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Original Contribution

Thymoquinone suppresses growth and induces apoptosis via generation of reactive oxygen species in primary effusion lymphoma

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ABSTRACT

We provide evidence that thymoquinone (TQ), a natural compound isolated from *Nigella sativa,* induces growth inhibition and apoptosis in several primary effusion lymphoma (PEL) cell lines. Our data demonstrate that TQ treatment results in down-regulation of constitutive activation of AKT via generation of reactive oxygen species (ROS) and it causes conformational changes in Bax protein, leading to loss of mitochondrial membrane potential and release of cytochrome *c* to the cytosol. This leads to activation of caspase-9, caspase-3, and polyadenosine 5′-diphosphate ribose polymerase cleavage, leading to caspase-dependent apoptosis. Pretreatment of PEL cells with *N*-acetylcysteine, a scavenger of ROS, prevented TQ-mediated effects. In addition, subtoxic doses of TQ sensitized PEL cells to TRAIL via up-regulation of DR5. Altogether, these findings demonstrate that TQ is a potent inducer of apoptosis in PEL cells via release of ROS. They also raise the possibility that incorporation of TQ in treatment regimens for primary effusion lymphomas may provide a novel approach to sensitizing malignant cells and provide a molecular basis for such future translational efforts.

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Primary effusion lymphoma (PEL) is a subtype of non-Hodgkin B cell lymphoma that mainly presents in patients with advanced AIDS, but is sometimes also found in human immunodeficiency virus-negative individuals [1,2]. PEL cells grow as a lymphomatous effusion in body cavities and are infected with Kaposi sarcoma-associated herpesvirus (KSHV/HHV8). Most cases show dual infection with Epstein–Barr virus (HHV4) [3]. Pleural and abdominal effusions from patients with AIDS PEL contain a number of cytokines, which serve as autocrine growth factors [4,5]. For example, IL-10 has been reported to serve as an autocrine growth factor for AIDS-related B cell lymphoma [6]. Recently, it has also been shown that PEL cells use viral IL-6 and IL-10 in an autocrine fashion for their survival and proliferation [6].

A number of constitutively activated signaling pathways play critical roles in the survival and growth of PEL cells. These include JAK/STAT, NF-κB, and PI3-kinase [7–10]. The KSHV protein K1 has been shown to regulate a number of survival proteins. Studies have shown that transgenic mice with K1 showed constitutive activation of NF-κB and Oct-2 as well as activation of Src-kinase Lyn [11]. Furthermore, K1 protein activates the PI3-kinase/AKT pathway in B lymphocytes and protects cells from FKHR- and FAS-mediated apoptosis [12]. A study by Cannon et al. also demonstrated that vGPCR induces activation of the transcriptional factors AP1 and CREB in PEL cells [13].

Thymoquinone (TQ) is a bioactive compound isolated from *Nigella sativa* Linn [14]. Extracts prepared from *N. sativa,* known as black seed, have been used for medical purposes for centuries for a number of diseases [14,15]. TQ is the active ingredient of black seed and has been shown to possess antitumor activities against a broad spectrum of cancer cells, including colon, ovarian, lung, osteosarcoma, and myeloblastic leukemia [16–20]. TQ has also been shown to inhibit chemically induced carcinogenesis in mice [21,22]. More recently, it was demonstrated that TQ augments the antitumor activity of standard cancer chemotherapeutic agents in pancreatic cancer cells in vitro and in vivo [23].

In this study, we investigated the antitumor activity of TQ against human primary effusion lymphoma cell lines. Our data provide the first evidence that TQ induces apoptosis of PEL cells via a mechanism involving generation of reactive oxygen species (ROS). We also examined the effects of TQ on extrinsic and intrinsic apoptotic pathways. Finally, we studied the effects of TQ in combination with TRAIL on PEL cell lines. Altogether, our data establish TQ as a potent inducer of apoptosis in PEL cells and identify the mechanisms by which such apoptosis is induced.

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Materials and methods

Cell culture

The human PEL cell lines BC-1, BC-3, BCBL-1, and HBL-6 were cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin, 100 U/ml streptomycin at 37 °C in an humidified atmosphere containing 5% CO_2. All the experiments were performed in RPMI 1640 containing 5% serum.

Reagents and antibodies

Thymoquinone, *N*-acetylcysteine (NAC), and Bax 6A7 monoclonal antibody were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Caspase-9 antibody and zVAD-fmk were purchased from Calbiochem (San Diego, CA, USA). Antibodies against phosphorylated (p) AKT, AKT, p-Bad, and cleaved caspase-3 were purchased from Cell Signaling Technologies (Beverly, MA, USA). Cytochrome *c*, β-actin, caspase-3, and polyadenosine 5′-diphosphate ribose polymerase (PARP) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). DR5 antibody was purchased from Cayman Laboratories (Ann Arbor, MI, USA). TUNEL assay kit was obtained from MBL (Watertown, MA, USA). Annexin V was purchased from Molecular Probes (Eugene, OR, USA). Apoptotic DNA ladder kit was obtained from Roche (Penzberg, Germany).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays

The antiproliferative effects of TQ against various PEL cell lines were determined by the MTT dye-uptake method as described earlier [24]. Briefly, 10^4 cells were incubated in triplicate in a 96-well plate in the presence or absence of the indicated test doses of TQ in a final volume of 0.20 ml for 24 h at 37 °C. Thereafter, 25 μl MTT solution (5 mg/ml in water) was added to each well. After 24 h incubation at 37 °C, 0.1 ml extraction buffer (20% SDS) was added, incubation was continued overnight at 37 °C, and then the optical density (OD) at 590 nm was measured. Cell viability was calculated as OD of the experiment samples/OD of the control (untreated) × 100. Replicates of 6 wells for each dosage including vehicle control were analyzed for each experiment.

Cell cycle analysis

Cell lines were treated with or without TQ for 24 h and the cells were washed once with phosphate-buffered saline (PBS) and resuspended in 500 μl hypotonic staining buffer (sodium citrate 250 mg, Triton X-100 0.75 ml, propidium iodide 25 μg, ribonuclease A 5 μg, and water 250 ml) and analyzed by flow cytometry as described previously [25].

TUNEL assay

Cell lines were treated with TQ as described in the legends. Apoptotic cells were measured using the TUNEL assay as described earlier [26]. Briefly, after 24 h of treatment with various concentrations of TQ, 1×10^6 cells were washed twice with PBS containing 0.2% bovine serum albumin (BSA) and fixed with 4% paraformaldehyde at 4 °C for 30 min. This was followed by two washes with PBS containing 0.2% BSA and the cells were permeabilized in 70% ethanol at −20 °C for 30 min. The cells were then washed twice and incubated with 30 μl TdT buffer (TdT buffer II, FITC–dUTP, and TdT in the ratio of 18:1:1) for 1 h at 37 °C. This was followed by two washes with PBS and the cells were resuspended in 500 μl of PBS. Stained cells were analyzed using FACScan flow cytometry equipped with a CellQuest data analysis program (Beckon–Dickinson, San Diego, CA, USA).

Annexin V staining

PEL cell lines were treated with various concentrations of TQ as described in the legends. Cells were harvested and the percentage apoptosis was measured by flow cytometry after staining with fluorescein-conjugated annexin V and propidium iodide (PI; Molecular Probes) as described previously [27]. We scored viable cells as those that are negative for annexin V and PI. Percentage of apoptosis was calculated from the reduction in the number of viable cells between the treated and the untreated samples. The amount of necrotic cells (annexin V negative, PI positive) was always minimal.

