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Thymoquinone suppression of the human hepatocellular carcinoma cell growth involves inhibition of IL-8 expression, elevated levels of TRAIL receptors, oxidative stress and apoptosis

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Abstract Hepatocellular carcinoma (HCC) is the fourth most common solid tumor worldwide. The chemokine interleukin-8 (IL-8) is overexpressed in HCC and is a potential target for therapy. Although the transcription factor NF-κB regulates IL-8 expression, and while thymoquinone (TQ; the most bioactive constituent of black seed oil) inhibits NF-κB activity, the precise mechanisms by which TQ regulates IL-8 and cancer cell growth remain to be clarified. Here, we report that TQ inhibited growth of HCC cells in a dose- and time-dependent manner, caused G2M cell cycle arrest, and stimulated apoptosis. Apoptosis was substantiated by activation of caspase-3 and -9, as well as cleavage of poly(ADP-ribose)polymerase. TQ treatments inhibited expression of NF-κB and suppressed IL-8 and its receptors. TQ treatments caused increased levels of reactive oxygen species (ROS) and mRNAs of oxidative stress-related genes, NQO1 and HO-1. Pretreatment of HepG2 cells with N-acetylcysteine, a scavenger of ROS, prevented TQ-induced cell death. TQ treatment stimulated mRNA expression of pro-apoptotic Bcl-xS and TRAIL death receptors, and inhibited expression of the anti-apoptotic gene Bcl-2. TQ enhanced TRAIL-induced death of HepG2 cells, in part by up-regulating TRAIL death receptors, inhibiting NF-κB and IL-8 and stimulating apoptosis. Altogether, these findings provide insights into the pleiotropic molecular mechanisms of TQ-dependent suppression of HCC cell growth and underscore potential of this compound as anti-HCC drug.

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Introduction

Thymoquinone (TQ; 2-isopropyl-5-methyl-1,4 benzoquinone) is the predominant bioactive constituent present in the volatile oil of black seed (*Nigella sativa*), commonly used as a condiment in the Middle East [1]. It has antioxidant effects and has been shown to protect against heart, liver, and kidney damage in animal studies [2]. Recent studies reported that TQ exhibited inhibitory effects on cell proliferation of many cancer cell lines, including colon, ovarian, lung, and myeloblastic leukemias [3]. Notably,

previous reports have shown that growth inhibitory effects of TQ are specific to cancer cells, with minimal toxicity to normal cells [4]. In addition, TQ has been shown to inhibit chemically induced carcinogenesis in mice [1, 5]. Here, we investigated TQ inhibition of HCC cell growth and elucidated the molecular mechanisms involved.

Hepatocellular carcinoma (HCC) is a highly progressive and lethal cancer, with average survival rates after the onset of symptoms of less than 1 year [6]. It is the fourth most common cause of cancer mortality worldwide and the third most common cancer in men [7]. It is also the second most common cancer affecting Saudi males [8]. HCC accounts for 80–90 % of liver cancers [9]. The rapid increases in rates of HCC in the United States and the developed world correlate with similar increases in the prevalence chronic hepatitis C virus infection [6]. Because almost all HCC patients have cirrhosis, chemotherapies or major resections are not well tolerated. Thus, new approaches to HCC therapy are urgently needed.

IL-8 (CXCL8) is a multifunctional chemokine that regulates human neutrophil functions, including chemotaxis, enzyme release and expression of surface adhesion molecules [10]. Ren et al. [11] have reported that the level of serum IL-8 was markedly elevated in patients with HCC compared with healthy subjects, and a high-serum IL-8 level significantly correlated with a more aggressive tumor phenotype in patients with resectable HCC. Akiba et al. [12] have also reported that IL-8 was overexpressed in HCC tumor cells compared with the normal livers, and HCC patients with high IL-8 expression had a significantly higher frequency of venous invasion, portal vein invasion and bile duct invasion than those with low IL-8 expression. This suggests that IL-8 could be a potential target for HCC therapy.

Materials and methods

Materials

Chemicals and supplies

Recombinant human TRAIL and IL-8 proteins were obtained from PeproTech EC Ltd (London, UK). MTT, propidium iodide, N-acetylcysteine and TQ were purchased from Sigma Aldrich (St Louis, MO, USA). DMEM/high glucose, FBS and 1 % penicillin/streptomycin were obtained from Hyclone Laboratories (Logan, UT, USA). Trizol reagent was purchased from Invitrogen (Carlsbad, CA, USA). Primers for real time PCR were obtained from Biolegio (Nijmegen, The Netherlands). High-capacity cDNA reverse transcription kit and SYBR Green Universal Master mix were purchased from Applied Biosystems® (Foster City, CA, USA). 2′,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA) was obtained from Molecular Probes (Eugene, Oregon, USA). Antibodies specific for the NF-κB related proteins were purchased from (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Antibodies specific for pro-caspase-3, cleaved caspase-9, cleaved poly(ADP-ribose) polymerase (PARP) and β-actin were purchased from Cell Signaling (Beverley, MA, USA). Annexin V-FITC kit was obtained from BioVision Research Products, Mountain View, CA, USA.

Cell lines

The HepG2 HCC cells were a kind gift from Dr. Ayman El-Kadi (University of Alberta, Edmonton, Alberta, Canada). The Huh-7 HCC cells were kindly provided by Dr. Charles Rice (Rockefeller University, New York, NY, USA) and maintained in culture essentially as described [13]. The cells were cultured in DMEM/high glucose supplemented with 10 % FBS, 2 mM L-glutamine and 1 % penicillin/streptomycin.

Methods

Measurement of viability of HCC cells by MTT assay

We conducted initial studies to examine the effects of TQ on viability of HCC cells. This was accomplished by testing the capacity of the reducing enzymes present in viable cells to convert 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT) to formazan crystals as previously described [14]. To test whether TQ can sensitize HCC cells to TRAIL-induced cell death, cells were incubated with TQ (12.5 μM) for 30 min at 37 °C, followed by addition of TRAIL (2.55 μM; 50 ng/ml) and incubated for additional 24 h at 37 °C, followed by MTT assay. We clarified the role of oxidative stress in TQ-induced HepG2 cell death by examining the effect of N-acetylcysteine (NAC) on TQ-induced inhibition of HepG2 cell proliferation. Cells were first treated with NAC (10 mM in PBS, pH 7.2) for 2 h, followed by addition of TQ (12.5, 25 or 50 μM) and incubated at 37 °C for 24 h.

TQ regulation of gene expression

RNA preparation and quantitative real-time PCR

To determine the effect of TQ on mRNA expression levels of IL-8 and its receptors, TRAIL death receptors, apoptosis, NF-κB and oxidative stress-related genes, we conducted quantitative real-time polymerase chain reaction (qRT-PCR) assay:

HepG2 cells were treated with TQ (12.5–100 μM) for 6 h, and total RNA was obtained from the cells using

