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Thymoquinone up-regulates PTEN expression and induces apoptosis in doxorubicin-resistant human breast cancer cells

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Abstract

The use of innocuous naturally occurring compounds to overcome drug resistance and cancer recalcitrance is now in the forefront of cancer research. Thymoquinone (TQ) is a bioactive constituent of the volatile oil derived from seeds of *Nigella sativa* Linn. TQ has shown promising anti-carcinogenic and anti-tumor activities through different mechanisms. However, the effect of TQ on cell signaling and survival pathways in resistant cancer cells has not been fully delineated. Here, we report that TQ greatly inhibits doxorubicin-resistant human breast cancer MCF-7/DOX cell proliferation. TQ treatment increased cellular levels of PTEN proteins, resulting in a substantial decrease of phosphorylated Akt, a known regulator of cell survival. The PTEN expression was accompanied with elevation of PTEN mRNA. TQ arrested MCF-7/DOX cells at G2/M phase and increased cellular levels of p53 and p21 proteins. Flow cytometric analysis and agarose gel electrophoresis revealed a significant increase in Sub-G1 cell population and appearance of DNA ladders following TQ treatment, indicating cellular apoptosis. TQ-induced apoptosis was associated with disrupted mitochondrial membrane potential and activation of caspases and PARP cleavage in MCF-7/DOX cells. Moreover, TQ treatment increased Bax/Bcl2 ratio via up-regulating Bax and down-regulating Bcl2 proteins. More importantly, PTEN silencing by target specific siRNA enabled the suppression of TQ-induced apoptosis resulting in increased cell survival. Our results reveal that up-regulation of the key upstream signaling factor, PTEN, in MCF-7/DOX cells inhibited Akt phosphorylation, which ultimately causes increase in their regulatory p53 levels affecting the induction of G2/M cell cycle arrest and apoptosis. Overall results provide mechanistic insights for understanding the molecular basis and utility of the anti-tumor activity of TQ.

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Conflict of interest statement

The authors declare that they have no competing interests.

Keywords

PTEN tumor suppressor; breast cancer; thymoquinone; apoptosis; cell signaling

1. Introduction

Breast cancer is one of the most common human malignancies and the second leading cause of cancer-related deaths in women [1]. Breast cancer patients show initial response to chemotherapeutic treatment; however, the majority of women eventually experience cancer treatment recalcitrance and emergence of drug resistant cells [2,3]. Doxorubicin, one of the clinically most important anti-neoplastic agents, possesses a wide spectrum of anti-cancer activity against various solid tumors, including breast cancer. However, the development of doxorubicin resistance limits its use in treating breast cancer patients [4]. Several mechanisms are responsible for doxorubicin resistance, including altered drug efflux, drug detoxification, enhanced DNA repair and disruption in apoptotic signaling pathways. Drug resistance is also associated with activation of signaling pathways such as the phosphatidylinositol-3 kinase (PI3K)/Akt or suppression of tumor suppressor genes, including PTEN [5].

The PI3K/Akt pathway is a pivotal signaling pathway, which controls cell growth, survival, proliferation and tumorigenesis [6,7]. PI3K-activated (phosphorylated) Akt promotes cell survival by inhibiting apoptosis through its ability to phosphorylate/inactivate downstream targets of apoptotic machinery, such as pro-apoptotic Bcl2 family member BAD and GSK-3β [8,9].The (PI3K)/Akt pathway is regulated by several critical upstream factors, e.g., tumor suppressor PTEN [10]. The tumor suppressor gene PTEN is one of the most common targets of mutation in human cancers, with a mutation frequency approaching that of p53. PTEN mutations have been found in glioblastomas, malignant melanomas, carcinoma of the prostate, breast, kidney, urinary bladder, uterus and other cancers, mostly in advanced stages of tumor progression [11,12]. Genetic mutation of PTEN resulted in increased Akt activity in many types of tumor [13]. On the other hand, overexpression of PTEN in cancer cells carrying mutant or deletion type PTEN was reported to inhibit cell proliferation and tumorigenicity via induction of cell cycle arrest and apoptosis [14]. PTEN exerts a tight regulatory control over the PI3K pathway and downstream functions, including activation of protein kinase B/Akt, cell survival, cell proliferation and cell arrest [14,15]. It has been reported that PTEN and p53 functions are mechanistically linked through MDM2 phosphorylation, which modulates the ubiquitination of p53 by the activation of PI3K/Akt pathway [16,17]. p53 functions as a transcription factor by binding to a p53-specific DNA sequence in responsive genes, which would increase the synthesis of p21, the most important checkpoint proteins involved in cell arrest at both G1 and G2/M.

Inhibition of PI3K/Akt pathway has been targeted as a strategy for drug development [18] and recently has drawn considerable attention for combination therapy alongside the use of naturally occurring innocuous dietary agents to achieve greater efficacy for drug-resistant cancer cells [19–21]. For instance, some of the dietary phytochemicals have been shown to downregulate PI3K/Akt pathway, thereby sensitizing drug-resistant cancer cells for low-dose chemotherapeutic drug-induced apoptosis. Dietary phytochemical thymoquinone (TQ) is the main active ingredient of the volatile oil of Nigella sativa Linn, known as the black seed. The black seed is an annual plant that has been widely used in the Indian subcontinent, Arabian countries and Europe for culinary and medicinal purposes [22,23]. Recently, Several clinical studies were conducted in humans receiving oral Nigella Sativa extract or black seeds for up to 8 weeks to evaluate the anti-inflammatory, antihypertensive, or antioxidant effects [24,25]. Most of the known biological activities of the seed have been

attributed to the active ingredient TQ. TQ has been shown to be safe on a wide variety of normal cells. The selective cytotoxicity of TQ for human cancer cells compared to primary mouse keratinocytes [23], mouse normal kidney cells [26], non-malignant fibroblasts [27] and normal human lung fibroblasts [28] has been reported in several studies. TQ has been used for decades as anti-oxidant, anti-inflammatory, and anti-neoplastic agent [29,30]. Previous studies have shown that TQ exhibits inhibitory effects on cell proliferation of many types of cancer cells [31] including non-Dox resistant breast cancer cells (MCF-7) [26,32,33]. TQ induces cell death and inhibits tumor growth by suppressing NF-κB, Akt activation, and extracellular signal-regulated kinase signaling pathways as well as angiogenesis [34,35]. We have previously reported that TQ induced apoptosis through activation of caspase pathway and mitochondrial events in p53-null myeloblastic leukemia HL-60 cells [36].