Measurement of ROS

ROS production was detected using dihydroethidium and high-performance liquid chromatography (HPLC) as described earlier [28,29]. Briefly, exponentially growing cells were treated with TQ alone or in the presence of 10 mM NAC for the indicated time periods. After treatment, the cells were washed three times with chilled Krebs–Hepes buffer. The cells were then exposed to 25 μM dihydroethidium dissolved in Krebs–Hepes buffer containing 0.1% dimethyl sulfoxide for 20 min at 37 °C. The cells were washed with Krebs–Hepes buffer and incubated for 1 h in Krebs–Hepes buffer at 37 °C. The cells were then spun at 1000 rpm for 10 min and resuspended in ice-cold methanol, homogenized, and filtered through a 0.22-μm filter. Separation of ethidium, oxyethidium, and dihydroethidium was performed using an Atlantis dC18 (4.6 × 150 mm, 5 μm) column equipped with a Waters Alliance 2695 separations module (Waters Associates, Milford, MA, USA).

Soft-agar colony assays

Soft-agar colony experiments were performed according to the manufacturer's protocol (Chemicon International, Temecula, CA, USA). Briefly, after treatment with TQ in the presence or absence of NAC, 2500 cells were plated in 0.5 ml culture medium containing 0.4% (v/v) top agar and 20% FBS layered over a basal layer of 0.8% (v/v) agar and 20% FBS in culture medium and allowed to grow for 4 weeks. After 4 weeks incubation, the cells were stained at a final concentration of 1 mg/ml with cell staining solution that was supplied with the kit.

Cell lysis and immunoblotting

Cells were treated with TQ as described in the legends and lysed as previously described [30]. Briefly, cell pellets were resuspended in phosphorylation lysis buffer (0.5–1.0% Triton X-100, 150 mM NaCl, 1 mM EDTA, 200 μM sodium orthovanadate, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 1.5 mM magnesium chloride, 1 mmol/L phenylmethylsulfonyl fluoride, and 10 μg/ml aprotinin). Protein concentrations were assessed by Bradford assay before the samples were loaded. Equal amounts of proteins were separated by SDS–PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon; Millipore, Billerica, MA, USA). Immunoblotting was performed with various antibodies and visualized using an enhanced chemiluminescence (Amersham, Arlington Heights, IL, USA) method.

Detection of Bax conformational changes

This assay was performed as described previously [31]. Briefly, after treatment with the indicated reagents for the indicated times, cells were harvested and washed with PBS, after which they were lysed with Chaps lysis buffer (10 mM Hepes, pH 7.4, 150 mM NaCl, 1% Chaps) containing protease inhibitors as described. Protein concentrations were assessed by Bradford assay and 500 μg of total protein was incubated with 2 μg of anti-Bax 6A7 monoclonal antibody for 2 h at 4 °C. After incubation, 25 μl of protein G beads was added into the reaction and incubated at 4 °C overnight on a shaker with gentle agitation. After washes in lysis buffer,

the samples were separated by SDS–PAGE, transferred, and immunoblotted using N20 Bax polyclonal antibody.

Assay for cytochrome c release

Release of cytochrome *c* from mitochondria was assayed as described earlier [32]. Briefly, cells were treated with and without TQ as described in the figure legends, harvested, resuspended in 5 volumes of a hypotonic buffer (20 mM Hepes–KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 20 μg/ml leupeptin, 10 μg/ml aprotinin, 250 mM sucrose), and incubated for 15 min on ice. The cells were homogenized by 15–20 passages through a 22-gauge needle, 1.5 in. long. The lysates were then centrifuged at 1000*g* for 5 min at 4 °C to pellet nuclei and unbroken cells. Supernatants were collected and centrifuged at 12,000*g* for 15 min. The resulting mitochondrial pellets were suspended in lysis buffer. Supernatants were transferred to new tubes and centrifuged again at 12,000*g* for 15 min and the resulting supernatants representing cytosolic fractions were separated. Twenty to twenty-five micrograms of proteins from the cytosolic fraction of each sample was analyzed by immunoblotting using an anti-cytochrome *c* antibody.

Measurement of mitochondrial potential using the JC-1 (5,5′,6,6′-teterachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide) assay

Cells (1×10^6) were treated with TQ for 24 h. The cells were washed twice with PBS and suspended in mitochondrial incubation buffer (Alexis Corp., Farmingdale, NY, USA). JC-1 was added to a final concentration of 10 μM and the cells were incubated at 37 °C in the dark for 15 min. The cells were washed twice with PBS and resuspended in 500 μl of mitochondrial incubation buffer and the mitochondrial membrane potential (% of green and red aggregates) was determined by flow cytometry as described previously [33].

Gene silencing using small interfering RNA (siRNA)

DR5 siRNA (Cat. Nos. S100056707 and S100056700 pooled) and scrambled control (Cat. No. 102781) siRNA were purchased from Qiagen (Valencia, CA, USA). For transient expression, cell lines were transfected using LipofectAMINE 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After the cells were incubated for 6 h, the lipid and siRNA complex was removed and fresh growth medium was added. Cells were treated 48 h after transfection for 24 h and specific protein levels were determined by Western blot analysis with specific antibodies against the targeted proteins and actin as a loading control.

Statistical analysis

Data are presented as means ± SD. Comparisons between groups were made with the paired Student *t* test. Values of $p<0.05$ were considered statistically significant.

Results

TQ causes a dose-dependent inhibition of proliferation and apoptosis of PEL cells

We sought to determine whether TQ treatment leads to inhibition of proliferation of various PEL cell lines. BC1, BC3, BCBL1, and HBL6 cells were cultured in the presence and absence of TQ at various concentrations (10, 25, 50 μM) for 24 h and proliferation was assessed using MTT assays. Fig. 1A shows that as the dose of TQ increased from 10 to 50 μM, cell growth inhibition increased in a dose-dependent fashion in all the PEL cell lines. The growth inhibition induced by TQ treatment was statistically significant ($p<0.001$) at most of the doses tested in all cell lines. In subsequent experiments, we sought to determine whether the suppressive effects of TQ on PEL cells reflect an induction of cell cycle arrest and/or apoptosis. PEL cells were treated with TQ for 24 h. Cells were stained and cell cycle fractions were examined by flow cytometry. As shown in Fig. 1C, the sub-G1 population of cells increased from 6.1% in control to 68.71% in TQ-treated BC1 cells. Similar results were obtained in BC3 cells (4.57 to 42.27%) and in HBL6 cells (3.89 to 49.35%). The increase in sub-G1 population was accompanied by decreases in cells in the G0/G1, S, and G2/M phases in TQ-treated PEL cells, features consistent with induction of apoptosis [30–34].

To further confirm that the increase in the sub-G1 population reflects induction of apoptosis, PEL cells were treated with 10 and 25 μM TQ and apoptosis induction was measured by annexin V/PI dual staining. As shown in Fig. 1B, treatment of PEL cells with TQ resulted in dose-dependent apoptosis in all cell lines. We also used DNA laddering assays for further confirmation of TQ-induced apoptosis in PEL cells. BC3 and BCBL1 cells were treated with TQ for 24 h and DNA was isolated. As shown in Fig. 1D, TQ caused a dose-dependent fragmentation of DNA, a characteristic of apoptotic cell death. Finally, trypan blue exclusion studies (Supplementary Fig. 1A) and TUNEL assays as shown in Supplementary Fig. 1B also indicated that TQ treatment in PEL cell lines resulted in apoptosis in a dose-dependent manner.