Table 1 Sequences of primers used in the study

Primer set	Sense primer 5′ → 3′	Antisense primer 5′ → 3′
IL-8	CTCTTGGCAGCCTTCCTGATT	TATGCACTGACATCTAAGTTCTTTAGCA
CXCR1	TGCATCAGTGTGGACCGTTA	TGTCATTTCCCAGGACCTCA
CXCR2	TGCATCAGTGTGGACCGTTA	CCGCCAGTTTGCTGTATTG
TRAIL R1	GATCGATGTGGTCAGAGCTGG	TGTGGATCGAGGCGTTCC
TRAIL R2	GTGCCCTTGACTCCTGGG	AAGGTGTCCCTGTGGCCC
Bcl-xS	CCCAGAAAGGATACAGCTGG	GCGAT-CCGACTCACCAATAC
P53	GCCCCCAGGGAGCACTA	GGGAGAGGAGCTGGTGTTG
Bcl-2	CATGTGTGTGGAGAGCGTCAA	GCCGGTTCAGGTACTCAGTCA
Bcl-xL	GGCGGCTGGGATACTTTTGT	CTCGGCTGCTGCATTGTTC
Survivin	GCCCAGTGTTTCTTCTGCTT	CCGGACGAATGCTTTTTATG
C-IAP	TCGCTTGAAAAGACTGGGCT	TTCTCTAGGGAGGTAGTTTTGATTT
X-IAP	CATTTCCAGATTGGGGCTCG	TGACCAGGCACGATCACAAG
ABIN-1	AGTGAACAAGCAGTGGGACC	GAGCTTGCGGTCAAAGTCAC
ABIN-2	AGGCTGACTGAGCGACTAGA	CTCCGCAGATGATGGGTCTC
NQO1	CGCAGACCTTGTGATATTCCAG	TGTTGCGCTCAATCTCCTCCT
HO-1	ATGGCCTCCCTGTACCACATC	CGTTTCTTCCATCCTTCCAGG
GAPDH	TGGGCTACACTGAGCACCAG	GGGTGTCGCTGTTGAAGTCA
β-Actin	CTGGCACCCAGGACAATG	GCCGATCCACACGGAGTA

TRIzol reagent according to the manufacturer's instructions and quantified by measuring the absorbance at 260 nm. The RNA quality was determined by measuring the 260/280 ratio. First strand cDNA synthesis was then performed using the High-Capacity cDNA reverse transcription kit, according to the manufacturer's instructions. Quantitative analysis of mRNA expression of target genes was performed by subjecting the resulting cDNAs to PCR amplification using 96-well optical reaction plates in the ABI Prism 7500 Real-Time PCR System. The reaction mix contained forward and reverse primers, SYBR Green Universal Master mix, nuclease-free water and cDNA sample. The sequences of primers used in the current study are listed in Table 1. Assay controls were incorporated onto the same plate, namely, no-template controls to test for the contamination of any assay reagents. Data were analyzed using the relative gene expression (ΔΔ CT) method, as described in Applied Biosystems User Bulletin No. 2. Briefly, data are presented as the fold change in gene expression normalized to the endogenous reference gene (glyceraldehyde-3-phosphate dehydrogenase "GAPDH" or β-actin) and relative to a calibrator.

Western blot analysis

The TQ regulation of NF-κB and caspase expression and PARP cleavage in HCC cells was investigated by Western blot. HCC cells were treated for 12 h (for NF-κB) or 24 h (for caspases and cleaved PARP) with different concentrations of TQ (0, 12.5, 25, and 50 μM). Approximately 6×10^6 cells were collected in 300 μl lysis buffer (50 mM HEPES, 0.5 M sodium chloride, 1.5 mM magnesium chloride, 1 mM EDTA, 10 % glycerol (v/v), 1 % Triton X-100, and 5 μl/ml of protease inhibitor cocktail). Total cellular proteins were obtained by incubating the cell lysates on ice for 1 h, with intermittent vortex mixing every 10 min, followed by centrifugation at $12{,}000\times g$ for 10 min at 4 °C. Then, 25 μg of protein from each treatment group was separated by 10 % sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then electrophoretically transferred to nitrocellulose membrane. Protein blots were then blocked overnight at 4 °C in blocking solution containing 0.15 M sodium chloride, 3 mM potassium chloride, 25 mM Tris-base (TBS), 5 % skim milk powder, 2 % bovine serum albumin and 0.5 % Tween-20. After blocking, the blots were washed several times with TBS-Tween-20 before being incubated with primary antibodies over-night in TBS solution containing 0.05 % (v/v) Tween-20 and 0.02 % sodium azide. Incubation with a peroxidase-conjugated anti-goat IgG secondary antibody was carried out in blocking solution for 2 h. The bands were visualized using the enhanced chemiluminescence method according to the manufacturer's instructions (GE Healthcare, Mississauga, ON, USA).The intensity of NF-κB p65 and p50, as well as IkB-α protein bands was quantified relative to the signals obtained for GAPDH protein, using ImageJ® image processing program (National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij).

Measurement of production of reactive oxygen species

The generation of intracellular reactive oxygen species (ROS) was evaluated based on the intracellular peroxide-dependent oxidation of 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) which forms a fluorescent compound, 2′,7′-dichlorofluorescein (DCF), as essentially previously described [15]. Briefly, HepG2 cells (10,000 cells/well) were cultured in 48-well culture plates. Twenty-four hour after the treatment with TQ at the indicated concentrations, the culture medium was removed; cells were washed twice with PBS and then incubated with 400 µl/well of PBS containing 10 µM of DCFH-DA at 37 °C for 10 min. The fluorescence intensity was monitored with a FLUOstar OMEGA microplate reader (BMG LABTECH Ltd., Germany) at an excitation wavelength of 485 nm and an emission wavelength of 520 nm. Results were expressed as percent of control.

Analysis of cell cycle and detection of apoptosis by flow cytometry

The influence of TQ on apoptosis and cell cycle in HepG2 cells was quantified with by flow cytometry following staining of cells with propidium iodide (PI) based on the procedure by Nicoletti et al. [16]. HepG2 cells (7×10^5) were left untreated or treated with TQ (6–100 µM) or treated with ethanol (solvent control). After different incubation times (6–18 h), cells were harvested by centrifugation and washed once with PBS. Then the cells were fixed in cold 70 % ethanol for 30 min, and the pellets were resuspended in hypotonic fluorochrome solution (50 µg/ml PI, 0.1 % sodium citrate and 0.1 % Triton X-100 in 1 ml of PBS). The cell suspensions were then treated with RNase for 30 min in dark. The red fluorescence of 10,000 events of PI-stained cells was acquired in FL4 Log channel through a 675-nm band-pass filter using Beckman Coulter flow cytometer (Cytomics FC 500, Beckman Coulter, Fullerton, CA, USA). The cell cycle phases were calculated, and the data were processed using CXP acquisition and analysis software.

To characterize the early apoptosis induced by TQ, Annexin V-FITC assay was used as previously described [17, 18]. Briefly, adherent cells grown in 6-well plates were labeled prior to harvesting. After medium removal, wells were washed once in PBS, before addition of binding buffer (BioVision Research Products, Mountain View, CA, USA). To each well, Annexin V-FITC (BioVision Research Products) was added, and plates were left again at room temperature for 10 min. Then, PI (BioVision Research Products) was added to each well, and plates were left at room temperature for 10 min. After labeling, all plates were washed twice in binding buffer to remove excess label, and cells harvested by scraping after adding binding buffer. Cells (10,000 events per sample) were immediately analyzed by flow cytometry on Cytomics FC 500 instrument, and the data were processed using CXP acquisition and analysis software. Annexin V-FITC positive/PI negative cells were considered as early apoptotic cells. Annexin V-FITC positive/PI positive cells were considered as late apoptotic (or necrotic) cells. Annexin V-FITC negative/PI positive cells were considered as necrotic cells. Annexin V-FITC negative/PI negative cells were considered as non-apoptotic live cells.

Data analysis

Data were expressed as the mean ± standard error (SEM) of the mean of n observations. Significant differences between individual treatment groups and corresponding solvent controls were analyzed by ANOVA followed by Tukey–Kramer. $*p < 0.05$ was accepted as a significant difference between each pair of compared groups.