To better understand the molecular mechanism by which TQ exerts its anti-neoplastic effects, we examined the anti-proliferative effects of TQ in doxorubicin-resistant human breast cancer cells. In this study, we investigated the potential mechanism by which TQ may regulate cell proliferation and apoptosis in doxorubicin-resistant human breast cancer MCF-7/DOX cells. We provide evidence that TQ transcriptionally upregulate PTEN, which led to phosphorylation of Akt and induction p53 protein and its transcriptional target p21, thus induces G2/M phase arrest and apoptosis in doxorubicin-resistant MCF-7/DOX cells. These observations provide a strong rationale towards designing TQ-based combinational therapeutic strategies for treating human breast cancer.

2. Materials and methods

2.1. Cell Lines and cultures

Doxorubicin-resistant human breast adenocarcinoma cell line, MCF-7/DOX, was generously provided by Dr. S A Salama (University of Texas-medical branch, TX). The cells were grown in DMEM medium supplemented with 10% fetal bovine serum, 50 IU/ml penicillin, 50 mg/ml streptomycin at 37°C in a humidified 5% CO_2 atmosphere.

2.2. Chemicals and reagents

TQ (Fig. 1) was prepared as a 10 mM stock solution in DMSO, and appropriate working dilutions were prepared with the cell culture medium immediately prior to the experiments. TQ, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], Hoechst dye and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Cell culture supplies were obtained from Life Technologies (Grand Island, NY). Antibodies against phospho-Akt [p-Akt (Ser473)], PARP, caspase-7, caspase-8, caspase-9, PTEN, Fas L, Bcl-2, Bax, and SignalSilence PTEN siRNA kit were purchased from Cell Signaling Technology (Danvers, MA). Antibodies against p53, p21 and β-actin were purchased from Neomarkers (Fremont, CA) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Antibody against γH2AX was purchased from Millipore (Billerica, MA). Horseradish peroxidase (HRP)-conjugated secondary antibodies, protease inhibitor cocktail tablets were from Roche (Indianapolis, IN). Chemiluminescence substrate kit was obtained from Pierce (Rockford, IL). DC Bio-Rad protein quantitation reagents were from Bio-Rad (Hercules, CA). The caspase inhibitor, z-VAD-FMK, was purchased from R&D systems (Minneapolis, MN). The MitoCapture apoptosis detection kit was made by Calbiochem (La Jolla, CA). Trizol Reagent, Invitrogen SuperScript III first strand-synthesis system and PTEN primers for RT-PCR were obtained from Invitrogen Corporation (Carlsbad, CA).

2.3. MTT assay for cell growth and cytotoxicity

The MTT assay measures the living cells' ability to uptake and convert soluble MTT into formazan crystals as described previously [37]. Exponentially growing MCF-7/DOX cells were seeded in 96-well plates at an initial density of 5×10^3/well, treated with different concentration of TQ and maintained in culture for indicated times and then phenol-free medium containing MTT (0.2 mg/ml) was added to the cultures for an additional 2 hours. The medium was replaced with acidified isopropanol (0.04 N HCl in isopropanol) and the plates were incubated at room temperature for 1 hour. The colorimetric absorbance of the samples was determined by Spectramax M5 microplate reader (Molecular Devices, CA). Cellular proliferation was expressed as a percentage of cell viability of TQ-treated cells relative to untreated controls.

2.4. DNA fragmentation analysis for detecting apoptosis

Exponentially growing MCF-7/DOX cells were treated with increasing concentrations of TQ and maintained in culture. After indicated time periods, all adherent and floating cells were recovered, centrifuged, washed once with PBS, resuspended in lysis buffer [20 mM EDTA, 100 mM Tris-HCl (pH 8.0), 1% SDS] containing 100 μg/ml RNase and incubated at 55°C overnight. Proteinase K (100 μg/ml, final concentration) was added to each sample and incubated at 37°C for 2 hours. DNA was precipitated with 2.5 volume of 100% ethanol and dissolved in TE pH 8. The DNA samples were separated on a 2% agarose gel, stained with ethidium bromide and visualized under UV light.

2.5. Flow cytometric analysis of cell cycle and apoptosis

Cell cycle distribution was determined as previously described [21]. TQ-treated cells were collected, washed with ice-cold PBS and fixed with 70% ice-cold ethanol overnight at −20°C. The cells were centrifuged at 1500 rpm for 5 minutes, uniformly resuspended in a mix of propidium iodide (50 μg/ml) and RNase A (100 μg /ml), and incubated at 37°C for 40 minutes. The cells were pelleted, washed and resuspended in PBS to a final concentration of 1×10^6/ml. The cell cycle distribution was analyzed using BD FACS Calibur (BD Biosciences, San Jose, CA).

2.6. Hoechst staining

The nuclear morphology of MCF-7/DOX cells was analyzed according to Lee *et al*, 1996 [38]. Cells, either TQ-treated or untreated, were fixed with formaldehyde and stained with Hoechst 33342 at 6.15 μg/ml for 15 min at room temperature. Cells were washed with and resuspended in PBS. The nuclear morphology of cells was observed by fluorescence microscopy. Cells with condensed or fragmented nuclei were considered apoptotic cells.

2.7. Immunofluorescence

The immunofluorescence double labeling was performed according to the method established in our laboratory [39]. Briefly, TQ-treated cells at different time points were washed twice with cold PBS, permeabilized with 0.5% Triton X-100/PBS for 8 minutes on ice and then fixed with 2% paraformaldehyde in 0.5% Triton X-100 at 40C for 30 min. After fixation, the coverslips were rinsed twice with cold PBS and blocked with 20% normal goat serum (NGS) in 0.1%Triton X-100/PBS washing buffer at room temperature for 2 h. Primary rabbit anti-γH2AX antibody (1:200 dilution) and goat anti-mouse FITC secondary antibody (1:200 dilution) were prepared in washing buffer containing 5% NGS and layered on the coverslips for 1 h at room temperature. Following each antibody incubation step, the cells were washed with 0.1% Tween-20 in PBS 4 times for 5 min each. Fluorescence images were captured with a Nikon Fluorescence Microscope E80i (Nikon, Tokyo, Japan) equipped with SPOT analysis software.