To confirm whether TQ is nontoxic to normal cells, we isolated peripheral blood mononuclear cells from whole blood of five normal individuals and treated them with 10 and 25 μM TQ for 24 h. The cells were harvested and stained with fluorescein-conjugated annexin V/PI and analyzed by flow cytometry. As shown in Supplementary Fig. 1C, the percentage of apoptosis ranged between 6 and 11%, suggesting that TQ is essentially nontoxic to normal cells in vitro. Recently, Banerjee et al. [23] also showed that TQ can be safely used in SCID mice at doses of 3 mg in combination with chemotherapeutic drugs without causing systemic toxicity. These data clearly suggest that TQ can be safely used in vivo without causing systemic toxicity.

We also confirmed that thymoquinone treatment was cytotoxic to PEL cells by clonogenic assay. The BC1 cell line was treated with 25 μM TQ and the cells were plated in agarose plates as described under Materials and methods. As shown in Supplementary Figs. 2A and B, TQ treatment efficiently blocked formation of colonies at 25 μM concentration, suggesting that TQ treatment blocks colony formation in PEL cell lines. Similar results were obtained with other PEL cell lines (data not shown).

TQ inhibits constitutive activation of PKB/AKT signaling pathways in PEL cells

In earlier studies, we had demonstrated that the AKT pathway is activated in PEL cells [9,24]. We sought to determine whether TQ modulates the AKT pathway. Using an antibody that recognizes the phosphorylated form of AKT on its activation site, serine 473, we sought to determine the effects of TQ on the constitutive activation status of AKT in PEL cell lines. BC1, BC3, and BCBL1 cells were treated in the presence or absence of 10 and 25 μM TQ for 24 h; the cells were lysed and proteins were separated by SDS–PAGE and immunoblotted with the anti-phospho-AKT antibody. As shown in Fig. 2A, TQ treatment resulted in suppression of AKT phosphorylation in all cell lines tested, raising the possibility that a major mechanism of TQ-mediated apoptosis of PEL cells involves AKT targeting.

Previous work in other systems has shown that the forkhead family of transcription factors are downstream targets of AKT and mediators of apoptosis [33]. Active FKHR transcription factors promote the transcription of genes involved in cell cycle arrest and apoptosis [35]. One mechanism by which AKT promotes cell survival is by phosphorylating FKHR transcription factors, which inactivates them and prevents apoptosis [35]. We thus studied the level of phosphorylation FKHR/FOXO1 in TQ-treated and untreated PEL cell lines. As shown in Fig. 2A,

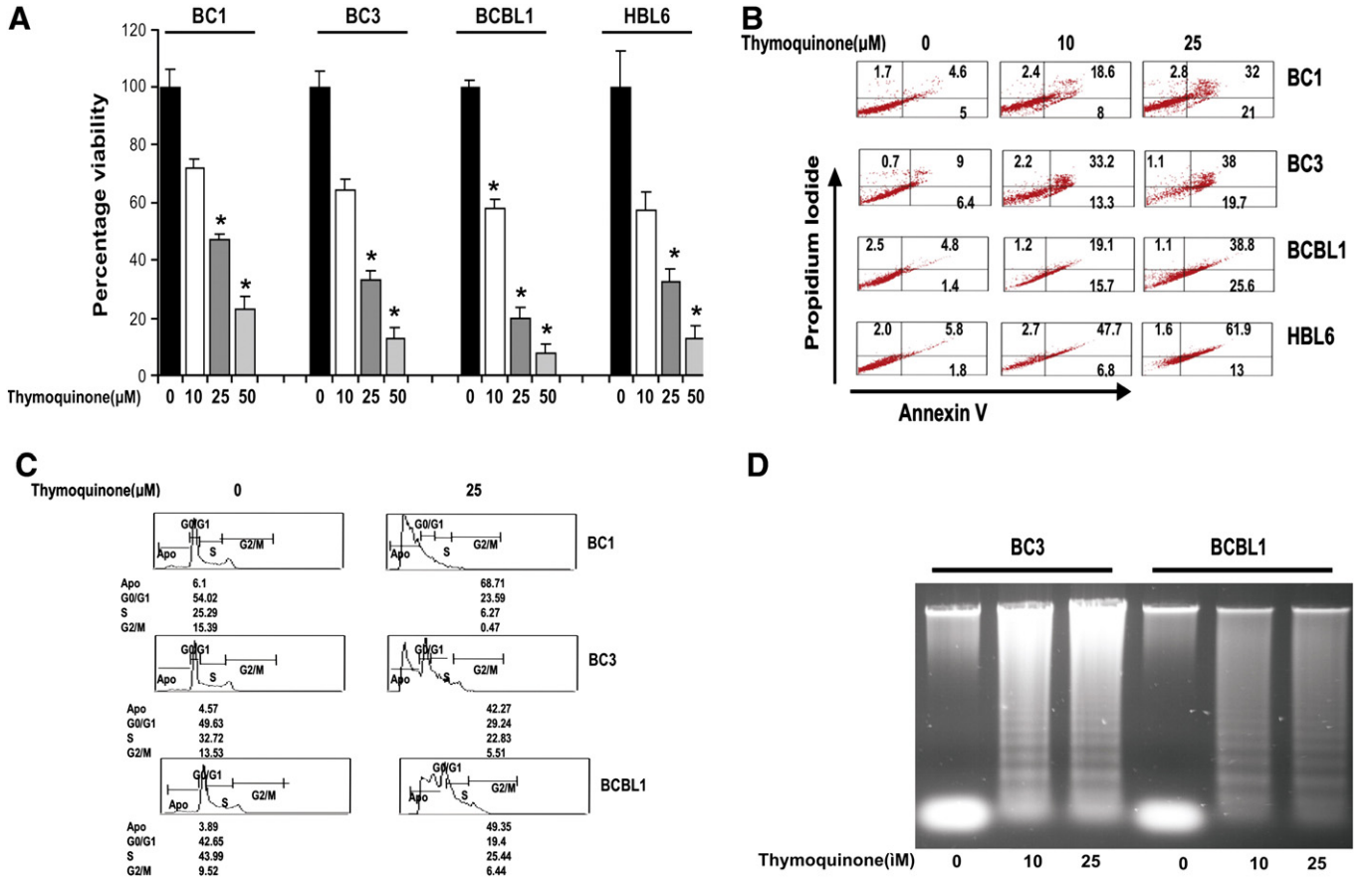

Fig. 1. (A) Thymoquinone inhibits the proliferation of PEL cells. BC1, BC3, BCBL1, and HBL6 cells were incubated with 10, 25, and 50 μM thymoquinone for 24 h. Cell proliferation assays were performed using MTT as described under Materials and methods. The graph displays the means ± SD (standard deviation) of three independent experiments with replicates of six wells for all the doses and vehicle control for each experiment. *$p<0.01$, statistically significant (Student's t test). (B) Thymoquinone-induced apoptosis detected by annexin V/PI dual staining. BC1, BC3, BCBL1, and HBL6 cells were treated with 10 and 25 μM thymoquinone for 24 h and cells were subsequently stained with fluorescein-conjugated annexin V and PI and apoptotic cells were analyzed by flow cytometry. A representative of four independent experiments is depicted. (C) Cell cycle analysis of PEL cells after thymoquinone treatment. BC1, BC3, and BCBL1 cells were treated with 25 μM thymoquinone for 24 h. Thereafter, the cells were washed, fixed, and stained with propidium iodide and analyzed for DNA content by flow cytometry as described under Materials and methods. A representative of four independent experiments is depicted. (D) Thymoquinone-induced DNA fragmentation of PEL cells. BC3 and BCBL1 cells were treated with the indicated doses of thymoquinone for 24 h and DNA was extracted and separated by electrophoresis on a 1.5% agarose gel.

constitutive phosphorylation of FKHR was seen in all PEL cells; this phosphorylation was inhibited by treatment with TQ.