Results and discussion

Effect of drugs on cell viability

NF-κB is a ubiquitous transcription factor that is activated in response to various inflammatory stimuli, carcinogens, tumor promoters and growth factors. Interestingly, NF-κB mediates up-regulation of the inflammatory cytokine IL-8 in many cancer cell types [19]. IL-8 is also overexpressed in HCC tumor cells compared with the normal livers [12]. TQ inhibits NF-κB activity in human chronic myeloid leukemia cells [20], and its inhibition of mammary tumor growth was found due, in part, to its inhibition of NF-κB activity [5], suggesting for its potential to inhibit HCC cell growth. Hence, we speculated that TQ will suppress HCC cell growth and that could involve reduced NF-κB activity and IL-8 expression. To test this possibility, we first conducted a dose escalation study utilizing MTT-based viability assays to examine HCC cell growth inhibitory effects of TQ. As shown in Fig. 1a, b, TQ induced a dose-dependent inhibition of both the HepG2 and Huh7 HCC cells, while exerting its maximal effect at a concentration of 16 µg/ml. We next examined whether TQ can enhance TRAIL-induced growth suppression of HCC cells. We treated HepG2 cells with TQ (2 µg/ml; 12.5 µM) or its vehicle control (ethanol) for 30 min followed by addition of TRAIL (50 µg/ml; 2.55 µM) or serum-free medium (SFM) for further 24 h. Percent specific death was determined by MTT assay essentially as in Fig. 1a. HCC cells treated with TRAIL or TQ alone exhibited only about 15 % or 25 % cell death, respectively (Fig. 1c). In contrast, TQ

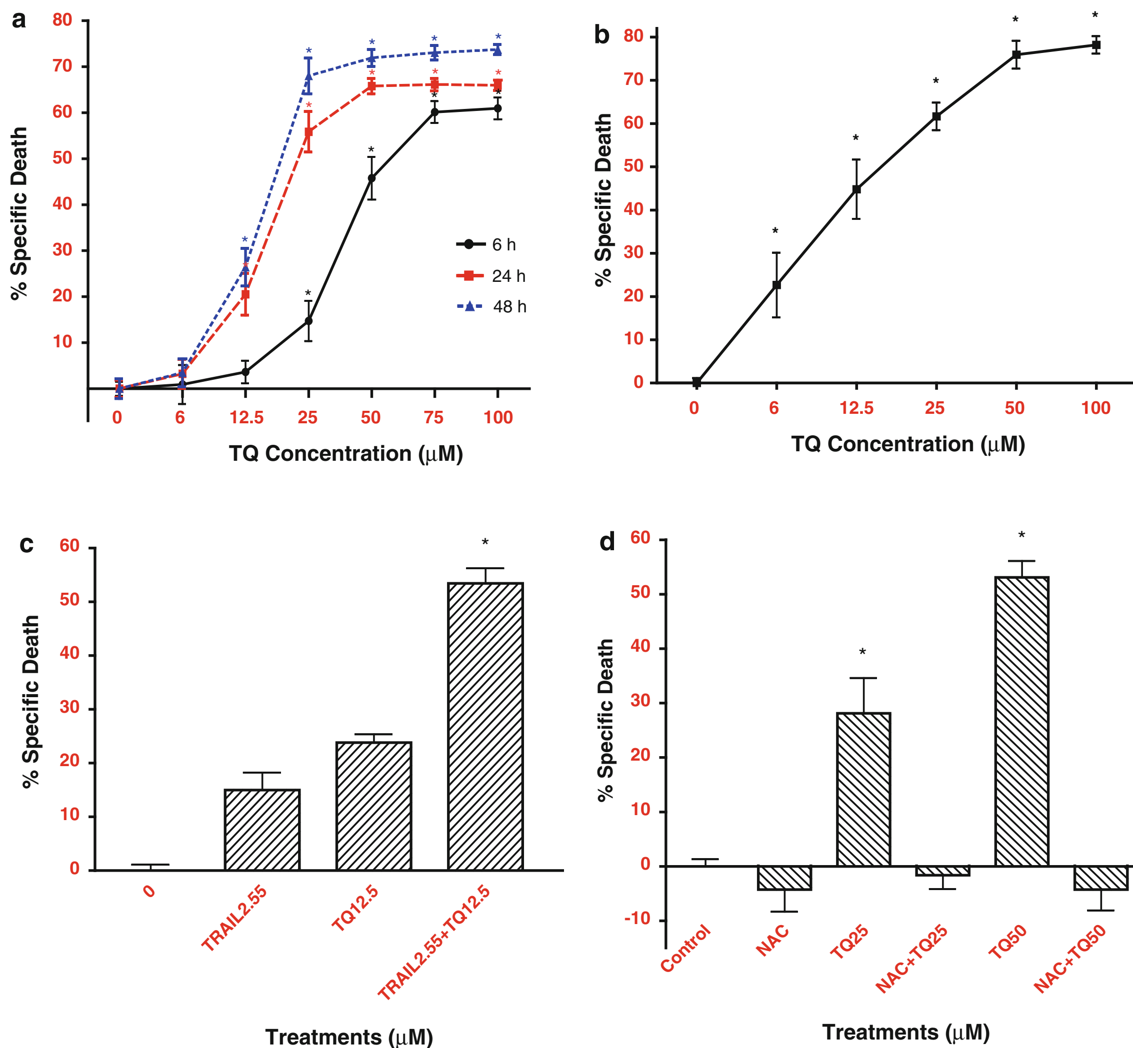

Fig. 1 HCC cell growth suppression by TQ, TRAIL, and NAC. Cells were treated with indicated concentrations of noted agents. Cell viability was determined by MTT assay as indicated in "Methods" section. At the end of the assay, the absorbance at 549 nm (**a**) was read on a microplate reader. % Specific death = A(untreated cells) − A(treated cells)/A(untreated cells) × 100. **a** TQ dose and time response in HepG2 cells. **b** TQ dose response in Huh-7 cells. **c** Effect of TQ and/or TRAIL on viability of HepG2 cells. **d** Effect of NAC and/or TQ on viability of HepG2 cells. Significant differences between individual treatments and control were analyzed by ANOVA followed by Tukey–Kramer. *$p < 0.01$

treatment was able to significantly ($p < 0.05$) enhance TRAIL-mediated cell death (54 %; Fig. 1c). These data indicate that TQ in combination with TRAIL can suppress growth of TRAIL-resistant HCC cells. Since previous studies (27) have shown that inhibition of NF-κB enhanced TRAIL-induced HCC cell death, it is likely that superior inhibitory effects of TQ+ TRAIL involve attenuation of NF-κB activity (see below).

Next, we studied the effect of NAC on TQ-induced cell death. We examined the potential of an antagonistic effect of NAC against TQ cytotoxicity. NAC is a well-known synthetic antioxidant that acts by raising the intracellular concentration of glutathione and/or acts as ROS scavenger [21]. NAC completely blocked TQ-induced HepG2 cell growth suppression (Fig. 1d), highlighting a role for the oxidative stress in the TQ-mediated HCC cell growth inhibition.

TQ causes G2M cell cycle arrest and apoptosis

We conducted flow cytometry analyses to measure DNA content of the HCC cells to determine TQ effects on cell cycle progression. The cellular DNA content is known to change during the four phases of the cell-division cycle. HepG2 cells are diploid (2n) in the G_0/G_1-phase (gap phase 0/1) and their DNA content doubles (4n) in the S-phase (synthesis phase). Finally, in mitosis (M-phase), cells are divided, and the resulting two daughter cells are again