2.8. Western blot analysis

The MCF-7/DOX cells were grown to 50% confluency and treated with TQ for the indicated time periods. The cells were then harvested, washed with PBS and lysed by boiling for 5 minutes in lysis buffer (10% Glycerol, 2% SDS in 62 mM Tris-HCl, pH 6.8) containing a cocktail of protease inhibitors. Equal amounts of total protein, as determined by DC protein assay, were separated on an 8–16% polyacrylamide gel and electrophoretically transferred to a PVDF membrane. After blocking with 5% non-fat dry milk in TBST buffer, the PVDF membranes were incubated with a specific primary antibody at 4°C overnight, washed three times, 10 min/each time with TBST buffer, and incubated again with an appropriate HRP-conjugated secondary antibody at 37°C for 1 hour. The membranes were washed with TBST buffer and examined by chemiluminescence detection.

2.9. Mitochondrial membrane potential

Mitochondrial membrane potential was examined using the MitoCapture mitochondrial apoptosis detection kit, according to the manufacturer's procedure. Cells were centrifuged at 600 g for 5 min. Cell pellets were resuspended in 1 ml of diluted MitoCapture solution, incubated at 37 °C for 15–20 min and then centrifuged again for 5 min. Pellets were resuspended in 1 ml pre-warmed incubation buffer and examined immediately by fluorescence microscopy.

2.10. Small RNA interference (RNAi) of PTEN expression

The SignalSilence PTEN siRNA kit was used to silence PTEN expression according to the manufacturer's protocol. Briefly, MCF-7/DOX cells were seeded in 12-well plates and grown for 24 hours to ~50% confluency. Before transfection, the medium was replaced with 500 µl of an antibiotic-free medium. To transfect siRNA, 2 µl of transfection reagent was added to 100 µl of serum-free medium in a sterile microfuge tube, followed by the addition of control or 3 µl PTEN siRNA (10 µM) to a final 50 nM concentration. This siRNA-transfection reagent mixture was incubated for 5 minutes at room temperature and then applied to cultured cells. After 48 hours of incubation, the medium was refreshed and the cells were treated with or without TQ for additional 48 hours. Finally, the cells were collected for Western blotting analysis.

2.11. Quantitative reverse transcription-polymerase chain reaction (RT-PCR)

MCF-7/DOX cells were treated with 50 µM TQ and maintained in culture for the indicated times. The cells were washed with PBS and total cellular RNA from various cell samples was isolated by using Trizol Reagent. The RT-PCR was performed by using the Invitrogen SuperScript III first strand-synthesis system as instructed by the manufacturer. Briefly, 1 µg total RNA, 2.5 µM Oligo$(dT)_{20}$ and 500 µM dNTP were incubated in a 10 µl reaction volume at 65 °C for 5 min followed by chilling on ice for 1 min. RT buffer [5 mM $MgCl_2$, 10 mM DTT, RNaseOUT (40 U/µl)] and SuperScript III RT (200 U/µl) were added to a final 20 µl volume for cDNA synthesis. For real-time RT-PCR, we used Power SYBR Green PCR Master Mix from Applied Biosystems (Foster City, CA) and the reactions were run on Roche Light cycler 480 (Applied Science, Indianapolis, IN). The following primers were used: GAPDH forward, 5´-GTGAAGCAGGCATC-3´; reverse, 5´-CGAAGGTGGAAGAG-3´ and PTEN forward, 5´-CTTCTCTTTTTTTTCTGTCC-3´; reverse, 5´-AAGGATGATGAGAATTT CAAGC A-3´. Real-time PCR was performed at 50 cycles (15s at 95°C, 56 s at 56°C and 45 s at 68°C). A melting point dissociation curve generated by the instrument was used to confirm the single product. Relative RNA levels expressed in arbitrary units were calculated using comparative method based on ΔCt and ΔΔCt values and normalized to GAPDH (endogenous control). All samples were run in triplicate.

2.12. Statistical analysis

GraphPad InStat software, version 3.06 (GraphPad, San Diego, CA), was used to compute statistical data. Data are expressed as mean ± SE of three to six independent experiments. Statistical comparisons were performed using unpaired Student's t-test test. The 0.05 level of probability was used as the criterion of significance.

3. Results

3.1. TQ inhibits the proliferation of MCF-7/DOX cells

MCF-7/DOX is a doxorubicin-resistant variant of human breast adenocarcinoma MCF-7 cell line [40,41]. We first examined the anti-proliferative effect of TQ on MCF-7/DOX cells. As measured by the MTT assay, the MCF-7/DOX cell proliferation following 12, 24 and 48 hours of exposure to 25, 50 or 100 μM TQ showed significant growth inhibition in TQ-treated cells compared to non-treated controls. The proliferation of TQ-treated cells decreased as a function of both TQ concentration and exposure time. For example, 12, 24 and 48 hours post-treatment with 100 μM TQ, cellular proliferation was approximately 70%, 50% and 35%, respectively (Fig. 2).

3.2. TQ induces apoptosis in MCF-7/DOX cells

We next examined the cellular apoptosis by agarose gel electrophoresis of DNA degraded by internucleosomal DNA fragmentation. Treatment of MCF-7/DOX cells with 50 and 100 μM TQ for 48 hours induced a dose-dependent DNA fragmentation, exhibiting a typical DNA ladder feature of apoptosis (Figure 3A). Consistent with DNA fragmentation, morphological changes e.g., fragmented nuclei and apoptotic bodies were seen when cells were stained with Hoechst dye and examined by fluorescence microscopy (Fig. 3B). TQ-induced apoptosis was further confirmed by flow cytometric assay, which showed a 25.6% sub-G1 population in TQ-treated cells as compared with 1.3% in untreated controls (Fig. 3C).

2.13. TQ disrupts mitochondrial membrane potential and activates caspase pathway

We further examined the changes in mitochondria membrane potential in TQ-treated MCF-7/DOX cells. The results from fluorescence microscopy showed that MCF-7/DOX cells lost their mitochondrial membrane potential following TQ treatment (Fig. 4A). In normal cells, the MitoCapture reagent accumulated and aggregated in the mitochondria, giving off a bright red fluorescence. In apoptotic cells, the MitoCapture reagent did not aggregate in the mitochondria, due to altered mitochondrial membrane potential, and thus remained in its monomeric form, generating a green fluorescence. About 25% of cells lost membrane potential, approximately the same as that of apoptotic cells examined by Hoechst staining (data not shown). These results indicate that loss of membrane potential occurs in the early stage of TQ-induced apoptosis in MCF-7/DOX cells.