We next determined the effects of TQ on the activation of GSK3, a kinase previously shown to be regulated by the PI3-kinase/AKT pathway, which plays an important role in the promotion of cell survival [9,24,36]. All PEL cell lines showed constitutive phosphorylation of GSK3, and TQ treatment caused dephosphorylation in all cell lines in a dose-dependent manner (Fig. 2A), further suggesting that AKT and its downstream effectors play important roles in TQ-induced apoptosis in PEL cell lines.

Another downstream target of AKT is the proapoptotic protein Bad [37]. Bad promotes cell death by interacting with antiapoptotic Bcl-2 members such as Bcl-xL, which allows the multidomain proapoptotic Bcl-2 family members Bax and Bak to aggregate and cause release of apoptogenic molecules (e.g., cytochrome c) from mitochondria to the cytosol, culminating in caspase activation and cell death [38]. TQ treatment of PEL cells also caused dephosphorylation of Bad (Fig. 2A), thereby allowing the apoptotic signal to reach the mitochondria.

TQ-induced ROS generation inhibits constitutive activation of p-AKT in PEL cells

A number of compounds used in chemotherapy induce cell death through the generation of ROS [39]. We examined whether ROS were also generated in PEL cells treated with TQ for various time periods and, if so, whether this is a mechanism for induction of TQ-induced apoptosis. Therefore, we used a dihydroethidium-based HPLC assay for detection of ROS release. As shown in Fig. 2B, TQ-mediated release of ROS increased in a time-dependent manner in BC1 cells treated with 25 μM TQ, starting as early as 2 h after treatment, peaking at 4 h, and diminishing after 8 h treatment with TQ (Fig. 2B). NAC is a widely used thiol-containing antioxidant that scavenges ROS in cells, thus affecting ROS-mediated signaling pathways. To confirm ROS release after treatment with TQ, we pretreated PEL cell lines with 10 mM NAC for 2 h followed by treatment with TQ for 24 h. As shown in Fig. 2C, the TQ-induced increase in ROS levels was blocked by pretreatment with NAC in PEL cell lines, demonstrating a requirement for ROS in the process. To confirm these data, we pretreated PEL cells with two additional inhibitors of ROS release, PEG–superoxide dismutase (SOD) and PEG–catalase, either alone or in combination, for 2 h followed by treatment with 25 μM TQ for 24 h. As shown in Supplementary Fig. 2C, TQ induced 65% apoptosis in PEL cells; however, pretreatment with NAC, PEG–catalase, and PEG–SOD followed by TQ treatment decreased the percentage of apoptosis to 20, 42, and 46%, respectively. Interestingly, pretreatment of BC1 cells with PEG–SOD and PEG–catalase together prevented TQ-mediated apoptosis (24%), suggesting the involvement of superoxide and hydrogen peroxide in TQ-induced apoptosis in PEL cells. We finally sought to determine whether inactivation of AKT and its downstream targets after TQ treatment is a direct consequence of ROS release. We pretreated BC1 cells with

