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Anticancer activity of thymoquinone in breast cancer cells: Possible involvement of PPAR-γ pathway

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ABSTRACT

Thymoquinone (TQ), an active ingredient of *Nigella sativa*, has been reported to exhibit anti-oxidant, anti-inflammatory and anti-tumor activities through mechanism(s) that is not fully understood. In this study, we report the anticancer effects of TQ on breast cancer cells, and its potential effect on the PPAR-γ activation pathway. We found that TQ exerted strong anti-proliferative effect in breast cancer cells and, when combined with doxorubicin and 5-fluorouracil, increased cytotoxicity. TQ was found to increase sub-G1 accumulation and annexin-V positive staining, indicating apoptotic induction. In addition, TQ activated caspases 8, 9 and 7 in a dose-dependent manner. Migration and invasive properties of MDA-MB-231 cells were also reduced in the presence of TQ. Interestingly, we report for the first time that TQ was able to increase PPAR-γ activity and down-regulate the expression of the genes for Bcl-2, Bcl-xL and survivin in breast cancer cells. More importantly, the increase in PPAR-γ activity was prevented in the presence of PPAR-γ specific inhibitor and PPAR-γ dominant negative plasmid, suggesting that TQ may act as a ligand of PPAR-γ. Also, we observed using molecular docking analysis that TQ indeed formed interactions with 7 polar residues and 6 non-polar residues within the ligand-binding pocket of PPAR-γ that are reported to be critical for its activity. Taken together, our novel observations suggest that TQ may have potential implication in breast cancer prevention and treatment, and show for the first time that the anti-tumor effect of TQ may also be mediated through modulation of the PPAR-γ activation pathway.

1. Introduction

Nigella sativa, commonly known as black cumin seed, has a long-term history in folk medicine with diverse therapeutic benefits including hypertension, headache, bronchial asthma, gastrointestinal problems and eczema [1]. The biological activity of this herb is mainly attributed to its main component in essential oil, known as thymoquinone (TQ) [2,3]. TQ has been reported to exhibit antioxidant [4], anti-inflammatory [5] and anticancer activity [6]. The cytotoxicity of this compound has been reported in many different types of malignancy including prostate cancer [7], osteosarcoma [8], fibrosarcoma [9], myeloblastic leukemia [10] and colorectal carcinoma [11], while having minimal toxicity in normal cells [12]. TQ has exhibited promising anti-tumor activity in murine tumor model xenografted with fibrosarcoma and squamous cell carcinoma [9], colon cancer [6] and prostate cancer [13]. Studies had shown that TQ was able to induce apoptosis via p53-dependent [11] and p53-independent pathways [10]. TQ could up-regulate p53 and p21 in HCT116 cells, resulting in the inhibition of anti-apoptotic Bcl-2 protein [11]. However, other study had shown that the shuttling of CHEK1 into the cell nuclei has indeed increased cell viability in p53-null HCT116 cells [14]. Furthermore, El-Mahdy et al. had reported that TQ could induce apoptosis in p53-null myeloblastic leukemia HL-60 cells through the activation of caspases 8, 9 and 3 [10]. Although the TQ-induced apoptotic pathway has been reported in many studies [15], the anticancer effect of this compound in breast adenocarcinoma is not fully understood.

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors [16,17]. Three types of PPARs (α, β or δ, γ) have been identified, each encoded by different genes and expressing differently in many parts of the human body [18,19].

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They exist as heterodimers with retinoid X receptor, and upon binding to agonist, increase the rate of transcription initiation [18]. Studies have shown that PPAR-γ plays an important role in cell proliferation, differentiation and apoptosis [19]. The anti-proliferative effect of PPAR-γ has been reported in many different cancer cell lines including breast [20–22], colon [23], prostate [24], and non-small cell lung cancer [25]. PPAR-γ was shown to induce G1/S arrest by up-regulating $p21^{WAF1/Cip1}$ [25] or $p27^{Kip1}$ [26], and down-regulating cyclin D1 [20]. Ligand activation of PPAR-γ can also induce apoptosis in prostate cancer [24], non-small cell lung cancer [25], and breast cancer [21,27,28]. In addition, ligand activation of PPAR-γ was reported to inhibit invasion and metastasis of breast cancer cells [29,30]. However, many studies and results from clinical trials have raised the question of the anticancer role of PPAR-γ. Both PPAR-γ-dependent and PPAR-γ-independent pathways of PPAR-γ ligands have been identified [31]. Abe et al. had shown that troglitazone, a PPAR-γ ligand, can inhibit KU812 leukemia cells independent of PPAR-γ involvement [32]. This was supported by another study showing that PPAR-γ antagonists (T0070907, BADGE, GW9662) could not block the proteolysis of ER-α in troglitazone-treated MCF-7 and ZR-75-1 breast cancer cells [33]. The same group had found that a biotinylated derivative of Delta2-troglitazone (devoid of PPAR-γ agonist activity) increased anti-proliferative effect in both MCF-7 and MDA-MB-231 cell lines [34]. Down-regulation of PPAR-γ in MCF-7 cells by RNAi was shown to inhibit cellular proliferation and apoptosis induction [35]. In addition to *in vitro* studies, administration of PPAR-γ ligands also produced controversial results in *in vivo* studies. Elstner et al. reported that the use of troglitazone could inhibit MCF-7 tumor growth in triple negative immunodeficient mice [27]. This was not in accord with the study by Lefebvre et al. showing that PPAR-γ ligands, including troglitazone and BRL-49,653, promoted the development of colon tumors in C57BL/6J-APCMin/+ mice [36]. Although several mechanistic pathways of TQ have been identified, the involvement of this compound in the PPAR-γ pathway remains to be clarified.

In this study, we investigated the anti-tumor activity of TQ in MCF-7, MDA-MB-231 and BT-474 breast cancer cells. As PPAR-γ is known to mediate anti-tumor activity in a variety of cancer types, we hypothesized that TQ may modulate the activity of PPAR-γ pathway in breast cancer cells and inhibit tumor cell growth. Our results demonstrated that TQ could induce apoptosis in MCF-7 cells through the activation of caspases and increase Bax/Bcl-2 ratio. Additionally, this compound was able to reduce the migration and invasion of MDA-MB-231 cells. Also, our molecular docking study demonstrated for the first time that TQ could make contact with amino-acids within the ligand binding pocket of PPAR-γ that are crucial for its activation. The activation of PPAR-γ was found to make an important role in TQ-induced apoptosis and reduced survivin level. Our study thus provided further evidence for the usefulness of TQ in cancer.