3.4. TQ treatment modulates pro-apoptotic and anti-apoptotic proteins in MCF-7/DOX cells

Bax/Bcl2 ratio represents a critical balance of regulatory pro-apoptotic and anti-apoptotic proteins in normal living cells. The increase in Bax/Bcl2 ratio leads to the release of Cytochrome c from the mitochondria, a decisive event in the apoptotic pathway. It is shown that TQ induces both p53-dependent and independent apoptosis by diminishing Bcl2 protein level [36,42]. We next determined whether the Bax/Bcl2 ratio changes upon the treatment of MCF-7/DOX cells with TQ. As seen in Fig. 4D, TQ treatment of MCF-7/DOX cells resulted in moderate decrease in Bcl2 and a significant increased level of Bax protein level. These results suggest that TQ induced-apoptosis is associated with a significant decrease of the Bcl2/Bax ratio in MCF-7/DOX cells.

3.5. Caspase activity is involved in TQ-induced apoptosis

To understand activation of the caspase cascade during TQ-induced apoptosis in MCF-7/DOX cells, we investigated various caspase-specific cleavages in TQ-treated cells. TQ treatment caused a dose–dependent proteolytic cleavage of caspases 3, 7 and 9, but not of caspase 8, as revealed by the appearance of the cleaved protein species (Fig. 4C). TQ also induced dose-dependent proteolytic cleavage of PARP, a known substrate of caspase-3. Treatment of cells with the general caspases inhibitor, z-VAD-FMK, markedly prevented proteolytic cleavage of PARP and caspases, in addition to attenuated TQ-induced DNA fragmentation (Fig. 4B). These results indicate the involvement of the caspase pathway in TQ-induced apoptosis.

3.6. TQ treatment arrests MCF-7/DOX Cells in G2/M phase and induces DNA damage

To test whether the anti-proliferative effect of TQ was related to cell cycle arrest, asynchronous MCF-7/DOX cells were treated with TQ and the cycle progression was examined by the flow cytometry (Fig. 5A). In untreated control, the percentage of cells in G_1, S and G_2/M phases were found to be 67%, 17.6% and 15%, respectively. TQ treatment resulted in a pronounced G2/M arrest in a time-dependent manner. The percentage of G_2/M phase cell population was found to be 32% following TQ treatment. Consistent with this observation, TQ treatment reduced the levels of Cdc25C and cyclin B1, while the p21 level was elevated at 12 hours and thereafter (Fig. 5B). To check whether TQ causes DNA damage in MCF-7/DOX cells, we performed the analysis of γH2AX activation as a sensitive indicator of direct or indirect damage induction by TQ. Cells were treated with TQ (100 μM) for various periods or exposed to UV irradiation (50 J) to serving as a positive reference control for γH2AX activation. As compared to significant γH2AX activation by high dose UV irradiation, a modest time-dependent increase in the phosphorylation of H2AX was observed following TQ treatment (Fig. 5D). In addition, immunofluorescent analysis confirmed the results of Western Blots as γH2AX-containing nuclear foci were evident in TQ-treated MCF-7/DOX cells (Fig. 5D).

3.7. PTEN and its downstream substrates are upregulated by TQ treatment

To investigate whether regulation of PI3K/Akt survival pathway is involved in TQ-induced apoptosis, we first analyzed the cellular level of PTEN and other regulatory proteins by Western blotting. The results showed a time-dependent increase of PTEN in MCF-7/DOX cells treated with TQ, as compared with that in untreated cells (Fig. 6A). The p-Akt level decreased while p53 level increased slightly upon TQ treatment. We further probed whether TQ treatment increases PTEN at transcription level using real-time RT-PCR (Fig. 6C). TQ treatment induced an increase in PTEN mRNA by 1.8, 2.0, 3.8, 5.9 and 7.9-fold after 1, 2, 4, 8 and 24 hours, respectively. Taken together, this data suggested that regulation of PTEN/Akt pathway is intimately associated with TQ-induced growth inhibition, apoptosis and G2/M arrest.

3.8. TQ-induced apoptosis in MCF-7/DOX cells was blocked by knocking-down PTEN

To assess the contribution of PTEN to the anti-proliferative effect of TQ in MCF-7/DOX cells, we tested the effect of siRNA-mediated PTEN silencing on cellular apoptosis and proliferation in TQ-treated MCF-7/DOX cells. The cells were transfected with PTEN siRNA before TQ treatment and the PTEN knockdown was confirmed by Western blotting (Fig. 7A). TQ-treated cells exhibited both PARP cleavage and decreased cell survival (Figs 7A and B). However, in siRNA-treated cells, TQ-induced PARP cleavage and cell death were dramatically blocked, indicating that PTEN knockdown overrode the effect of TQ and prevented TQ-treated cells from undergoing apoptosis. We surmised that upregulation of

PTEN by TQ eliminates survival signals mediated by p-Akt and consequently renders MCF-7/DOX cells susceptible to TQ-induced apoptosis.

4. Discussion

Acquired and de novo resistance remain a major clinical challenge in cancer treatment, restricting the successful use chemotherapeutic agents. The dose escalation necessary to overcome even a small increase in cellular resistance can cause severe cytotoxicity to normal tissues. Innocuous natural compounds are being increasingly explored to achieve greater efficacy for drug-resistant cancer cells. For instance, we reported that the dietary phytochemical tangeretin downregulates PIK3/Akt pathway and sensitizes cisplatin-resistant human ovarian cancer cells to low-dose cisplatin-induced cell death [21]. In the current study, we examined the effectiveness of TQ on growth inhibition and apoptosis in doxorubicin-resistant human breast adenocarcinoma MCF-7/DOX cells. We have found that TQ treatment greatly inhibits the proliferation of MCF-7/DOX cells. Our results show that the inhibitory effect of TQ on cell proliferation is associated with DNA damage, G2/M phase arrest, inhibition of PI3K/Akt pathway, and upregulation of PTEN expression.