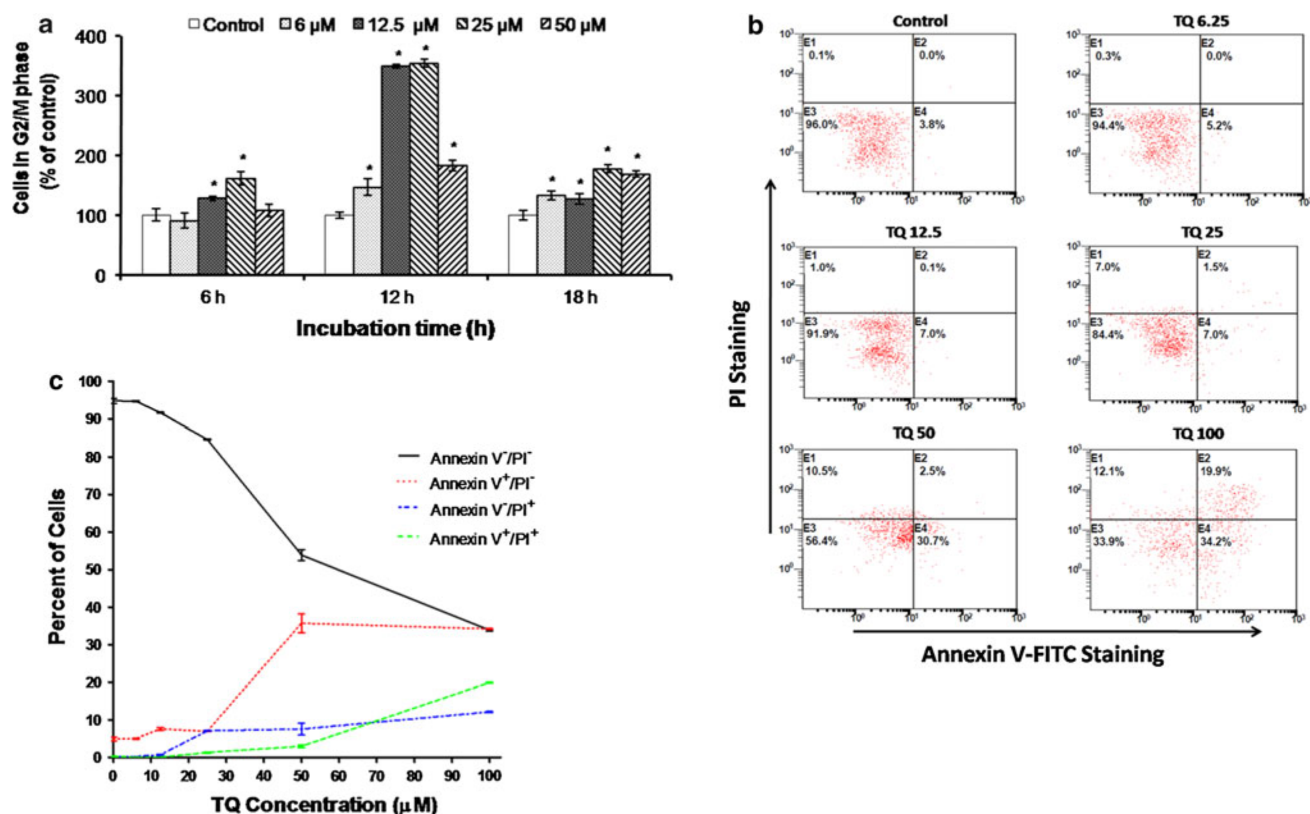

Fig. 2 TQ treatment induces cell cycle arrest and apoptosis. **a** Cells were treated with increasing concentrations of TQ (6–100 μM) for 6–18 h. The amount of cellular DNA was assessed by DNA staining with PI and subsequent flow cytometry. Data are presented as mean ± SEM of percent of PI stained control-treated cells. **b**, **c** HepG2 cells were treated with indicated concentrations of TQ for 6 h. Cells were then labeled with Annexin V and PI, and analyzed by flow cytometry; **b** *Numbers* in the respective quadrants indicate the percentage of cells present in this area; **c** Quantitative assessment of the Annexin V assay results. Annexin V^-/PI^-: live cells; Annexin V^+/PI^-: early apoptotic cells; Annexin V^-/PI^+: necrotic cells; Annexin V^+/PI^+: late apoptotic (or necrotic) cells. Significant differences between individual treatments and control were analyzed by ANOVA followed by Tukey–Kramer. *$p < 0.05$ compared with control (0 μM)

diploid (2n). Cellular DNA amount can be visualized by DNA staining with PI and subsequent flow cytometry. Solvent control-treated HepG2 cells displayed the normal cell cycle distributions and were near to those in the SFM untreated cells at all sampling times. On the other hand, 12 or 18 h incubations with TQ caused significant accumulation of cells in G_2/M-phase, relative to the solvent controls ($p < 0.05$). Moreover, HepG2 cells treated with 12.5 and 25 μM TQ have undergone G_2/M cell cycle arrest after 6 h incubation as evident by the appearance of more cells in G_2/M phase, relative to those in the solvent control group ($p < 0.01$) (Fig. 2a). Such G_2 arrest has been proposed to be due to induction of G_2 checkpoint machinery that allows damaged DNA to be repaired before cells move to the next cell cycle stage [22]. However, if DNA damage is extensive, cell death by apoptosis will be ensue.

To determine whether TQ induced apoptosis, HepG2 cells were exposed to TQ for 6 h, followed by incubation with Annexin V and PI as described in Methods. As shown in Fig. 2b, c, TQ treatment increased the percent of Annexin V-FITC positive/PI negative cells (early apoptotic) and Annexin V-FITC positive/PI positive cells (late apoptotic or necrotic) cells in a dose-dependent manner. These data indicate that TQ inhibits HCC cell growth in part by inducing apoptosis within only six hours of treatment, even at very low concentration [12.5 μM (2 μg/ml)]. These findings are also consistent with our MTT data, which showed that TQ induced dose-dependent reduction of HepG2 cell viabilities within 24 h, starting from the 12.5 μM (2 μg/ml) concentration (see Fig. 1a). Thus, our data corroborate previous reports showing that TQ induces apoptosis in several human cancer cell lines [23, 24].

Effect of TQ on expression of NF-κB

NF-κB family is comprised several structurally related proteins that form homodimers and heterodimers and include NFκB-1 (p50), NFκB-2 (p52), p65 (RelA), c-Rel,

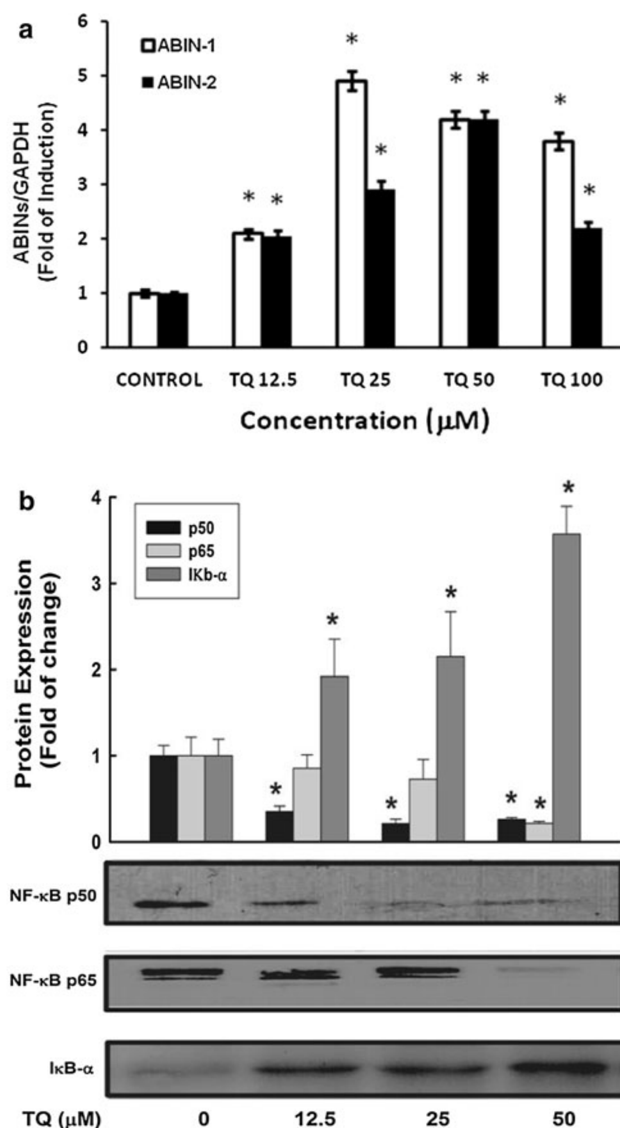

Fig. 3 TQ regulation of NF-κB signaling. **a** HepG2 cells were treated with noted concentrations of TQ for 6 h. The mRNA amounts of ABIN-1 and -2 were quantified using qRT-PCR and normalized to GAPDH housekeeping gene as in "Methods" section. The *values* in histogram represent mean of fold change ±SEM ($n = 6$). *$p < 0.05$ compared with control (0 μM). **b** HepG2 cells were treated for 12 h with noted concentrations of TQ. NF-κB p65 and p50 subunits and IκB-α protein levels were determined by Western blot analysis as described in "Methods" section. The *values* in histogram represent mean of fold change ± SEM ($n = 6$). *$p < 0.05$ compared with control (0 μM)