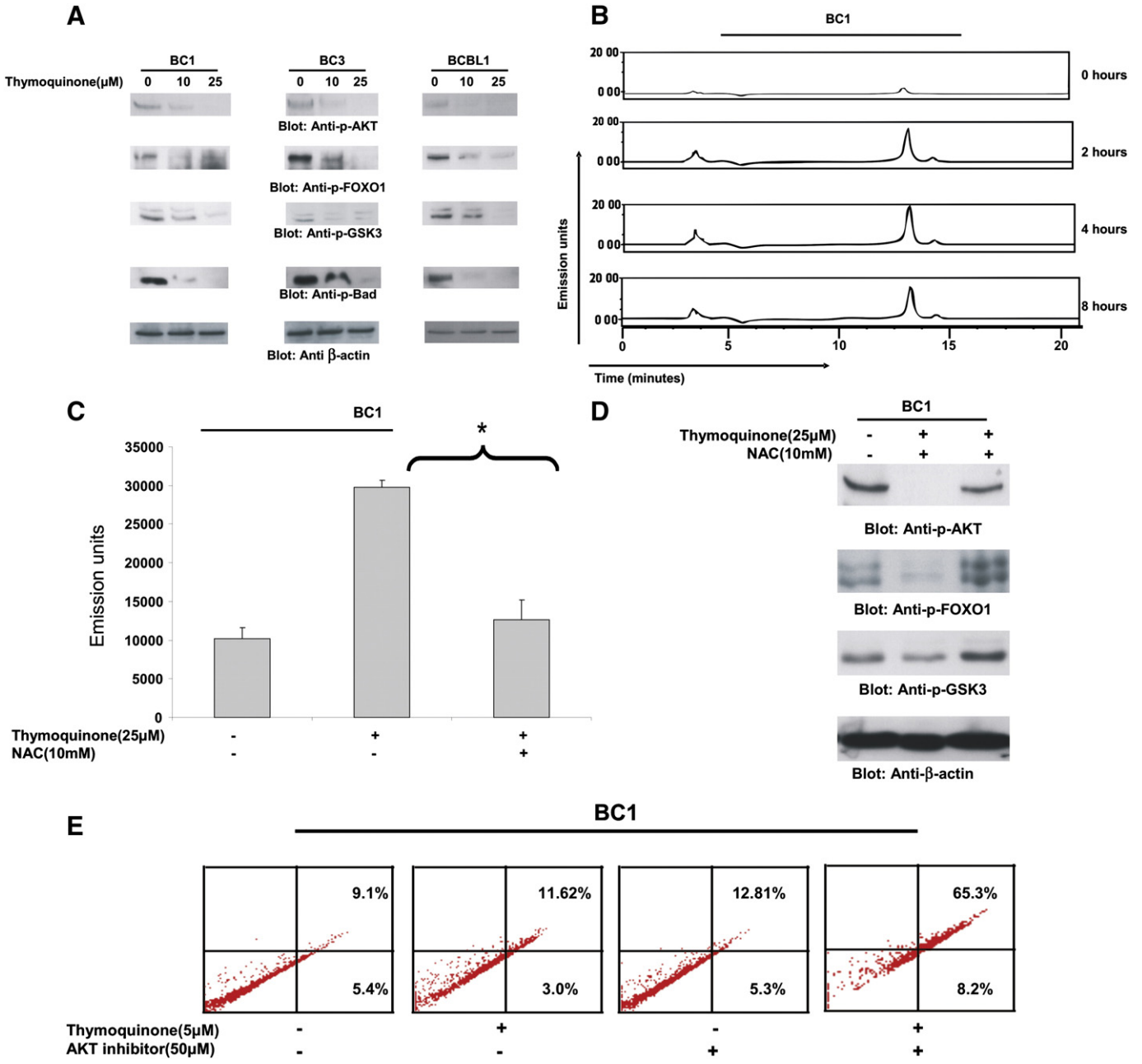

Fig. 2. (A) Thymoquinone treatment dephosphorylates AKT and its downstream targets in PEL cells. BC1, BC3, and BCBL1 cells were treated with 10 and 25 μM thymoquinone for 24 h. After cell lysis, equal amounts of proteins were separated by SDS–PAGE, transferred to Immobilon membrane, and immunoblotted with antibodies against p-AKT, p-FOXO1, p-GSK3, p-Bad, and β-actin as indicated. (B) Thymoquinone increases ROS generation in PEL cells. BC1 cells were incubated in the absence or presence of 25 μM thymoquinone for the indicated time periods. After being washed with Krebs–Hepes buffer, cells were incubated with 25 μM dihydroethidium as described under Materials and methods. Cells were resuspended in methanol, homogenized, filtered, and immediately analyzed using a Waters Alliance 2695 separations module. (C) NAC inhibits release of ROS in thymoquinone-treated PEL cells. BC1 cells were pretreated with 10 mM NAC for 2 h followed by treatment with 25 μM thymoquinone for 24 h. After being washed with Krebs–Hepes buffer, the cells were incubated with 25 μM dihydroethidium as described under Materials and methods. Cells were resuspended in methanol, homogenized, filtered, and immediately analyzed by HPLC, and fluorescence detector response (emission unit) was plotted as a bar graph. *Statistically significant ($p<0.05$). (D) Effect of NAC on thymoquinone-induced dephosphorylation of AKT and its downstream targets in PEL cells. BC1 cells were pretreated with 10 mM NAC for 2 h followed by treatment with 25 μM thymoquinone for 24 h. After cell lysis, equal amounts of proteins were separated by SDS–PAGE, transferred to Immobilon membrane, and immunoblotted with antibodies against p-AKT, p-FOXO1, p-GSK3, and β-actin as indicated. (E) TQ and AKT inhibitor synergistically induce apoptosis in PEL cells. BC1 cells were treated with 5 μM thymoquinone in the presence or absence of 50 μM AKT inhibitor for 24 h. After treatment, the cells were stained with fluorescence-conjugated annexin V/PI and analyzed by flow cytometry.

10 mM NAC for 2 h and subsequently treated them with 25 μM TQ for 24 h. The cells were lysed and proteins were separated on SDS–PAGE and immunoblotted with p-AKT, p-FOXO1, and p-GSK3 antibodies. As shown in Fig. 2D, pretreatment of BC1 cells with NAC abrogated TQ-induced inhibition of constitutively active p-AKT, p-FOXO1, and p-GSK3, strongly suggesting that ROS regulate the TQ-mediated suppression of AKT activity, directly or indirectly.

We also investigated whether antioxidant protection prevented TQ-mediated inhibition of clonogenic survival of PEL cells. BC1 cells were either pretreated with 10 mM NAC for 2 h followed by treatment with 25 μM TQ and cells were plated for colony formation. After 4 weeks, cells were stained and analyzed. As shown in Supplementary Figs. 2A and B, TQ treatment inhibited colony formation; however, pretreatment with NAC salvaged BC1 cells from TQ-induced inhibition

of colony formation. These data clearly suggest that TQ-induced cytotoxicity in PEL cells is ROS dependent.

To better understand the role of TQ-induced inactivation of AKT leading to apoptosis, we treated PEL cell lines with either an AKT direct inhibitor (50 μM) or TQ (5 μM) alone at suboptimal doses or a combination of both the inhibitors for 24 h, after which the cells were stained with fluorescent annexin V/PI and analyzed for apoptosis. As shown in Fig. 2E, neither AKT inhibitor nor TQ could induce apoptosis alone; however, a synergistic apoptotic response was recorded when both the drugs were given together. These data clearly suggest that TQ was acting via the AKT pathway and synergized the action of the AKT inhibitor to induce apoptosis.

TQ treatment suppresses Bcl-2 protein expression and increases Bax:Bcl-2 ratio in PEL cells

The Bcl-2 family of proteins plays a central regulatory role via its interacting pro- and antiapoptotic members that integrate a wide variety of upstream survival and distress signals to decide the fate of the cells [40]. We therefore evaluated the effect of TQ treatment on the expression of two major members of the Bcl-2 family of proteins, Bax and Bcl-2, in BC1 cell line. As shown in Fig. 3A, TQ treatment caused a time-dependent decrease in Bcl-2 expression as well as an increase in Bax protein level. Densitometric analysis revealed an increase in the Bax:Bcl-2 ratio (Fig. 3A), which has been previously shown to correlate with apoptosis [24].

TQ-mediated ROS release causes Bax conformational changes leading to activation of mitochondrial apoptotic pathway

As it is known that dephosphorylation of Bad as well as down-regulation of Bcl-2 plays an important role in activation of Bax and apoptosis, we examined the effects of TQ treatment on Bax activation and determined whether it correlates with release of ROS or caspase activation. BC1 and BC3 cells were pretreated with either NAC or zVAD-fmk for 2 h followed by treatment with 25 μM TQ. As seen in Fig. 3B, NAC pretreatment blocked conformational changes in Bax protein, whereas zVAD-fmk pretreatment was unable to inhibit such changes, indicating that Bax activation is due to TQ-mediated ROS release and upstream of caspase activity.

In subsequent studies, we sought to determine the effects of TQ on mitochondrial membrane potential. PEL cells were treated with TQ for 24 h and labeled with JC-1 dye, and mitochondrial membrane potential was measured by flow cytometry. As shown in Fig. 3C, TQ treatment of PEL cells resulted in loss of mitochondrial membrane potential as measured by JC-1-stained green fluorescence depicting apoptotic cells. To test whether TQ-mediated apoptosis caused release of cytochrome *c* from mitochondria in PEL cells, BC1 and BC3 cells were treated in the presence or absence of TQ for 24 h and cytosol-specific, mitochondria-free lysates were prepared as described under Materials and methods. Our data demonstrate that cytochrome *c* was released into the cytosol after TQ treatment (Fig. 3D), supporting the notion that TQ-induced apoptosis in PEL cells involves signaling at the mitochondrial level.

TQ-induced signaling causes caspase-9/3 activation and PARP cleavage in PEL cells

Because caspases are important mediators of apoptosis induced by various apoptotic stimuli [41], we investigated whether TQ treatment also caused their activation. BC1 and BC3 cells were treated with 10 and 25 μM TQ for 24 h and immunoblotted with anti-caspase-9, anti-caspase-3, and anti-PARP antibodies. As shown in Fig. 4A, TQ treatment of PEL cells induced activation and cleavage of caspases-9