Multiple factors contribute to development of drug resistance of cancer cells, including reduction of intracellular drug accumulation, increase in DNA damage repair, constitutive activation of PI3K/Akt signaling, activation of the Ras and MAPK pathways, dysfunction of the tumor suppressor gene p53 [43]. In acquiring resistance to chemotherapeutic drug doxorubicin, MCF-7/DOX cells exhibit a considerable dysregulation of the miRNAome profile and epigenetic changes in DNA methylation and histone modification [40,41]. These changes confer a characteristic gene expression profile that is consistent with the cancerous phenotype of the MCF-7/DOX cells. For instance, anti-apoptotic proteins BCL6, NOTCH1 and K-RAS were upregulated, whereas PTEN, BRCA1 and RB1 were shown to be downregulated in MCF-7/DOX cells. In our study, TQ was able to upregulate and thereby restore the normal function of the very upstream factor, PTEN, in MCF-7/DOX cells. The upregulated PTEN can function towards cell cycle arrest and apoptosis through several regulatory axes of the signaling pathway. It has been shown that PTEN over-expression subdues constitutive Akt activation and sensitizes receptor mediated drug-induced apoptosis in cancer cells, and suppresses tumor formation in animal models [10,44]. PTEN also exerts positive regulation of p53 level and function [45,46]. For instance, PTEN has been shown to inhibit PI3K/Akt signaling that promotes the nuclear translocation of MDM2, the major regulator of p53. The ability of PTEN to restrain MDM2 in the cytoplasm gives rise to the cellular contents and transactivation of the p53, promoting p21 induction and cell cycle arrest. Consistent with these observations, we observed a decrease in p-Akt but an increase in p53 and p21 level in TQ-treated MCF-7/DOX cells (Figs. 5 and 6). Additionally, we observed a G2/M arrest in TQ-treated cells. Perhaps, such an effect is due to DNA damaging effect of TQ, as it might be metabolized to reactive species and increase oxidative stress [47]. Our observation that TQ induced DNA damage (Fig. 5C&D) came in accordance with Roepke *et al.*, 2007 and Gurung *et al.*, 2010 who reported TQ-induced damage in Osteosarcoma cell and in glioblastoma cells, respectively [28,48]. It is worthy to note that DNA damage causes a decrease in Cyclin B1 expression, and such a repression is considered as one of the mechanisms by which the p53 inhibits G2/M transition [49]. Accordingly, we have shown in Fig. 5B that TQ caused Cyclin B1 repression.

Generally, two pathways are involved in apoptosis: the extrinsic pathway, activated by cell surface death receptors, and the intrinsic or mitochondrial pathway, which is usually activated by DNA damage and is controlled by the tumor suppressor gene p53 [50]. In our study, TQ-induced apoptosis was completely abolished by the general caspase inhibitor, z-VAD-FMK, indicating that the apoptosis in MCF-7/DOX cells is mediated through a

caspase-dependent mechanism. Furthermore, the caspase-3, -7, -9, but not caspase-8, were activated upon TQ treatment, and, PARP was cleaved in a TQ dose-dependent manner. Therefore, TQ-induced apoptosis in MCF-7/DOX cells occurs through the intrinsic pathway, possibly triggered by DNA damage caused by TQ induced reactive species and oxidative stress. To support this view, TQ was shown to disrupt mitochondrial membrane potential in MCF-7/DOX cells and change the Bax/Bcl2 ratio by increasing Bax while decreasing Bcl2 expression. It has been known that the Bax/Bcl2 ratio plays an important role in the release of Cytochrome c from mitochondria, which is critical event in caspase inactivation. It was also reported that TQ-treatment abolishes Bcl2 in a p53-dependent manner [42]. In our experiments, both p53 and its downstream target p21 were increased by TQ treatment. We surmise that TQ-induced apoptosis is also regulated by p53 in MCF-7/DOX cells.

Our results showed for the first time that TQ induces apoptosis in doxorubicin-resistant breast cancer cells through up-regulation of PTEN at transcription level. The up-regulated PTEN, in turn, inhibited the PI3K/Akt pathway and induced p53 and p21 protein expression, thereby inducing G2/M cell cycle arrest and apoptosis. How TQ exerts its regulatory effects on PTEN transcription, however, remains largely to be established. A recent study revealed that dietary phytochemicals were able to inhibit the activity of DNA methyltransferase(s) and reactivate epigenetically silenced genes [51]. It would be interesting to learn whether TQ treatment affects cellular gene expression profile by resetting DNA methylation and histone modifications, which could be altered during the development of drug resistance. In time, answers to these questions could open promising new avenues, *via* modulation of epigenetic traits of cancer cells, as potential chemotherapy or chemoprevention targets.

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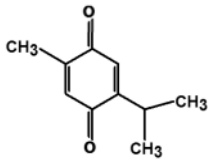

Fig. 1. Structure of TQ (2-isopropyl-5-methyl-1,4 benzoquinone)

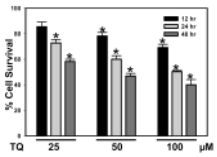

Fig. 2. Anti-proliferative effect of TQ on MCF-7/DOX cells

Exponentially growing MCF-7/Dox cells were seeded in 96-well plates. The cells were treated with indicated concentrations of TQ, maintained in culture for 12, 24 or 48 hours and incubated in medium containing MTT for additional 2 hours. Cell growth was determined by MTT assay, assessing the ability of cells to convert the soluble MTT into an insoluble formazan precipitates in cells. Values represent mean ±SE of n=3. *: Denotes significance at $p<0.05$