2. Materials and methods

2.1. Reagents

RPMI1640, DMEM and fetal bovine serum (FBS) were purchased from Hyclone (Loughborough, UK). Trypsin EDTA, trypan blue, thiazoyl blue tetrazolium bromide (MTT), crystal violet, thymoquinone, 5-fluorouracil and paclitaxel were purchased from Sigma–Aldrich (St. Louis, MO, USA), while doxorubicin was purchased from Euroasian Chemical Private Ltd. (Mumbai, India). Dimethyl sulfoxide was purchased from MP Biomedicals (Solon, OH, USA). GSK0660 and GW0742 were purchased from Tocris Bioscience (Ellisville, MO, USA). 15d-PGJ2 and GW9662 were purchased from Cayman (Michigan, USA). Antibiotics–antimycotics were purchased from Gemini Bio-products (West Sacramento, CA, USA). Antibodies to Bcl-2, Bcl-xL, Bax, PPAR-γ, cyclin D1 and caspase 7 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), while survivin, caspase 8, caspase 9 and β-actin were purchased from Cell Signaling (Beverly, MA, USA). Chicken anti-rabbit IgG HRP-conjugated and chicken anti-mouse IgG HRP-conjugated antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.1.1. Cell lines

Three different breast cancer cell lines (MCF-7, MDA-MB-231 and BT-474), each with distinct characteristics, were used to assess their sensitivity to the anti-proliferative effect of TQ. MCF-7 and BT-474 are estrogen receptor (ER)-positive, but the former lack HER-2 expression as compared to the latter [37]. MDA-MB-231 is ER-negative [37] and has low HER-2 expression. MCF-7 expresses wild-type p53, while MDA-MB-231 and BT-474 express mutant p53. All three cell lines express PPAR-γ protein [38,39]. These cell lines were purchased from ATCC (Manassas, VA, USA). MCF-7 and MDA-MB-231 cells were cultured in RPMI1640 supplemented with 10% FBS and 1% antibiotic. BT-474 cells were cultured in DMEM medium supplemented with 12.5% FBS and 1% antibiotics. All cells were maintained at 37 °C and 5% CO_2 in a humidified atmosphere.

2.1.2. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay

The anti-proliferative effect of TQ was determined by MTT assay as described previously [15]. Briefly, breast cancer cells were seeded at a density of about 1×10^4 cells per well in a 96-well microtiter plate followed by overnight incubation. These cells were exposed to the indicated concentrations of TQ for 12, 24 and 48 h. The cells were then treated with MTT solution (5 mg ml^{-1}) for 4 h, at the end of which viable cells were measured by their ability to reduce the yellow dye, MTT, to a purple formazan product. The medium was removed and replaced with 100 μl DMSO to dissolve the formazan crystal formed. After 1 h, the absorbance (570 nm) of each well was read from an absorbance reader (Tecan infinite M200, Männedorf, Switzerland).

2.1.3. Flow cytometry analysis

The effect of TQ on the cell cycle was determined using flow cytometry analysis as described previously [40]. Briefly, cells were seeded at a density of about 2×10^5 cells per well in a 6-well microtiter plate followed by overnight incubation. Cells were then starved for 24 h in serum-free medium before treatment with the indicated concentrations of TQ for 12, 24 or 48 h. The cells were detached and fixed with 70% ethanol for at least 2 h before staining with propidium iodide (Sigma–Aldrich, St. Louis, MO, USA) mixed with RNase (Sigma–Aldrich, St. Louis, MO, USA). The cells were kept under dark conditions and subject to flow cytometry analysis (CyAn™ ADP from Beckman Coulter, Brea, CA, USA) after 20 min.

The procedure of annexin V-propidium iodide staining was carried out according to the manufacturer's protocol (Miltenyi Biotech, Bergisch Gladbach, Germany). After TQ treatment for 12 h, cells were trypsinized, washed with binding buffer, and resuspended in annexin V-FITC-added binding buffer for 15 min under dark conditions. The cells were then washed and stained with propidium iodide-added binding buffer. The cell samples were analyzed immediately by flow cytometry.

2.1.4. Wound healing assay

The migration of cells was investigated using 'wound-healing' assay as described previously [13]. Briefly, cells were seeded in a 6-well microtiter plate until about 80% confluence. A 'wound' was created using pipette tip and rinsed with PBS to remove detached

cells. Medium with the indicated concentrations of TQ was added for 48 h incubation. The microscopic observation of the cells was recorded at 0, 24 and 48 h.

2.1.5. Invasion assay

The invasiveness of breast cancer cells was investigated with BD Matrigel™ Invasion Chamber 24-well Plate 8 micron (BD Biosciences, San Diego, MA, USA) as described previously [41]. Briefly, the chamber was allowed to rehydrate in serum-free media for 2 h before being placed in 24-well microtiter plates filled with 5% FBS media. A total of 6×10^4 cancer cells in serum-free media were added into the top of each chamber. The cells were allowed to settle for 4–6 h before incubated with the indicated concentrations of TQ for 24 h or 48 h. The media was removed and the cells fixed with 4% paraformaldehyde for 30 min. The migratory cells, which attached to the bottom of the chamber, were stained with 0.5% crystal violet solution for 15 min. The observation of the migratory cells was recorded with 20× light microscopy (Olympus BX51, Shinjuku, Japan). The percentage of the migratory cells in treated wells was normalized against untreated cells.

2.1.6. Real-time RT-PCR

The mRNA expression of various genes was examined using real-time RT-PCR as described previously [21]. Briefly, cells were seeded at a density of about 2×10^5 cells per well in a 6-well microtiter plate followed by overnight incubation. After treatment with TQ, total cellular RNAs were isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA). TaqMan® Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) was used for reverse transcription (RT) reaction of RNA to cDNA. Briefly, 1 μg of total RNA was mixed with RT buffer, $MgCl_2$, dNTPs, random hexamers, RNase inhibitors, RTase and H_2O to make a total volume of 10 μl. The RT reaction was carried out over a PCR thermal cycle (25 °C for 10 min, 37 °C for 60 min and 95 °C for 5 min). Expression levels were determined using TaqMan® Universal Master Mix 2.0 (Applied Biosystems, Foster City, CA, USA) mixed with target primer probes (purchased as kits from Applied Biosystems). Reactions were carried out in duplicate and the mRNA expression was normalized against 18S rRNA as an internal control.