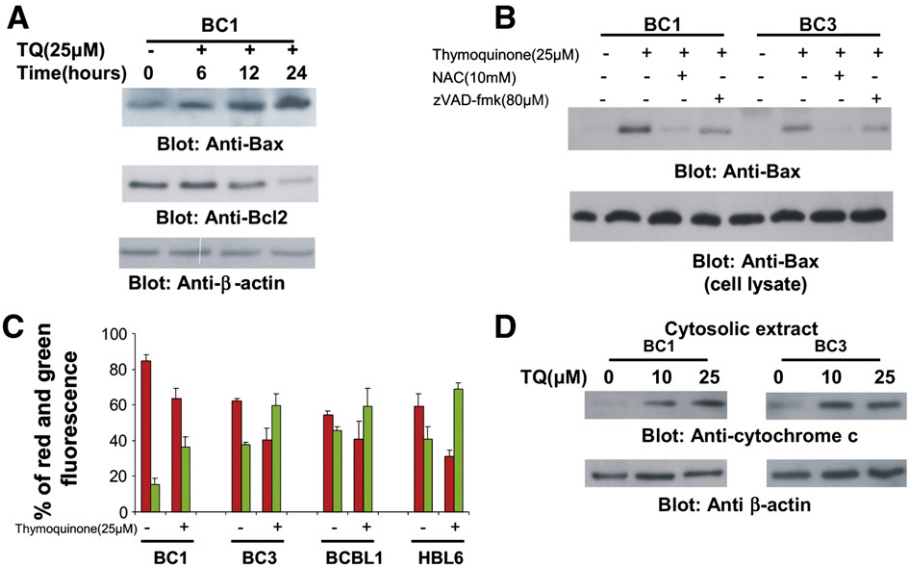

Fig. 3. Thymoquinone-induced mitochondrial signaling pathway in PEL cells. (A) Thymoquinone treatment causes alterations in Bax:Bcl-2 ratio. BC1 cells were treated with 25 μM thymoquinone for various time periods. Cells were lysed and equal amounts of proteins were separated by SDS–PAGE, transferred to Immobilon membrane, and immunoblotted with antibodies against Bax, Bcl-2, and β-actin as indicated. The data obtained from the immunoblot analyses of Bax and Bcl-2 were used to evaluate the effect of thymoquinone on Bax:Bcl-2 ratio. The densitometric analysis of the Bax and Bcl-2 bands was performed using AlphaImager Software (San Leandro, CA, USA), and the data (relative density normalized to β-actin) were plotted as Bax:Bcl-2 ratio. (B) Effects of NAC and zVAD-fmk on thymoquinone-induced Bax activation in BL cells. BC1 and BC3 cells were pretreated with either 10 mM NAC or 80 μM zVAD-fmk for 2 h and subsequently treated with 25 μM thymoquinone for 8 h. Cells were lysed with 1% Chap lysis buffer and the lysates were immunoprecipitated with anti-Bax 6A7 antibody. The proteins were separated by SDS–PAGE, transferred to PVDF membrane, and immunoblotted with Bax rabbit polyclonal antibody. Cell lysates were also immunoblotted to ensure equal loading. (C) Loss of mitochondrial membrane potential by thymoquinone treatment of PEL cells. PEL cells were treated with and without 25 μM thymoquinone for 24 h. Live cells with intact mitochondrial membrane potential and dead cells with lost mitochondrial membrane potential was measured by JC-1 staining and analyzed by flow cytometry as described under Materials and methods. The graph displays the means ± SD (standard deviation) of three independent experiments. (D) Thymoquinone-induced release of cytochrome *c*. BC1 and BC3 cells were treated with and without 10 or 25 μM thymoquinone for 24 h. Mitochondrial free cytosolic fractions were isolated as described under Materials and methods. Cell extracts were separated on SDS–PAGE, transferred to PVDF membrane, and immunoblotted with an antibody against cytochrome *c*. The blots were stripped and reprobed with an antibody against actin for equal loading.

and -3. As expected, PARP was also cleaved, a hallmark of cells undergoing apoptosis. To address whether ROS release plays a role in TQ-mediated caspase activation, BC1 and BCBL1 cells were pretreated with NAC or zVAD-fmk for 2 h, followed by treatment with TQ for 24 h. As shown in Fig. 4B, pretreatment of PEL cells with NAC or zVAD-fmk abrogated caspase and PARP activation induced by TQ, clearly indicating that caspases play a critical role in TQ-induced apoptosis via release of ROS in PEL cells.

TQ-induced ROS generation regulates up-regulation of DR5

Recent studies have shown that DR5 expression is up-regulated by ROS generated by a number of compounds [30,42]. In view of these findings, we sought to determine whether TQ-generated free radicals modulate the expression of DR5 in PEL cells. First, we carried out immunoblotting experiments to investigate the induction of DR4 and DR5 proteins by TQ. BC1 cells were treated with TQ for various time periods and cell lysates were separated by SDS–PAGE and immunoblotted with an antibody against DR5. As shown in Fig. 5A, TQ treatment of PEL cells selectively up-regulated DR5 in a time-dependent manner. When the blots were probed with an antibody against DR4, no increases in protein levels were detectable (data not shown). We then examined whether TQ treatment of PEL cells regulated DR5 expression at the transcriptional level. RT-PCR analysis demonstrated that TQ enhanced DR5 up-regulation at the mRNA level in a time-dependent manner (data not shown), indicating a transcriptional regulatory mechanism. To investigate whether ROS generation is directly associated with TQ-induced DR5 up-regulation, we assessed DR5 expression in the BC1 cell line pretreated with either NAC or 80 μM zVAD-fmk for 2 h followed by treatment with TQ. Treatment with TQ significantly increased DR5 protein levels, whereas pretreatment with NAC markedly inhibited TQ-induced DR5 up-regulation (Fig. 5B). Interestingly, pretreatment with the pancaspase inhibitor zVAD-fmk followed by TQ treatment did not alter the expression level of DR5, suggesting no active role of caspases in the regulation of DR5 expression. Taken together, these data clearly indicate that ROS generation is critical for TQ-induced DR5 up-regulation.

Up-regulation of DR5 does not play a role in TQ-induced apoptosis

DR5 up-regulation is important in activating the extrinsic pathway of apoptosis; however, its role in TQ-induced apoptosis of PEL cells has not been elucidated. To address this issue, BC1 and BC3 cells were transfected with either scrambled siRNA or siRNA specific for DR5 and, after treatment with TQ, apoptosis was evaluated by annexin V/PI dual staining. Surprisingly, silencing of DR5 by siRNA did not prevent the apoptotic effect of TQ in PEL cells (Fig. 5C). In addition, down-regulation of TQ-induced DR5 by siRNA transfection also did not prevent the activation of caspases-8 and -3 as well as cleavage of PARP, strongly suggesting that even though TQ causes up-regulation of DR5, DR5 does not play an active role in the TQ-induced apoptosis in PEL cell lines (Fig. 5D).

TQ sensitizes TRAIL-mediated apoptosis in PEL cell lines

TQ treatment at higher doses has a potent apoptotic effect in PEL cell lines; however, lower doses of TQ failed to induce apoptosis alone. To examine whether up-regulation of DR5 in PEL cell lines by TQ resulted in augmentation of apoptosis when combined with TRAIL, BC1 and BC3 cells were treated with subtoxic doses of TQ and TRAIL either alone or in combination for 24 h and apoptosis was assessed by flow cytometry. Figs. 5E and F show that neither TQ at 5 μM nor TRAIL (25 and 50 ng) treatment alone induced apoptosis in PEL cells.

