancer cells and may not fully explain the mechanisms of its anticancer effects. Although TQ has been shown to be a potent anticancer agent, studies to elucidate its molecular mechanism of action have been lacking. Our current findings are an initial attempt towards that direction and more rigorous studies to completely understand the molecular mechanisms will need to be carried out in the future. We showed that TQ-induced growth inhibition in PC-3 and C4-2B prostate cancer cells is due to the generation of ROS. Our data suggest that up-regulation of GADD45α and AIF and down-regulation of Bcl-2-related proteins might play a significant role in inhibition of cell growth by TQ in PC-3 cells. Our present study also demonstrates that TQ-induced growth inhibition in C4-2B cells is ROS-dependent and not AR-dependent. In conclusion, the present study offers novel insight into the mechanism of TQ-induced growth inhibition by ROS generation and apoptosis in human prostate cancer cells, which suggests that TQ has the potential to be used as a new therapeutic strategy for prostate cancer.

Author contributions: All authors participated in the design of the studies, interpretation of the data and review of the manuscript. Mr Padma S Koka was primarily involved in carrying out the research towards this project.

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