2.1.7. Western blot analysis

The protein expression was investigated using immunoblotting as described previously [15]. Briefly, cells were seeded at a density of about 2×10^5 cells per well in a 6-well microtiter plate followed by overnight incubation. Cells were exposed with the indicated concentrations of TQ before whole cell lysate extraction. Cell lysates were resolved by 12% SDS/PAGE gel and electroblotted onto nitrocellulose membrane. The membrane was probed with primary antibodies against interest target protein. After overnight incubation, the immunoblot was washed, exposed to HRP-conjugated secondary antibodies for 1 h, and examined with ECL Western blotting analysis system (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

2.1.8. Molecular docking study

An X-ray crystallography structure of PPAR-γ (PDB ID: 2Q5S) was obtained from the RCSB Protein Data Bank. This structure was selected because it has a resolution of 2.05 Å, with a *R* value of 0.199 and *R* free value of 0.245. The protein file contains two copies of PPAR-γ. The first copy, chain A, was removed because it contains less residues than the second copy, chain B. The ligand associated with chain A and all water molecules were also removed. The remaining protein chain and its associated ligand were then processed using the default settings for the "Protonate 3D" feature in Molecular Operating Environment (MOE) software. This is to add hydrogen atoms and determine the ionization state of the residues. Molecular docking of TQ to PPAR-γ was then performed using the Dock feature in MOE. The default "Triangle Matcher" algorithm was used to generate 1000 different poses for TQ. London dG scoring function was used to rank these poses. The top 10 poses were retained and further refined by energy minimization. The MMFF94x force field with calculation of implicit solvation energy using Generalized Born model was used for the energy minimization. Side chains of residues within 6 Å from TQ were allowed to move during energy minimization. After energy minimization, the pose with the best interaction energy with PPAR-γ was retained.

2.1.9. Luciferase assay

The activity of PPAR-γ was investigated using luciferase assay as described previously [21]. Briefly, cells were seeded at density of about 6×10^4 cells per well in 12-well microtiter plate followed by overnight incubation. The cells were incubated in DMEM medium for at least 1 h before transfection with pPPRE-tk-Luc (three PPREs from rat acyl-CoA oxidase promoter under the control of the Herpes simplex virus thymidine kinase promoter) and Renilla plasmids as an internal control. For PPARs study, the cells were transfected with each of GAL4-PPAR-α LBD, GAL4-PPAR-γ LBD and GAL4-PPAR-δ LBD plasmids (a generous gift from Dr. Javier F. Piedrafita, Torrey Pines Institute for Molecular Studies, San Diego, CA, USA), together with GAL4-Luc and Renilla plasmids. For dominant negative transfection, the cells were transfected with PPAR-γ mutant {(PPARγC126A/E127A) (PPAR-γDN) containing amino acid substitutions in the DNA binding domain that abolish binding to PPAR-γ response elements, was kindly provided by Dr. Christopher K. Glass, UCSD, San Diego, CA, USA} or pCMX-mPPARγ plasmid (a cDNA clone encoding the mouse PPARγ was a generous gift from Dr. Ronald M. Evans, The Salk Institute for Biological Studies, San Diego, CA, USA), together with pPPRE-tk-Luc and Renilla plasmid as an internal control. Cells were transfected with the indicated plasmids using calcium phosphate transfection kit (Clontech, Mountain View, CA, USA) for 12–14 h before recovery with normal RPMI1640 medium for at least 6 h. After TQ treatment, the cells were harvested using ice-cold reporter lysis buffer. The lysate was then centrifugated at 12,000 rpm for 3 min. The supernatant was mixed with luciferase substrate solution (Promega, WI, USA) for luciferase reading, followed by stops & glow buffer (Promega, WI, USA) for Renilla reading. Bioluminescence generated was measured using Sirius luminometer v3.1 (Berthold, Munich, Germany). The luciferase reading obtained was normalized to the corresponding Renilla reading and to the protein amount.

2.2. Statistical analysis

Data were shown in bar graph form, expressed as means ± S.E.M. from at least three independent experiments and statistically determined by ANOVA test or Student's *t*-test, with $p < 0.05$ considered significant.

3. Results

3.1. Anti-proliferative effect of TQ on breast cancer cell lines

The ability of TQ to inhibit the proliferation of breast cancer cells (MCF-7, MDA-MB-231 and BT-474) was determined by MTT assay. TQ suppressed the growth of all three breast cancer cell lines in a dose- and time-dependent manner (Fig. 1A). IC_{50} values of TQ in MCF-7 cells after 12 h, 24 h and 48 h of exposure were determined as 48, 40 and 32 μM, respectively. IC_{50} values after 12 h, 24 h and 48 h TQ exposure in MDA-MB-231 cells were 24, 14, and 11 μM, respectively. IC_{50} values after 12 h, 24 h and 48 h TQ exposure in BT-474 cells were 38, 18, and 21 μM, respectively.