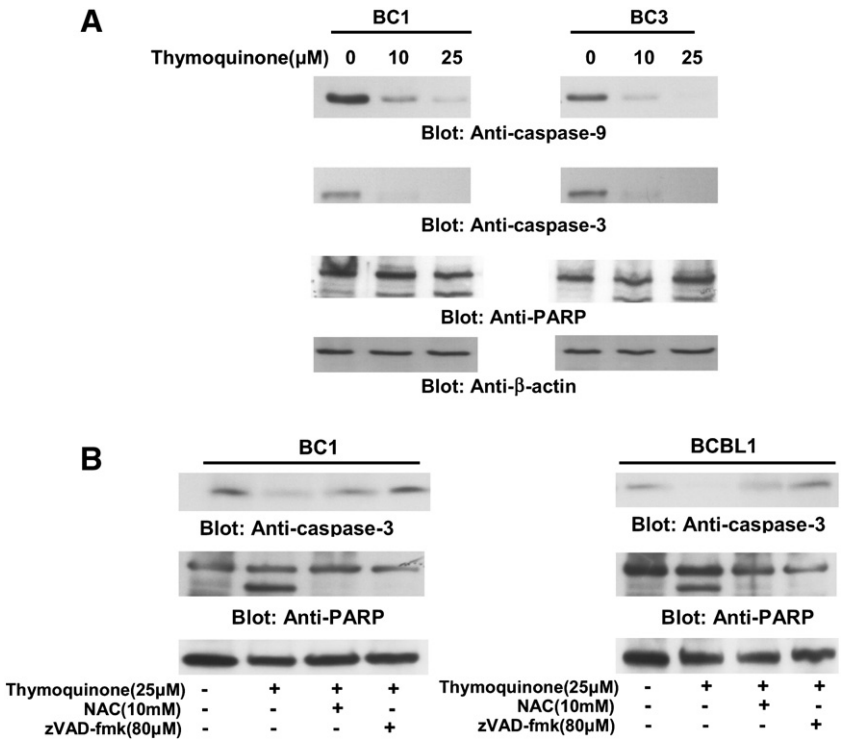

Fig. 4. (A) Activation of caspases-9 and -3 and cleavage of PARP induced by thymoquinone treatment in PEL cells. BC1 and BC3 cells were treated with and without 10 and 25 μM thymoquinone for 24 h. The cells were lysed and equal amounts of proteins were separated by SDS–PAGE, transferred to PVDF membrane, and immunoblotted with antibodies against caspase-9, caspase-3, PARP, and β-actin. (B) Effects of NAC and zVAD-fmk on thymoquinone-induced activation of caspase-3 and PARP. BC1 and BCBL1 cells were pretreated with either 10 mM NAC or 80 μM zVAD-fmk for 2 h and subsequently treated with 25 μM thymoquinone for 24 h. Cells were lysed and equal amounts of proteins were separated by SDS–PAGE, transferred to PVDF membrane, and immunoblotted with antibodies against caspase-3, PARP, and β-actin.

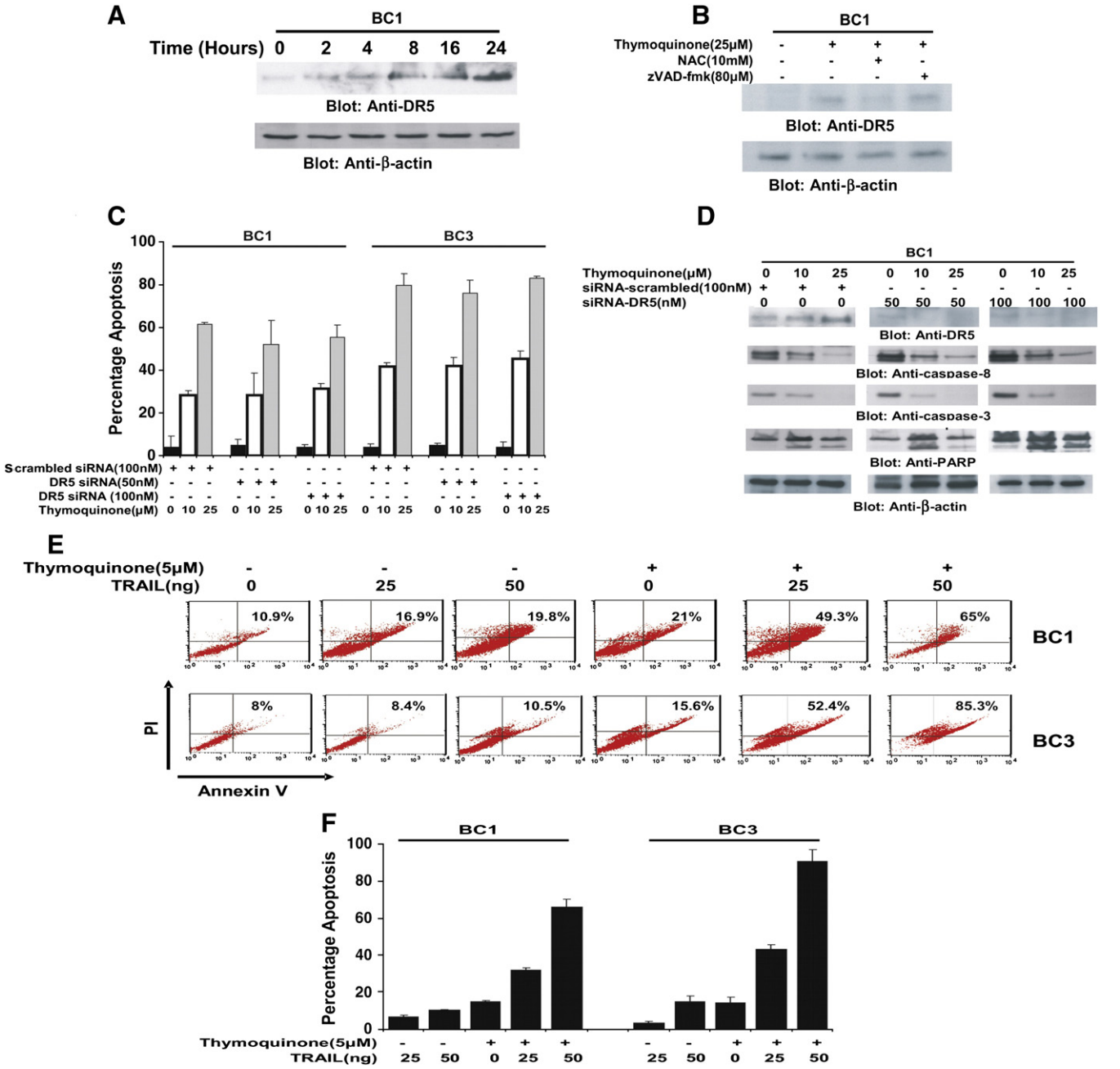

Fig. 5. (A) Thymoquinone induces up-regulation of DR5 expression. BC1 cells were treated with 10 μM thymoquinone for the indicated time periods. After cell lysis, equal amounts of proteins were separated by SDS–PAGE, transferred to Immobilon membrane, and immunoblotted with antibodies against DR5 and β-actin. (B) NAC inhibits up-regulation of DR5 in thymoquinone-treated PEL cells. BC1 cells were pretreated with either 10 mM NAC or 80 μM zVAD-fmk for 2 h and subsequently treated with 25 μM thymoquinone for 24 h. The cells were lysed and equal amounts of proteins were separated by SDS–PAGE, transferred to PVDF membrane, and immunoblotted with antibodies against DR5 and β-actin. (C) DR5 knockdown by siRNA does not inhibit thymoquinone-induced apoptosis in PEL cells. BC1 and BC3 cells were transfected with 50 or 100 nM siRNA specific against DR5 or with scrambled siRNA using LipofectAMINE for 48 h as described under Materials and methods. After 48 h of transfection, the cells were treated with 10 and 25 μM thymoquinone for 24 h, after which the cells were stained with fluorescence-conjugated annexin V/PI and analyzed by flow cytometry. The graph displays the means ± SD of three independent experiments. (D) DR5 siRNA expression does not inhibit thymoquinone-induced caspase activation or PARP cleavage in PEL cells. BC1 cells were transfected with 50 or 100 nM siRNA specific against DR5 or with scrambled siRNA using LipofectAMINE for 48 h, after which the cells were treated with 10 and 25 μM thymoquinone for 24 h. The cells were lysed and equal amounts of proteins were separated by SDS–PAGE, transferred to PVDF membrane, and immunoblotted with antibodies against DR5, caspase-8, caspase-3, PARP, and β-actin. (E) Combination of thymoquinone and TRAIL synergistically induces apoptosis in PEL cells. BC1 and BC3 cells were treated with 5 μM thymoquinone in the presence or absence of 50 or 100 ng TRAIL for 24 h. After treatment, the cells were stained with fluorescence-conjugated annexin V/PI and analyzed by flow cytometry. (F) The bar graph displays the means ± SD of three independent experiments.