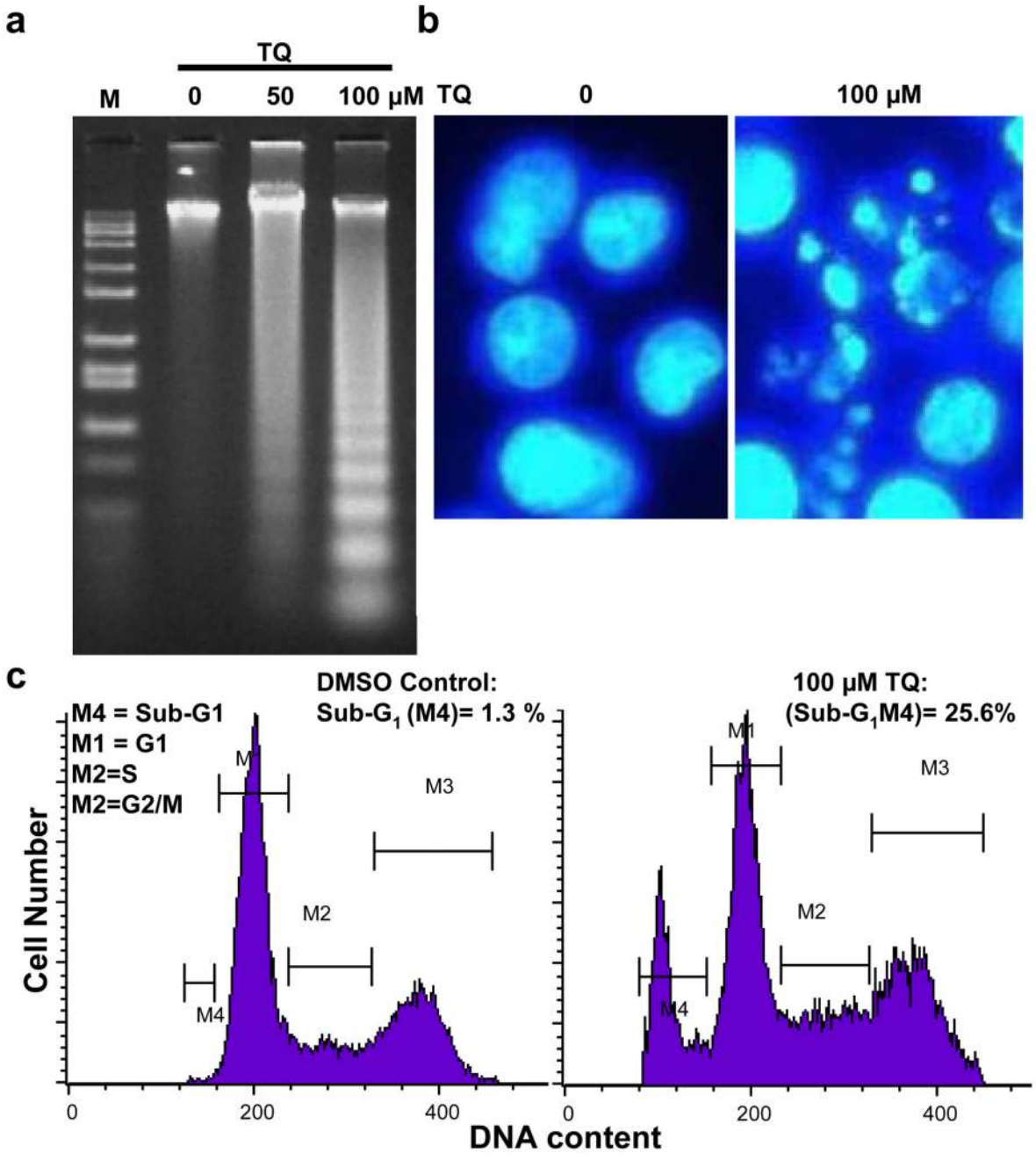

Fig. 3. TQ induces apoptosis in MCF-7/DOX cells

(A) Gel analysis of DNA fragmentation patterns of TQ-treated or untreated cells. (B) Morphologic examination of TQ-treated and untreated cells by Hoechst 33342 staining. (C) Flow cytometric analysis of cell populations at sub-G0/G1 (sub-G1) phase.