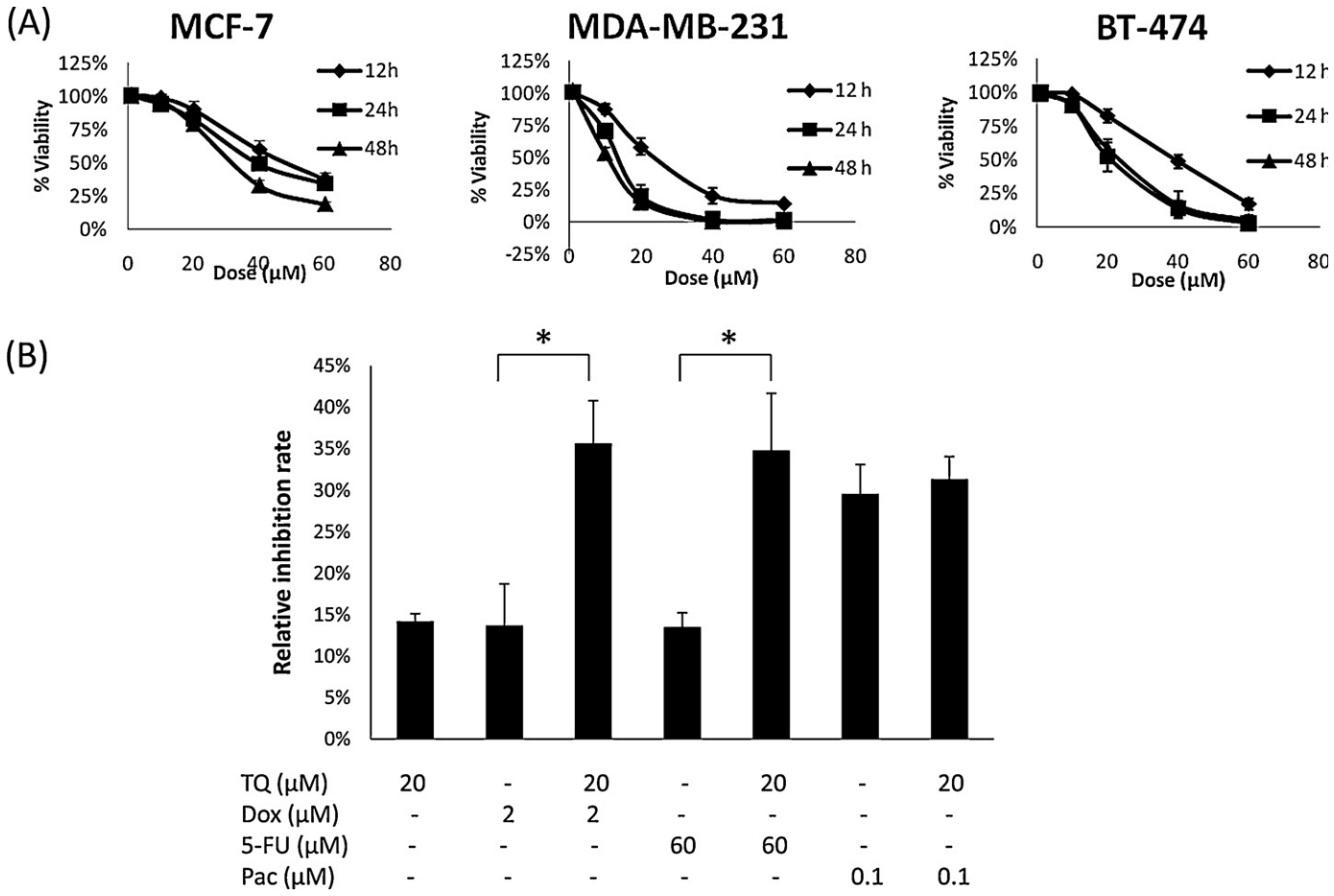

Fig. 1. Anti-proliferative effect of TQ on breast cancer cells. (A) Dose–response curve of TQ treatment after 12 h, 24 h and 48 h exposure in MCF-7, MDA-MB-231 and BT-474 breast cancer cells. Cell viability was determined by MTT assay and is reported as the percentage of viable cells relative to the control. Values are means ± S.E.M. of three independent experiments. (B) Growth inhibition rate of TQ alone or in combination with various anticancer agents. MCF-7 cells were treated with TQ alone or in combination with anticancer agents for 24 h before MTT solution is added. The data was expressed as the percentage of dead cells relative to the control. Values are means ± S.E.M. of three independent experiments. $^{*}p < 0.05$.

Overall, IC_{50} value of TQ was found to be <50 μM, suggesting that TQ could exert strong anti-proliferative effect in breast cancer cells at low doses. The MCF-7 cell line was noted to be the least sensitive to TQ-induced growth inhibition, while MDA-MB-231 was the most sensitive.

We next investigated whether TQ potentiates cytotoxicity of chemotherapeutic agents including doxorubicin, 5-fluorouracil and paclitaxel in breast cancer MCF-7 cells. Growth inhibition rate was obtained using MTT assay and calculated as the percentage of dead cells versus control. Following 24 h treatment, we found that TQ significantly enhanced the cytotoxic effect of doxorubicin and 5-fluorouracil, with no enhancement of the paclitaxel effect (Fig. 1B). 20 μM of TQ was found to up-regulate the cytotoxicity of doxorubicin by 2.6 times (from 14% to 36%) and of 5-fluorouracil by 2.7 times (from 13% to 35%). These results support the conclusion that TQ potentiates the cytotoxicity of chemotherapeutic agents.

3.1.1. TQ induced apoptosis in MCF-7 cells through the activation of caspases and increased Bax/Bcl-2 ratio

Although TQ has been reported to inhibit the growth of MCF-7 breast cancer cells [42], its mechanism of action was unclear; hence we have used these cells for most of the mechanistic experiments as described below. In order to study the chronology of the cellular and molecular events after TQ treatment, we utilized flow cytometry analysis on TQ-treated MCF-7 cells at different concentrations and exposure periods. Fig. 2A shows that TQ (80 μM) induced subG1 accumulation after 24 h exposure, and also after 48 h exposure at both 60 and 80 μM. Interestingly, 12 h exposure did not result in substantial subG1 accumulation in contrast to the MTT assay which showed that TQ had induced strong cytotoxicity as early as 12 h. This might be because nuclear fragmentation is considered to be a feature of late apoptosis. Another apoptosis marker, phosphatidylserine exposure, was examined by flow cytometry with annexin V-FITC staining. Results revealed that TQ was able to increase annexin V positive cells in a dose-dependent manner (Fig. 2B), indicating induction of apoptosis. The data are consistent with the results from the MTT assay. Together, these results support the conclusion that TQ induces cell death through apoptosis.

We next examined the mechanistic pathway of apoptosis in TQ-treated MCF-7 cells using immunoblotting assay. Following 12 h exposure, TQ was shown to down-regulate procaspases 8, 9 and 7 in a dose-dependent manner (Fig. 2C). This suggests that cleavage events had occurred, indicating the activation of caspases 8, 9 and 7. In addition, TQ was found to down-regulate the antiapoptotic Bcl-2 gene product, with a slight increase in Bax expression. Together, Bax/Bcl-2 ratio was increased suggesting that this was one of the mechanisms of TQ-induced apoptosis. No significant difference was found in the protein levels of tumor suppressor, p53 after TQ exposure (Fig. 2C), suggesting that the effect of TQ might be independent of p53 activation in MCF-7 cells.