and RelB. NF-κB commonly refers to a p50-RelA heterodimer, which is one of the most avidly forming dimers and is the major NF-κB complex in most cells [25]. NF-κB has been shown to mediate the upregulation of IL-8 in a variety of many cancer cell types [26]. In addition, NF-κB has been found to be a critical TRAIL-induced anti-apoptotic factor in liver cancer cell lines, and inhibition of NF-κB unmasks the TRAIL-induced apoptotic signaling cascade and thus, sensitizes human HCC cells to TRAIL-induced apoptosis [27]. In light of this information, we proposed that TQ can inhibit NF-κB activity and, in turn, repress IL-8 expression, and enhance TRAIL-induced apoptosis of HCC. Figure 3a shows that TQ significantly upregulated mRNA expression of A20-binding inhibitor of NF-κB activation- (ABIN-) 1 and 2 at all concentrations used. ABIN proteins are known to negatively regulate NF-κB activation by tumor necrosis factor (TNF) and several other stimuli [28]. Therefore, the increase of ABIN-1 and -2 proteins by TQ reveals that TQ inhibits canonical NF-κB signaling as an early response. These results were corroborated by western blot analysis (Fig. 3b), demonstrating a significant down-regulation of expression of NF-κB p50 at all concentrations tested by approximately 60–70 %, and a decrease of NF-κB p65 protein levels at the higher concentration (50 μM). TQ also elevated expression of the NF-κB inhibitory protein IκBα (Fig. 3b), a protein that is known to interact with NF-κB, and block its ability to enter the nucleus and bind to DNA [25]. Consistent with our hypothesis, these data demonstrate, for the first time, which TQ inhibits NF-κB expression in liver cancer cells.

Regulation of HCC gene expression by TQ

Effect of TQ on the expression of IL-8 and its receptors

Since TQ inhibits NF-κB activity in HCC cells (Fig. 3b), we next clarified whether TQ will also inhibit IL-8 expression in HCC cells. HepG2 cells were treated for 6 h with the increasing concentrations of TQ, thereafter, mRNA levels of IL-8 and its receptors were determined by qRT-PCR as described in Methods. Figure 4a shows that TQ significantly decreased the mRNA expression of IL-8. Although lower dose of TQ [12.5 μM (2 μg/ml)] resulted in a small (about 20 %) and insignificant ($p > 0.05$) reduction of IL-8 mRNA level, a higher dose of TQ [25–100 μM (4–16 μg/ml)] elicited a significant ($p < 0.05$) and robust (approximately 60 %) decrease in the IL-8 mRNA levels.

To further clarify whether TQ suppressed IL-8 signaling, we investigated whether TQ also affected expression of IL-8 receptors. HepG2 cells were treated for 6 h with the increasing concentrations of TQ, and the mRNA levels of IL-8 receptors were determined by qRT-PCR. As shown in Fig. 4b, c, TQ inhibited the expression of both receptors, although at different potencies. TQ suppressed CXCR1 more potently than CXCR2, as it significantly down-regulated the expression of CXCR1 at a relatively lower concentration (50 μM; Fig. 4b), when compared with the CXCR2 levels that were not affected at this dose (Fig. 4c). Although the TQ dose of 100 μM suppressed mRNA levels of both the receptors, mRNA levels of CXCR1 had greater

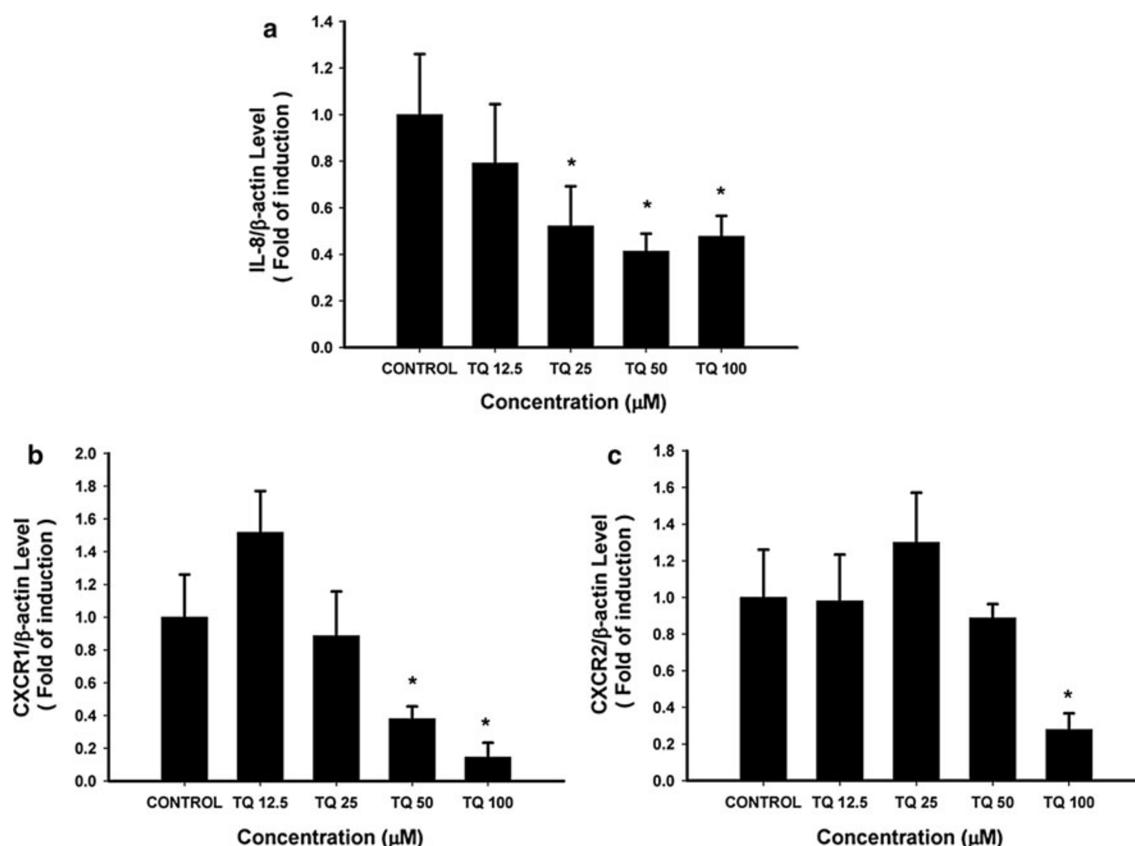

Fig. 4 TQ inhibits expression of IL-8 and its receptors. HepG2 cells were treated for 6 h with noted TQ concentrations. The amounts of IL-8 (**a**), CXCR1 (**b**) and CXCR2 (**c**) mRNAs, were quantified using qRT-PCR and normalized to β-actin housekeeping gene as in "Methods" section. Duplicate reactions were performed for each experiment and the *values* in the histograms represent mean of fold change ± SEM ($n = 6$). *$p < 0.05$ compared with control (0 μM)

reduction when compared with the mRNA levels of CXCR2, 90 versus 70 %, respectively. Collectively, these data indicate that TQ strongly affects IL-8 expression as well as its ability to signal via its receptors, particularly the CXCR1. To the best of our knowledge, this is the first report that demonstrates such inhibitory effect of TQ on IL-8 and its receptors. Together with our current data and available reports in the literature [20], we propose that TQ likely inhibits NF-κB and this, in turn, leads to the inhibition of IL-8 expression and consequent inflammatory signaling.