However, a combination treatment of TQ and TRAIL resulted in strong induction of apoptosis (from 10.9 to 49.3% at concentrations of 5 μM TQ and 25 ng TRAIL and 65% at concentrations of 5 μM TQ and 50 ng TRAIL) in the BC1 cell line. Similar results were obtained in the BC3 cell line, from 8 to 52.4 and 85.3%, respectively. These data suggest a vital role for TQ in combination with TRAIL in PEL cell lines.

Discussion

Induction of apoptosis in malignant cells is a very important mechanism of action of some chemopreventive agents [43]. Thymoquinone, an active constituent of black seed, has well-documented proapoptotic properties in a variety of cell types, including cells of

hematopoietic origin [17–20]. Previous studies have demonstrated that TQ induces apoptosis in various cancer cell types [44,45]. We now provide evidence that TQ induces cell death and apoptosis in PEL cell lines in a dose-dependent manner. We found that all PEL cell lines studied express constitutively phosphorylated/activated AKT and its downstream target molecules, FOXO1, GSK3, and Bad. TQ treatment of PEL cell lines suppressed p-AKT in all cell lines via generation of ROS, and pretreatment of cells with NAC, a scavenger of ROS, abrogated the TQ-induced suppression of p-AKT. Furthermore, TQ-mediated ROS-induced apoptosis occurred via modulation of Bax, suggesting that Bax plays a critical role in TQ-induced apoptosis.

TQ mediated its apoptotic effects in PEL cells via Bax conformational changes that led Bax to translocate to the mitochondrial membrane, causing changes in the mitochondrial membrane potential. Our data also demonstrate that pretreatment of PEL cells with a universal caspase inhibitor, zVAD-fmk, did not block TQ-dependent conformational changes in Bax protein in PEL cell lines, indicating that the TQ-mediated Bax conformational change occurred upstream of caspase activation. However, pretreatment with NAC blocked Bax conformational changes after TQ treatment in PEL cell lines, suggesting that Bax conformational changes and oligomerization are mediated by ROS.

There are reports indicating that overexpression of Bax enhances cytochrome *c* release from mitochondria to the cytosol [46], and direct addition of recombinant Bax protein to isolated mitochondria has been previously shown to induce cytochrome *c* release [47]. Our results establish that TQ induces the loss of mitochondrial potential in PEL cells. Loss of mitochondrial membrane potential is one of the main mechanisms responsible for cytochrome *c* release in response to various cytotoxic stimuli. More recently, ample evidence suggests [48,49] that some apoptogenic agents induce Bax translocation to the mitochondrial membrane, followed by cytochrome *c* release. In the cytosol, cytochrome *c* plays a key role in the formation of the apoptosome complex by activating the binding of procaspase-9 and Apaf-1 in the presence of ATP. The formation of the apoptosome then causes cleavage of caspase-9 that propagates the death signal by activating caspase-3 and causing cleavage of PARP. Activation and cleavage of PARP is the hallmark of apoptosis that in turn causes DNA fragmentation and cell death.

There are studies that have shown that ROS release sensitizes cancer cells to TRAIL-induced apoptosis via up-regulation of DR5 in other tumors, including renal cancer and astrocytoma [50,51]. In concordance with these studies, our data also showed that TQ treatment of PEL cells caused up-regulation of DR5 via generation of ROS. However, up-regulation of DR5 did not play a role in TQ-induced apoptosis in these cell lines. We confirmed these findings by showing that, even after knockdown of expression of DR5 in PEL cell lines by specific siRNA, TQ was able to induce efficient apoptosis as was evident by flow cytometry results. These data were further confirmed by immunoblotting results that showed activation of caspases-8 and -3 and cleavage of PARP in TQ-treated PEL cells even after DR5 expression was knocked down. These results clearly suggest that even though TQ treatment causes up-regulation of DR5 in PEL cells, DR5 does not play a role in TQ-induced apoptosis. Up-regulation of DR5 by TQ treatment does give an added attractive target for inducing more potent apoptosis without causing toxicity by using a combination treatment with subtoxic doses of TQ and TRAIL. This allows us to utilize the intrinsic as well as the extrinsic apoptotic pathway to induce apoptosis. The up-regulation of DR5 hence sensitizes PEL cells to TRAIL-induced apoptosis.

Based on our findings, we propose a model (Fig. 6) in which thymoquinone treatment of PEL cells leads to a release of ROS. ROS release causes inactivation of AKT and its downstream targets such as p-Bad, leading to an imbalance between Bcl-2 and Bax proteins. This imbalance causes activation of the mitochondrial-dependent apoptotic pathway, ultimately leading to DNA damage and apoptosis in PEL cells treated with TQ. On the other hand, we also propose that ROS release secondary to TQ treatment causes up-regulation of DR5, thereby opening up another avenue to target these cells in combination with TQ and DR5 to induce apoptosis.

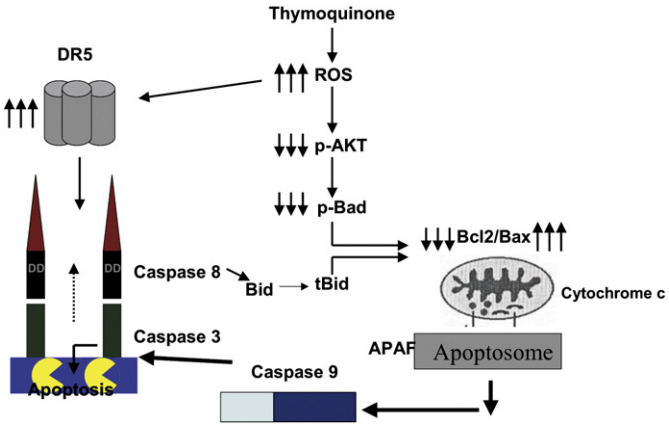

Fig. 6. Schematic diagram depicting thymoquinone-induced apoptosis in PELs.

Clinically, resistance to apoptosis by chemotherapeutic agents is a frequent problem that emerges during the management of malignancies. The cytotoxic inducing ability of TQ in cancer could make it a potentially effective chemopreventive and/or therapeutic agent for the treatment of various tumors. Furthermore, the combination of subtoxic doses of TQ and TRAIL resulted in considerable apoptosis in PEL cells. PEL is a very aggressive lymphoma; thus, it is possible that combinations of TQ with chemotherapeutic agents may provide a novel therapeutic approach to sensitize these cells and increase clinical response. Based on our data, studies in that direction are warranted.

Supplementary materials related to this article can be found online at doi:10.1016/j.freeradbiomed.2010.12.034.

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