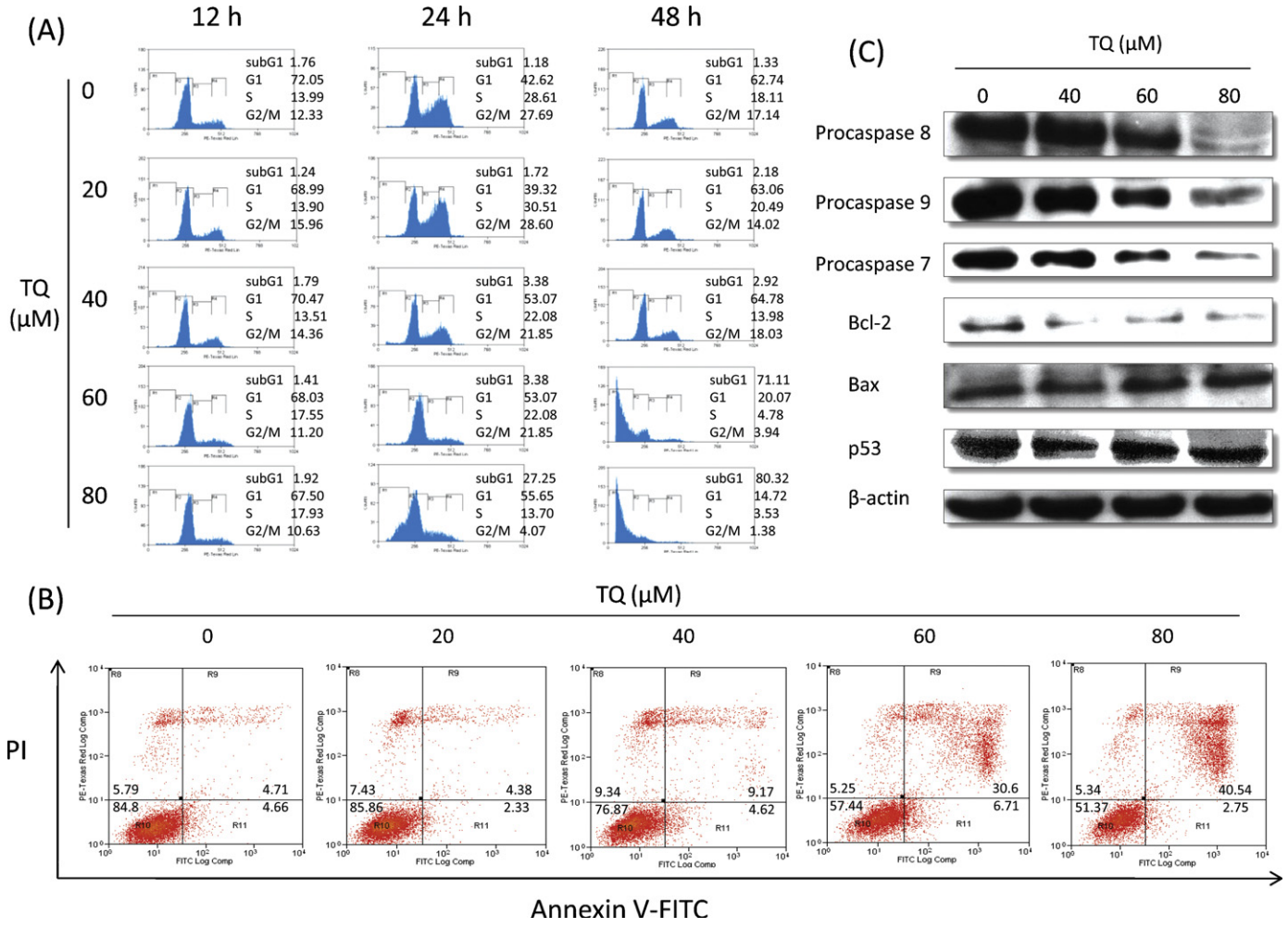

Fig. 2. TQ induces apoptosis in MCF-7 cells through the activation of caspases and down-regulation of Bcl-2. (A) Dose- and time-dependent effects of TQ on cell cycle of MCF-7 cells. Cells were exposed to the indicated concentrations of TQ for 12 h, 24 h or 48 h followed by propidium iodide staining. The data are representative of three independent experiments. (B) TQ induces apoptosis in a dose-dependent manner. Cells were treated with the indicated concentrations of TQ for 12 h followed by annexin V-propidium iodide staining. The data are representative of three independent experiments. (C) Western blot analysis of various targets in TQ-treated cells. Cells were treated with the indicated concentrations of TQ for 12 h. Whole-cell extracts were resolved on SDS-PAGE gel and probed with the indicated antibodies. The data are representative of at least three independent experiments.

3.1.2. Effect of TQ on the migration/invasion of breast cancer cells

To ascertain the inhibitory effect of TQ on breast cancer metastasis, we investigated the effects of TQ on the migration potential of MCF-7 and MDA-MB-231 cells, using the 'wound-healing' assay. A 'wound' was created with a pipette tip and the migration of breast cancer cells to fill up the 'wound' was recorded by microscopic observation. 48 h after the 'wound' was created, vehicle-treated cells had almost completely filled in the cleared area, both for MCF-7 and MDA-MB-231 cells. The migration of the cancer cells was reduced with TQ-enriched medium, in a dose-dependent manner (Fig. 3A).

The ability of TQ to reduce the invasiveness of breast cancer cells was further investigated by invasion assay. We found that TQ significantly inhibited MDA-MB-231 cell invasion in a dose-dependent manner (Fig. 3B). However, TQ shown little effect in MCF-7 cells, which might be explained by poor invasiveness nature of this cell line.

3.1.3. TQ increased PPAR-γ and PPAR-β/δ activity in MCF-7 cells

We first explored the possibility of the role of TQ on PPARs in MCF-7 breast cancer cells by using luciferase reporter assay. Cells were transfected with each of PPARs plasmids, including GAL4-PPAR-α LBD, GAL4-PPAR-γ LBD and GAL4-PPAR-δ LBD plasmids, together with GAL4-Luc and Renilla plasmids as internal control. Cells were treated with TQ and then extracted the whole-cell lysate for analysis by luciferase assay. Fig. 4A shows that TQ induced the activities of PPAR-β/δ and PPAR-γ but had no significant effect on PPAR-α. We next examined the specificity of TQ on the activity of PPAR-β/δ and PPAR-γ using respective agonist and antagonist. We found that GSK0660, an antagonist of PPAR-β/δ, was not able to reverse TQ-induced PPAR-β/δ activity, suggesting that the inducible effect of TQ is not specific (Fig. 4B). However, the antagonist of PPAR-γ, GW9662, was found to significantly reverse TQ-induced PPAR-γ activity (Fig. 4C).

3.1.4. TQ induced PPAR-γ activity and reduced its related anti-apoptotic targets at both mRNA and protein levels

Due to the specificity in PPAR-γ activity, and PPAR-γ has been extensively shown to be associated with anticancer effects in a variety of cancer types; hence we chose to focus on this isoform and its association with the inhibitory effects of TQ. We measured the dose- and time-dependent effects of TQ on PPAR-γ activity in MCF-7 cells. The cells were pre-transfected with pPPRE-tk-Luc and Renilla plasmids, and then were treated with different concentrations of TQ for 6 h, 8 h and 18 h. After normalization with the vehicle control, we found that TQ significantly increased PPAR-γ