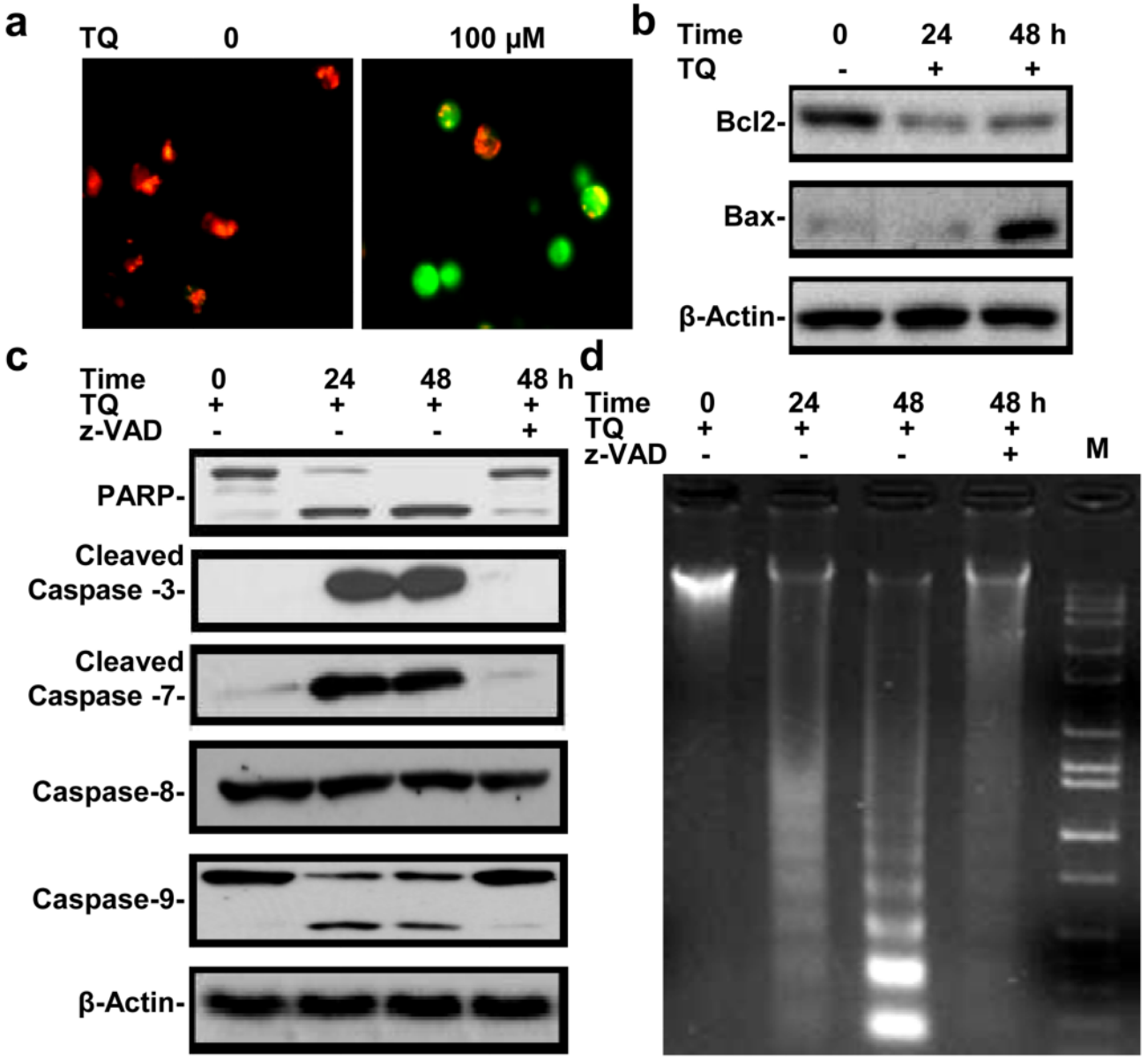

Fig. 4. TQ alters mitochondrial membrane potential and activates caspase pathway

(A) MCF-7/DOX cells were incubated with 100 μM TQ for 48 hours and incubated with MitoCapture reagent at 37°C. In healthy cells, MitoCapture accumulates and aggregates in mitochondria, giving off a bright red fluorescence; in apoptotic cells, MitoCapture does not aggregate in mitochondria and thus remains in its monomeric form, generating green fluorescence. (B) MCF-7/Dox cells were pre-treated with Z-VAD-FMK and then incubated with 100 μM TQ for the indicated time points. DNA fragmentation was analyzed using agarose gel electrophoresis. (C) MCF-7/Dox cells were treated with or without general caspase inhibitor z-VAD-FMK for 1 hour and then incubated with100 μM TQ for 24 or 48 hours. PARP, Caspase-3, -7, -8 and 9 were examined by Western blot analysis. (D) Cells were treated with 100 μM TQ for 24 or 48 hours. Cellular Bax and Bcl2 were analyzed by

Western blotting and the Bcl2/Bax ratio was calculated. Values represent mean ±SE of n=3.
*: Denotes significance at $p<0.05$

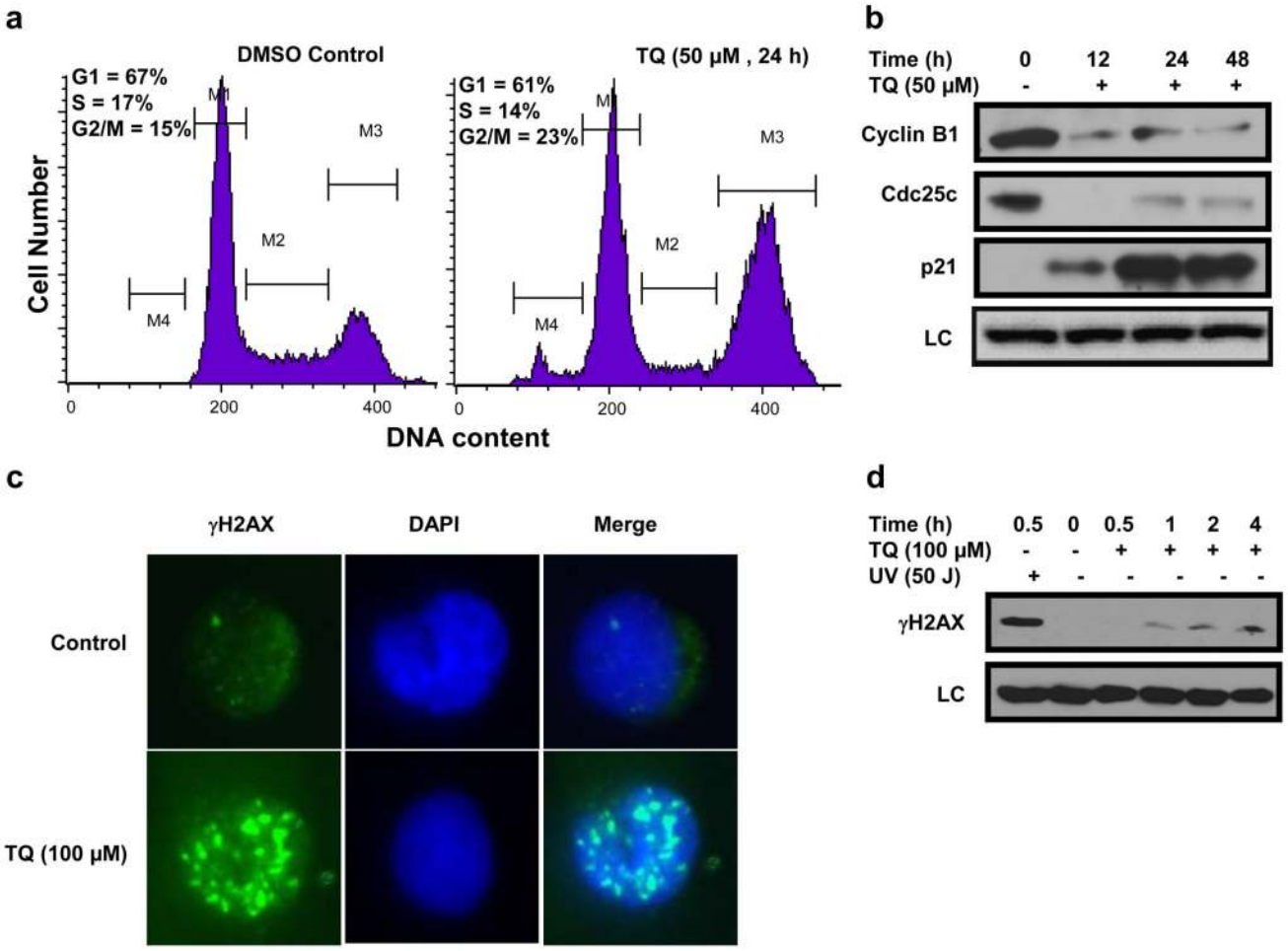

Fig. 5. TQ induces DNA damage and G2/M arrest in MCF-7/DOX cells

Cells were treated with TQ or its vehicle for 24 hours. (A) Cell cycle distribution was analyzed using flow cytometry; (B) cellular levels of Cyclin B1, Cdc25C and p21 were determined by Western blot analysis. (C) γH2AX foci formation in TQ-treated cells compared to control untreated cells. Slides were immuno-stained with γH2AX antibody and nuclei were counter stained with DAPI (blue fluorescence) and examined by fluorescence microscopy. (D) γH2AX level was determined using Western blot analysis.