TQ regulation of genes transducing apoptosis signaling

TQ is known to induce apoptosis in cancer cells without significant cytotoxicity to normal cells [29]. Moreover, our data strongly suggest that TQ inhibited HCC cell growth in part by promoting apoptosis. Although, TQ inhibited expression of NF-κB subunits, the mechanisms of TQ inhibition of HCC growth have yet to be fully clarified. Moreover, since IL-8 is known to prevent apoptosis in cancer cells [30], it is likely that inhibition of IL-8 activity by TQ contributes to apoptosis of HCC cells. To further elucidate molecular mechanisms of apoptosis signaling by TQ, we next determined alterations in levels of pro- and anti-apoptotic genes using qRT-PCR. Specifically, we examined expression of the pro-apoptotic genes p53, Bcl-xS and TRAIL receptors in TQ-treated HCC cells. TQ increased the expression of TRAIL receptors, although at different potencies. TQ was relatively more potent inducer of TRAIL-R2 than TRAIL-R1, as it up-regulated the expression of TRAIL-R2 at lower concentration (12.5 μM; Fig. 5b), while TRAIL-R1 expression was not affected (Fig. 5a). However, both death receptors were up-regulated by higher TQ concentrations. This clearly suggests that TRAIL receptors, at least partly, contribute to TQ-induced HCC cell growth inhibition by likely activating the extrinsic pathway of apoptosis in TQ-treated HepG2 cells. Our data support recent findings showing that DR5 (TRAIL-R2) is selectively up-regulated by TQ in human primary effusion lymphoma cells [31]. Interestingly, Choi et al. [32] reported that DR5 ligation by TRAIL not only leads to apoptosis of human glioma cells but also induces expression of IL-8. Therefore, even though the HepG2

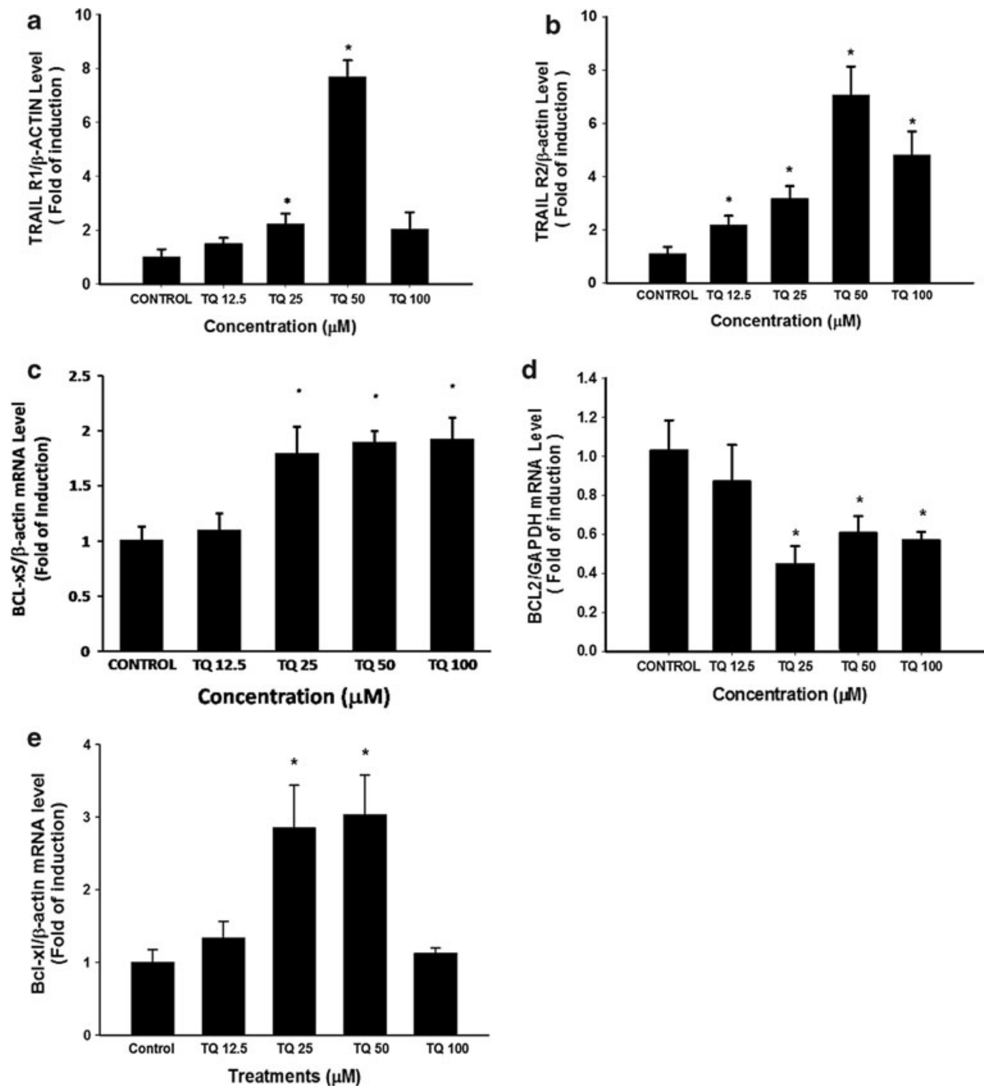

Fig. 5 Effect of TQ on mRNA expression of apoptosis-related genes. HepG2 cells were treated for 6 h with noted concentrations of TQ. The mRNA amounts of TRAIL-R1 (**a**), TRAIL-R2 (**b**), Bcl-xS (**c**), Bcl-2 (**d**) and Bcl-xL (**e**) were quantified using qRT-PCR and normalized to β-actin or GAPDH housekeeping gene as in "Methods" section. Duplicate reactions were performed for each experiment and the values in each histogram represent mean of fold change ± SEM ($n = 6$). *$p < 0.05$ compared with control (0 μM)

HCC cells have constitutive level of IL-8, TRAIL could further induce expression of IL-8 in these cells and diminish TRAIL-induced apoptosis, with consequent emergence of TRAIL-resistant HCC cells. For this reason, IL-8 inhibition in combination with TRAIL would be a superior anti-HCC therapy. Our data, thus, far strongly suggest that TQ inhibition of IL-8 expression and signaling (Fig. 4) together with up-regulation of TRAIL receptors (Fig. 5a, b) are critical for its superior HCC cell growth suppression when combined with TRAIL.

We further investigated whether TQ induced additional pro-apoptotic signaling in HCC cells. Figure 5c shows that TQ also caused elevated expression of the pro-apoptotic protein Bcl-xS, indicating a role for the intrinsic apoptosis pathway in TQ-induced HCC cell growth inhibition. To the best of our knowledge, this is the first study that examines the effect of TQ on Bcl-xS expression, revealing that this protein is a novel TQ-regulated pro-apoptotic protein. However, TQ did not stimulate the expression of p53 in HepG2 cells (data not shown). TQ regulation of apoptosis involving p53 is controversial. Gali-Muhtasib et al. [33] have previously found that TQ triggers apoptotic cell death in cancer cells via a p53-dependent mechanism, while El-Mahdy et al. [23] showed that TQ induced apoptosis in p53-null cancer cells. Although, the HepG2 cells harbor wild-type p53 [34], while the Huh-7 cells express mutant p53 [35], TQ suppressed growth of both the HCC cells (Fig. 1). These observations strongly suggest that TQ inhibited growth of HCC cells in a p53-independent manner. Taken together, although TQ stimulated expression of TRAIL receptors, it is likely that HCC growth inhibitory effects of TQ are transduced independent of the p53.