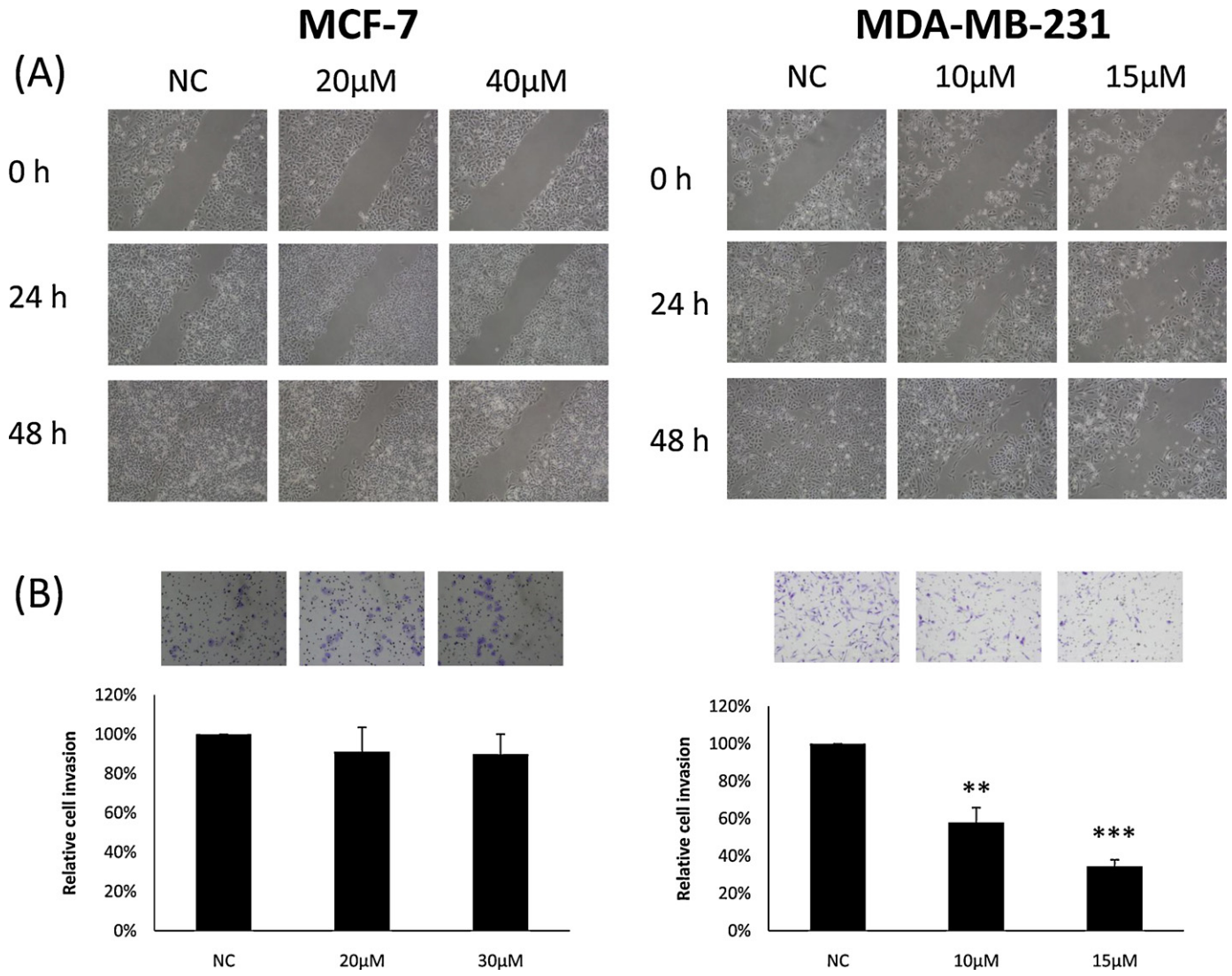

Fig. 3. TQ suppresses the migration/invasion of breast cancer cells. (A) Microscopic observation of the growth of MCF-7 and MDA-MB-231 cells after TQ exposure for 0 h, 24 h and 48 h. A 'wound' was created with a pipette tip before treatment with TQ-enriched culture medium. The data are representative of three independent experiments. (B) The cell invasion assay for evaluating the inhibitory effect of TQ on breast cancer cell invasion. MDA-MB-231 and MCF-7 cells were treated with the indicated concentrations of TQ for 24 h and 48 h, respectively. The cells were fixed with 4% paraformaldehyde before staining with 0.5% crystal violet as described in Section 2. The percentage of the migratory cells of the treated group was normalized against the untreated group. Values are means ± S.E.M. of two or three independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$.

activity in a dose-dependent manner after 6 h, 8 h and 18 h exposure (Fig. 5A).

We next examined the molecular docking of TQ to PPAR-γ using the Dock feature in MOE as described in Section 2. The complex of TQ and PPAR-γ had interaction energy of −18.06 kcal/mol. We found that TQ was able to form interactions with 7 polar residues and 6 non-polar residues in the receptor (Fig. 5B, left). Among these interactions, Ser289, His323, His449, Tyr473 and Phe363 were previously found in earlier studies to be important for binding and activity [43–45].

To elucidate the mechanistic pathway of PPAR-γ, we have investigated the effects of TQ on the mRNA and protein expressions of PPAR-γ-related genes in MCF-7 cells. We found that TQ significantly decreased mRNA levels of Bcl-2, Bcl-xL and survivin in a time-dependent manner, while no change occurred in cyclin D1 (Fig. 5C). Interestingly, TQ also increased PPAR-γ transcription which is quickly faded after 8 h of treatment. In addition, TQ also down-regulated Bcl-2, Bcl-xL and survivin protein levels time-dependently in MCF-7 cells (Fig. 5D), while no change occurred in cyclin D1, in accord with the corresponding mRNA results. We also found that PPAR-γ protein level was slightly increased after 6 h exposure to TQ (Fig. 5D), which are in agreement with our mRNA results shown in Fig. 5C. Altogether, these results suggest the involvement of the PPAR-γ pathway in the apoptotic effect of TQ.

3.1.5. GW9662, a pharmacological PPAR-γ inhibitor, reversed the effect of TQ on cell apoptosis, and survivin expression level but not Bcl-2 and Bcl-xL

To determine if the increase in cell apoptosis and the decrease in PPAR-γ-related genes are due to TQ-induced PPAR-γ activation, we utilized GW9662, a pharmacological PPAR-γ inhibitor, to block the activation of the PPAR-γ pathway. MCF-7 cells were pre-treated GW9662 for 2 h followed by TQ exposure for the indicated periods. As shown in Fig. 6A, GW9662 significantly reversed TQ-induced PPAR-γ activity. TQ-induced apoptosis was also reversed by GW9662, though not completely (Fig. 6B). This suggests that PPAR-γ is involved in TQ-induced apoptosis, at least to a certain extent. Moreover, the treatment of GW9662 was also reversed the decrease in mRNA and protein levels of survivin by TQ (Fig. 6C and D). A slight reverse in the decrease of Bcl-xL protein level was also