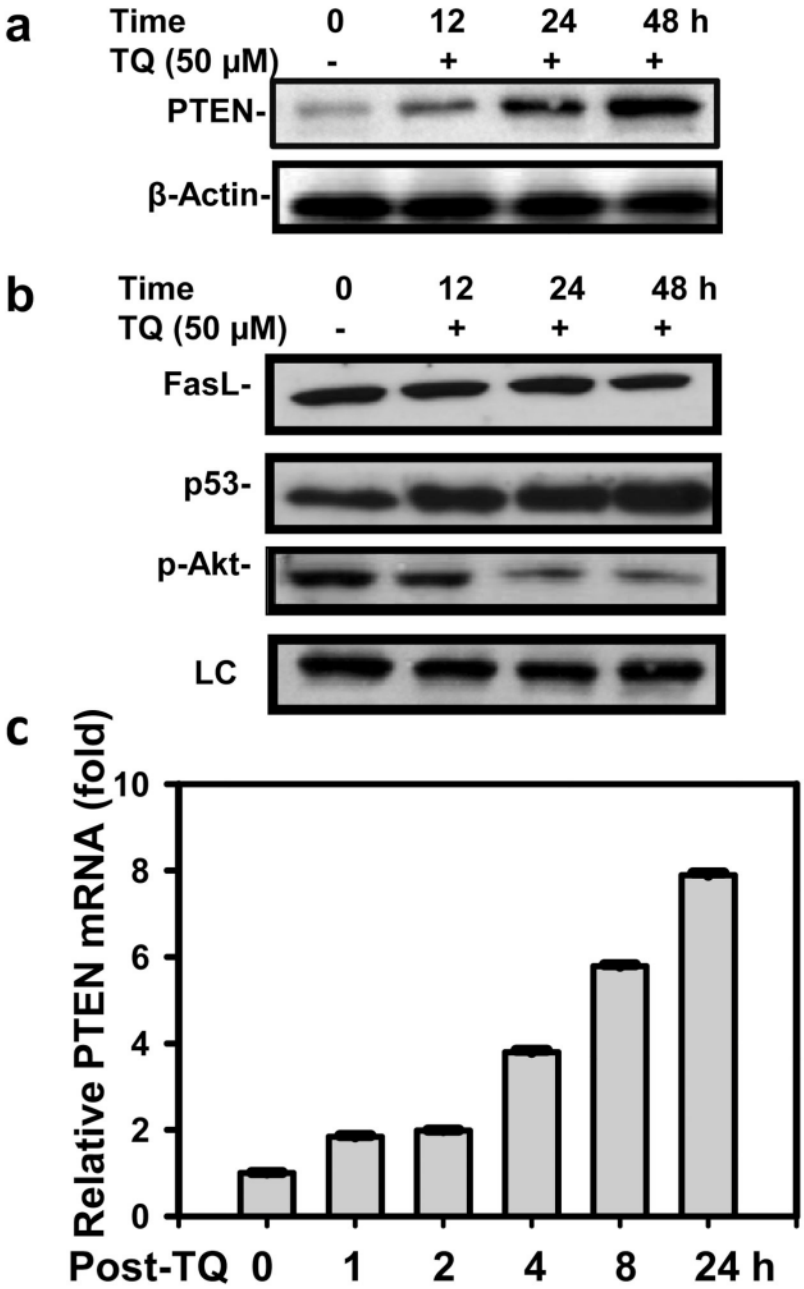

Fig. 6. TQ up-regulates PTEN in MCF-7/DOX cells

Cells were treated with 50 μM TQ or its vehicle for different time periods. (A) PTEN protein expression was determined by Western blot analysis. (B) Western blot analysis of the downstream targets of PTEN, p-Akt, p53 and Fas L in the100 μM TQ treatment and untreated cells. (C) Total RNA was extracted from the cells after TQ treatment, and, the PTEN mRNA was detected by real-time RT–PCR assay using gene-specific primers as described in 'Materials and methods'. The levels of mRNA transcripts were expressed in relative (fold) to DMSO-treated unirradiated cells as control.

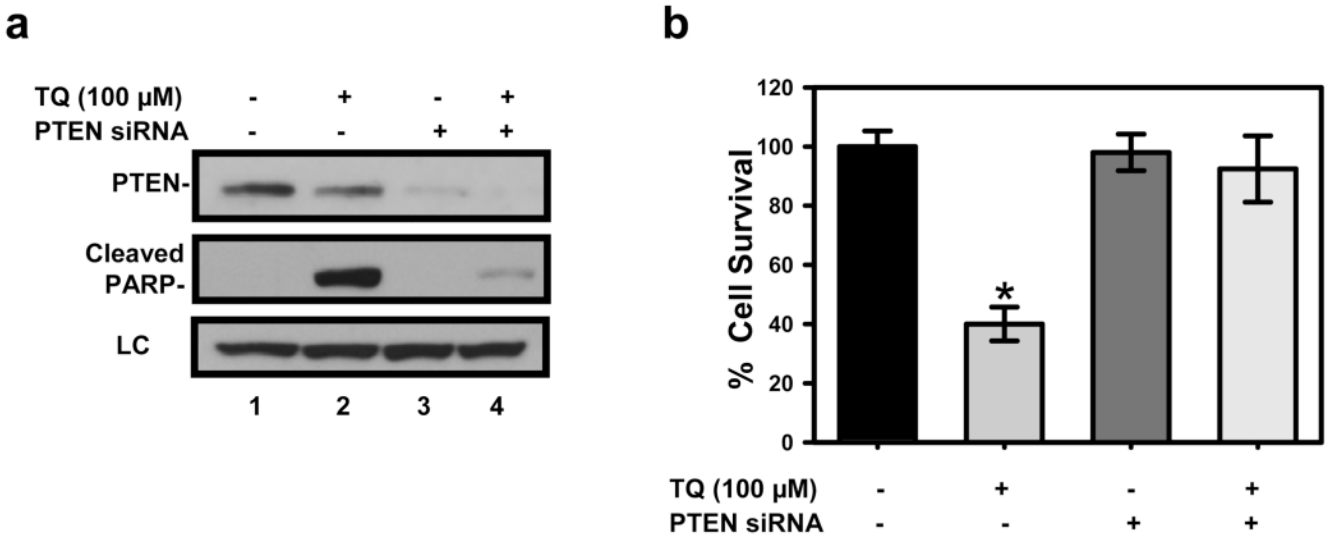

Fig. 7. Silencing PTEN by siRNA hinders the ability of TQ to induce apoptosis in MCF-7/DOX cells

Cells were transfected with 50 nM PTEN siRNA for 24 hours and then treated with TQ for 48 hours. (A) Cellular levels of PTEN and PARP proteins were determined by Western blotting. (B) Cell survival after siRNA-mediated PTEN Silencing and TQ treatment was determined using MTT assay. Values represent mean ±SE of n=3. *: Denotes significance at $p<0.05$