We further examined whether TQ also regulated expression of the anti-apoptotic genes Bcl-2 and Bcl-xL, as well as the IAP family member survivin. Although TQ treatments caused decreased the expression of Bcl-2

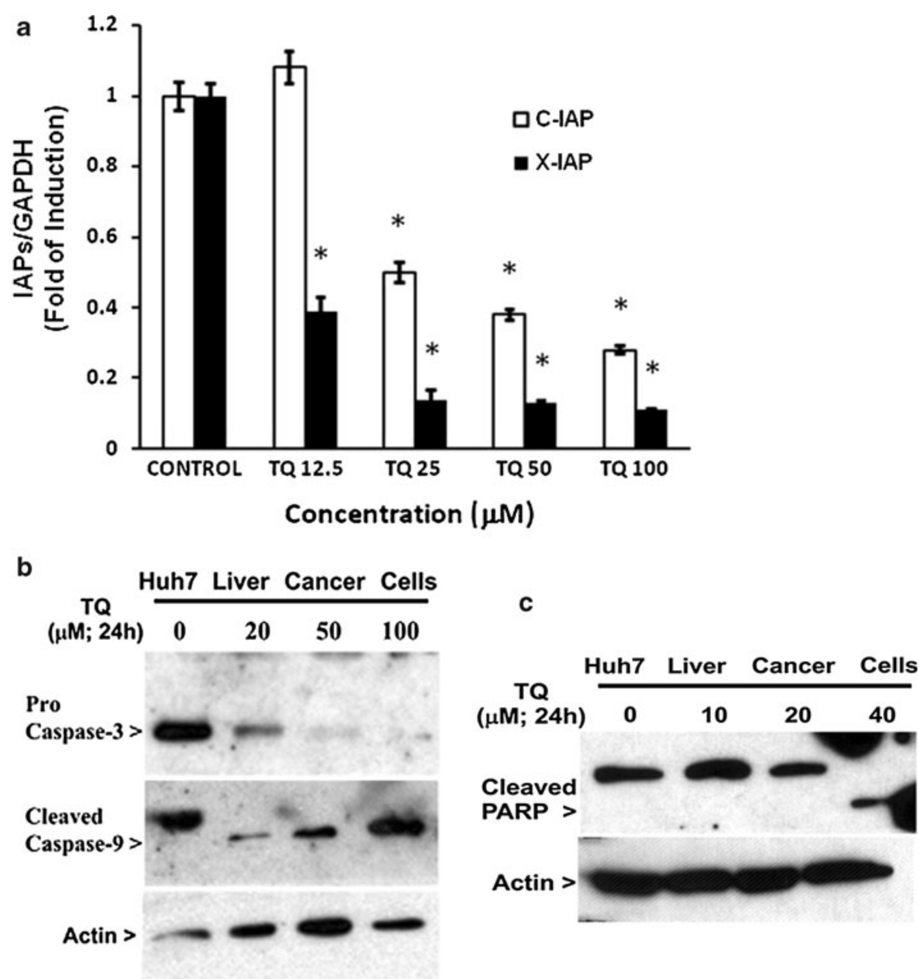

Fig. 6 TQ inhibits expression of IAPs and enhances expression of caspases and PARP. **a** HepG2 cells were treated with noted concentrations of TQ for 6 h. The mRNA amounts of C-IAP and X-IAP were quantified using qRT-PCR and normalized to GAPDH housekeeping gene as in "Methods" section. The *values* in histogram represent mean of fold change ±SEM. ($n = 6$).*$p < 0.05$ compared with control (0 μM). **b**, **c** Huh-7 cells were treated for 24 h with indicated concentrations of TQ. Pro-caspase-3 and cleaved caspase-9 (panel B) as well as cleaved PARP (panel C) protein levels were determined by Western blot analysis as described in "Methods" section

(Fig. 5d), expression of survivin was not significantly affected (data not shown), indicating that survivin was likely not involved in the TQ-induced HepG2 cell growth suppression. Although the down-regulation of Bcl-2 by TQ is consistent with previous reports [33, 36], our RT-PCR results intriguingly revealed that TQ enhanced mRNA levels of the anti-apoptotic gene Bcl-xL (Fig. 5e), which could be perceived as a potential mechanism for development of resistance to TQ-induced growth inhibitory effects. The fact that TQ decreased expression of the anti-apoptotic gene Bcl-2 (Fig. 5d) and increased expression of the pro-apoptotic gene Bcl-xS (Fig. 5c), both effects are known to target cells to apoptosis, would suggest that even in the presence of antagonistic effect of Bcl-xL increase (Fig. 5e), the net effect of TQ presence is likely increased mitochondrial, intrinsic apoptosis in the HCC cells. Indeed, it is well known that the ratio between pro- and anti-apoptotic proteins determines, in part, the susceptibility of cells to a death signal [37]. To further explore the effect of TQ on IAP expression, we analyzed expression of two more IAPs, namely C-IAP and X-IAP. RT-PCR results showed decreases in C-IAP and X-IAP mRNA levels in TQ-treated cells (Fig. 6a). Since IAPs are known to be induced by NF-κB [38], our results reveal that TQ-induced down-regulation of NF-κB expression (Fig. 3b) is associated with suppression of IAPs expression, thereby increasing the sensitivity of HCC cells to TQ-induced apoptosis (Fig. 2). Collectively, we may conclude that even though TQ appears to induce both extrinsic and intrinsic pathways of apoptosis of HepG2 cells, the extrinsic pathway is more likely to be involved in TQ-induced cell death.

To further substantiate our observation of induction of apoptosis by TQ (see Fig. 2), we determined whether and to which extent caspases were involved in transducing TQ effects. Huh7 cells were either untreated or treated with TQ for 24 h, and levels of pro-caspase-3, cleaved caspase-9 and PARP proteins were analyzed. TQ treatment resulted in a significant increase in apoptosis demonstrated by a decrease in the expression of pro-caspase-3, suggesting that TQ stimulated activation of caspase 3 (Fig. 6b). TQ also enhanced the expression of caspase 9 and cleavage of PARP (Fig. 6b, c).