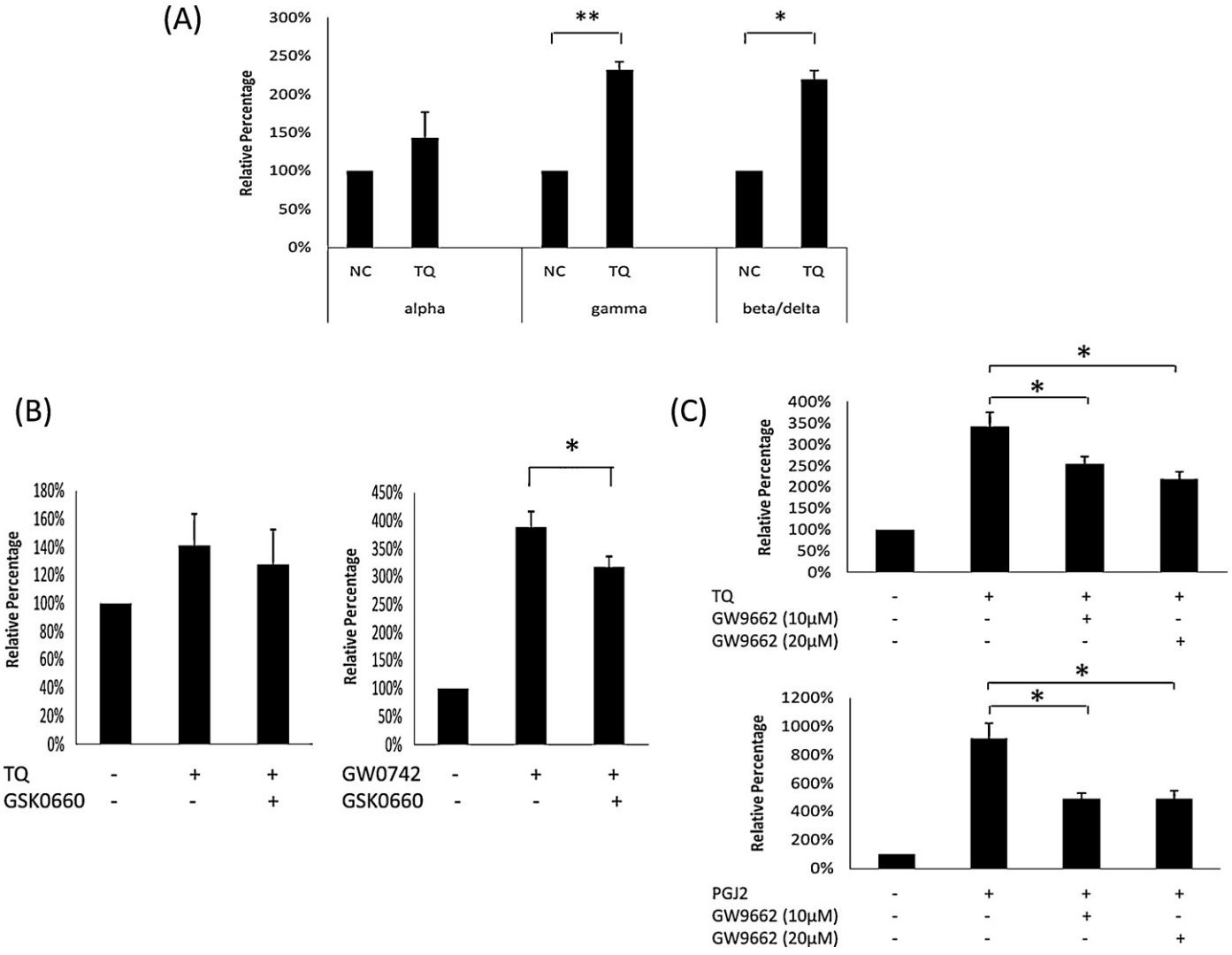

Fig. 4. The involvement of TQ in the PPARs in MCF-7 breast cancer cells. (A) Effect of TQ in PPARs. Cells were transfected with each of GAL4-PPAR-α LBD, GAL4-PPAR-γ LBD and GAL4-PPAR-δ LBD plasmids, together with GAL4-Luc and Renilla plasmids for 12–14 h before treatment with 40 μM TQ for 18 h. The data are expressed as percentages of the respective PPAR activity relative to the control. Values are means ± S.E.M. of two or three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$. (B) The inhibitor of PPAR-β/δ, GSK0660, could not block TQ-induced PPAR-β/δ activity. Cells were transfected with GAL4-PPAR-δ LBD plasmids together with GAL4-Luc and Renilla plasmids for 12–14 h. The cells were pre-treated with 50 μM GSK0660 for 4 h before treatment with 40 μM TQ or 10 μM GW0742, a PPAR-β/δ agonist, both for 18 h. The data are expressed as percentages of the PPAR-β/δ activity relative to the control. Values are means ± S.E.M. of two or three independent experiments. $^{*}p < 0.05$. (C) TQ-induced PPAR-γ activity could be blocked by GW9662, an inhibitor of PPAR-γ. Cells were transfected with GAL4-PPAR-γ LBD plasmids together with GAL4-Luc and Renilla plasmids for 12–14 h. The cells were pre-treated with 10 μM or 20 μM GW9662 for 2 h before treatment with 40 μM TQ or 20 μM PGJ2, a PPAR-γ agonist, both for 18 h. The data are expressed as percentages of the PPAR-γ activity relative to the control. Values are means ± S.E.M. of two or three independent experiments. $^{*}p < 0.05$.

observed following GW9662 treatment; however no significant change in Bcl-2 level was found. Interestingly, GW9662 also decreased the expression of PPAR-γ mRNA level in MCF-7 cells.

3.1.6. PPAR-γ dominant negative impaired the effect of TQ on the PPAR-γ pathway

In addition to the pharmacological approach, we also investigated the induction of PPAR-γ activity by TQ through genomic approach. We over-expressed faulty PPAR-γ nuclear receptor to reduce the chance of TQ binding. Fig. 7A shows that the induction of PPAR-γ activity in MCF-7 cells by TQ was significantly reversed after transfection with PPAR-γ dominant negative. Also, the decreases in both mRNA and protein levels of survivin by TQ treatment were reversed with PPAR-γ dominant negative transfection (Fig. 7B and C). Together, these results suggest that TQ could reduce survivin expression through the activation of PPAR-γ pathway.

4. Discussion

Breast cancer remains one of the highest death cases among different kinds of cancer [46]. Although many different types of cytotoxic drug have been developed for clinical uses, it is important to note that cancer chemotherapy is always accompanied with adverse effects which may be fatal in some cases. For example, doxorubicin has been associated with cardiotoxicity and congestive heart failure [47]. In addition, the cure rate of chemotherapy is also limited by the development of drug resistance. As such, there is increasing interest in natural products to complement conventional medicine. In the present study, we report the anticancer activity of TQ, a compound isolated from *N. sativa* (an herb used in Ayurvedic medicine), for its effects in breast cancer cell lines and its possible mechanism(s) of action.