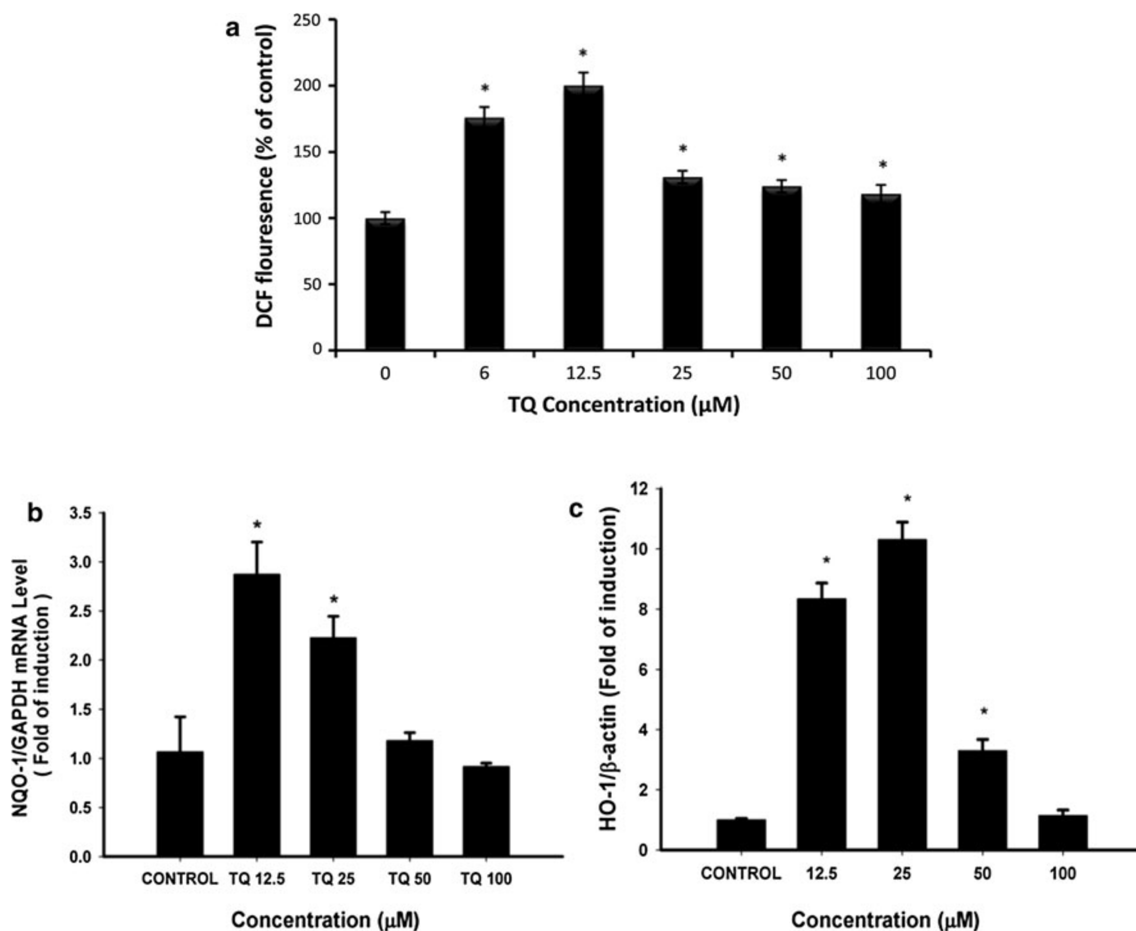

Fig. 7 TQ induces oxidative stress in HCC cells. HepG2 cells were treated for 6 h with indicated concentrations of TQ. Production of ROS (**a**) was then assessed by DCF assay as indicated in the "Methods" section. Significant differences between individual treatments and control were analyzed by ANOVA followed by Tukey–Kramer. $*p < 0.05$. The mRNA amounts of NQO1 (**b**) and HO-1 (**c**) were quantified using qRT-PCR and normalized to GAPDH or β-actin housekeeping gene as in methods. Duplicate reactions were performed for each experiment and the *values* in each histogram represent mean of fold change ± SEM ($n = 6$). $*p < 0.05$ compared with control (0 μM)

TQ induces oxidative stress in HCC cells

ROS production by many anticancer agents has been reported to be responsible for induction of cancer cell apoptosis in vitro and in vivo [39]. Thus, we next examined whether TQ stimulated ROS production in HCC cells. As shown in Fig. 7a, TQ (6–100 μM) significantly increased the ROS accumulation. Of note is the fact that TQ induced significantly higher levels of ROS at all concentrations tested ($p < 0.05$). The decline of the DCF fluorescence level with the increasing TQ concentrations may be explained by the significant reductions of the cell survival as seen in MTT assays (Fig. 1a). Accordingly, at higher TQ concentrations, reduced ROS levels could be due to reduced number of viable cells.

Similar to other quinones, TQ is considered as a redox-cycler. It has been reported to be metabolized in vivo to semiquinone radical (one-electron reduction) by oxidoreductases such as NADPH-cytochrome c reductase or to dihydrothymoquinone (two-electron reduction) by cellular oxidoreductases such as NAD(P)H:quinone oxidoreductase 1 (NQO1). While the former acts as a pro-oxidant by the generation of toxic ROS, the latter molecule acts as superoxide anion and general free radical scavenger [40, 41]. It appears from the above results that the one-electron reduction mechanism is more predominant in TQ-treated HepG2 cells than the two-electron reduction; thereby semiquinone free radical is generated that, in turn, enhances ROS generation. As noted above, TQ inhibited mRNA expression of Bcl-2. This could be due to elevated ROS generation following TQ exposure, since Bcl-2 has been shown to be down-regulated in response to rapid increases in oxidative stress [42].

Excessive ROS that are generated under certain pathological conditions act as mediators of apoptotic signaling pathway [43]. To further substantiate the involvement of

oxidative stress in TQ-induced apoptosis, we explored the effect of TQ treatment on the expression of oxidative stress genes. HepG2 cells were treated for 6 h with the increasing concentrations of TQ; thereafter, NQO1 and hemeoxygenase 1 (HO-1) mRNA levels were determined by qRT-PCR. Figure 7b shows that TQ at low concentrations (12.5 and 25 μM) induced NQO1 mRNA levels by approximately three- and two-fold, respectively, whereas higher concentrations did not significantly alter NQO1 levels. NQO1, also known as DT-diaphorase, is a cytosolic enzyme that plays an essential role in the detoxification of quinones through two-electron reduction process, thereby protecting cells against ROS, carcinogenicity and other toxicities caused by quinones [44, 45]. TQ has been presumed to promote oxidative/electrophilic stress via quinone-quinol redox cycles [46]. Hence, the induction of NQO1 by TQ at low concentrations seems to be a mechanism by which HepG2 cells would likely protect themselves against TQ-oxidative stress, whereas the significant growth inhibitory effects of the higher TQ concentrations would involve impairment of such induction. NQO1 is known to be highly expressed in tumor compared to normal tissue [47], and HepG2 cells are known to over-express NQO1, relative to primary human hepatocytes [48]. Further, De Haan et al. [49] have reported that there is a critical threshold level of NQO1 above which a further increase does not offer additional protection against quinone cytotoxicity. Therefore, TQ-induced upregulation of NQO1 does not likely protect HepG2 cells from TQ cytotoxicity, since in our study, the TQ concentrations that upregulated NQO1 (12.5 and 25 μM) effectively inhibited HepG2 cell growth (Fig. 1a). On the other hand, TQ induced HO-1 mRNA at all concentrations tested, with the exception that higher concentrations (100 μM) that failed to induce HO-1 mRNA level. As it could be the case for expression of many genes, the effect of TQ on gene transcription was impaired by its cytotoxicity at high concentrations (Fig. 7c). HO-1 is a stress responsive protein that responds to many kinds of chemically or physiologically induced oxidative stress in various cells and tissues [50]. Since HepG2 cells express low levels of HO-1 [51], TQ-dependent induction of HO-1 could likely be a response of the HepG2 cells to TQ-induced oxidative stress. Our results are in agreement with previous reports showing that TQ induces oxidative stress-mediated apoptosis in various cancer cell lines [31, 46].

Conclusion

In the light of our data presented here, we conclude that TQ inhibits HCC cell growth and IL-8 expression by novel mechanisms. TQ also enhanced TRAIL-induced cell death of HCC cells. G2M cell cycle arrest coupled with reduced levels of NF-κB subunits, IL-8, and its receptors, and upregulation of TRAIL death receptors, increased oxidative stress, activation of caspases 9 and 3, and apoptosis contributed to robust inhibition of HCC cell growth in the combined presence of TQ and TRAIL. The fact that NAC abrogated TQ-induced cytotoxicity in HCC cells further substantiate an important role for oxidative stress in TQ-mediated HCC cell growth suppression. Since up-regulation of IL-8 has been associated with a more aggressive HCC phenotype [11], and TQ is shown in this study as a novel regulator of IL-8, we recommend clinical trials to examine the effectiveness of TQ as an adjuvant therapy for HCC and other tumors expressing high levels of IL-8.

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Conflict of interest We have no personal or financial conflict of interest and have not entered into any agreement that could interfere with our access to the data on the research, or upon our ability to analyze the data independently, to prepare manuscripts, and to publish them.

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