Although TQ has been shown to suppress the growth of many different types of tumor cells [7–11], the inhibitory effect of this

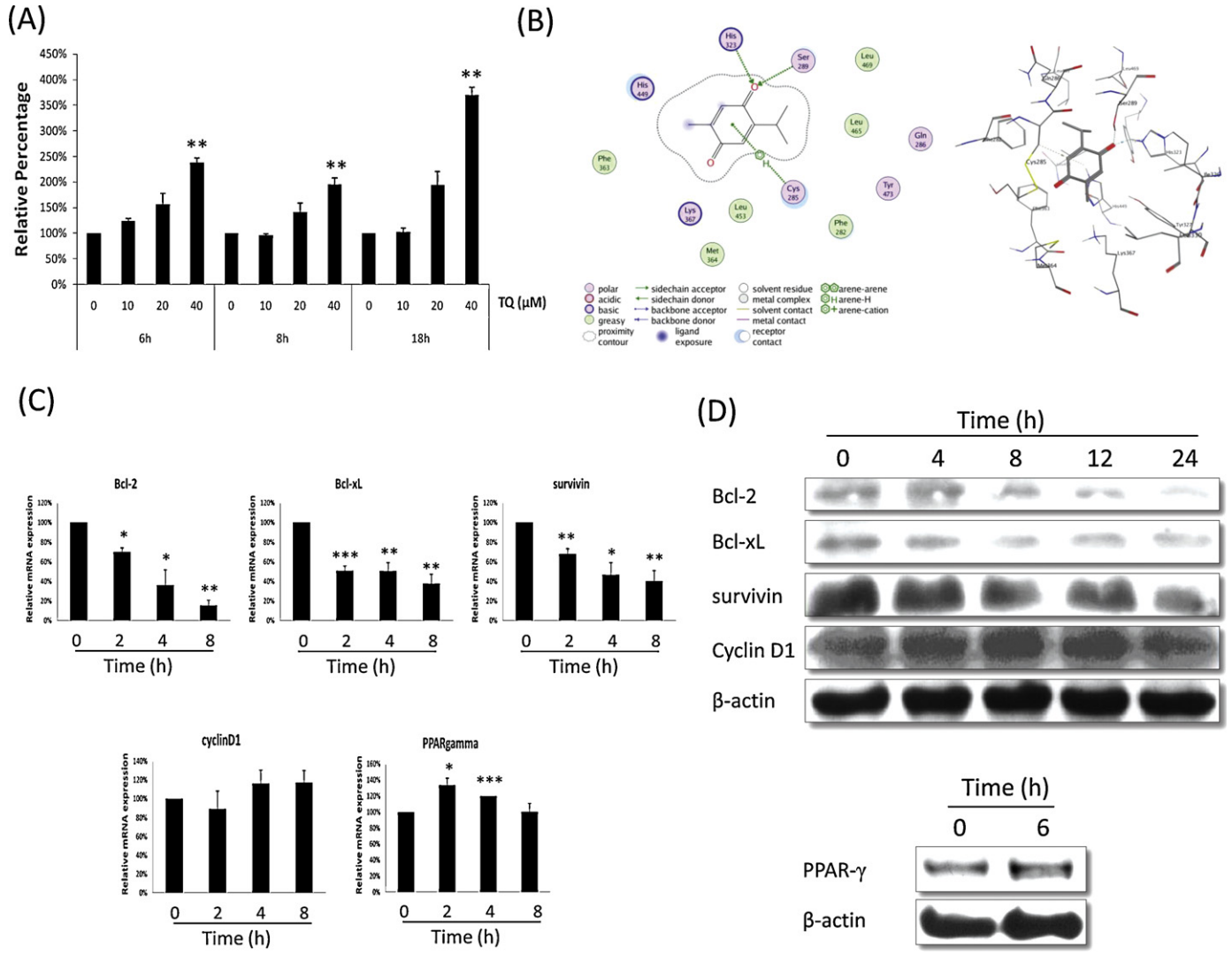

Fig. 5. Effect of TQ in the PPAR-γ pathway in MCF-7 breast cancer cells. (A) TQ increases PPAR-γ activity in a dose- and time-dependent manner. Cells were transfected with pPPRE-tk-Luc and Renilla plasmids for 12–14 h before treatment with the indicated concentrations of TQ for 6, 8 and 18 h. The data are expressed as percentages of the PPAR-γ activity relative to the control. Values are means ± S.E.M. of two or three independent experiments.$^{**}p < 0.01$. (B) The ligand interaction map of TQ inside PPAR-γ (*left*) and 3D conformational structure of TQ inside PPAR-γ (*right*). (C) TQ suppresses various PPAR-γ-regulated genes at the mRNA level. Cells were treated with 40 μM of TQ for 0, 2, 4 and 8 h. Total RNA was isolated and analyzed by real time RT-PCR as described in Section 2. Values are means ± S.E.M. of two or three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. (D) TQ suppresses PPAR-γ-regulated genes at the protein level. Cells were treated with 40 μM of TQ for 0, 4, 6, 8, 12 and 24 h. Whole-cell extracts were resolved on SDS-PAGE gel and probed with the indicated antibodies. The data are representative of at least three independent experiments.

compound in breast cancer is not well documented. Our data reveal that TQ inhibits the growth of the breast cancer cell lines-MCF-7, MDA-MB-231 and BT-474, in a dose- and time-dependent manner. We also found that TQ potentiated the cytotoxic effect of chemotherapeutic agents such as doxorubicin and 5-fluorouracil in MCF-7 cells. The ability of TQ to combine with chemotherapeutic agents to produce synergistic cytotoxicity has been reported in other cell lines, including KBM-5 [48], HL-60 [42] and NCI-H460 [49]. In addition, TQ was shown to ameliorate cisplatin-induced nephrotoxicity [50] and doxorubicin-induced cardiotoxicity [51] in animal models. Together, these results suggest that TQ can be used as a complement to conventional chemotherapeutic drugs for the purpose of enhancing the anti-tumor effect and/or reducing toxicity of the latter.

To understand the mechanism of how TQ produce inhibitory effect in breast cancer cells, we utilized different molecular techniques to label and measure selected markers. In the present study, we demonstrate that TQ was able to induce apoptosis in MCF-7 cells dose-dependently. This compound was found to trigger the activation of caspases 8, 9, and 7 in MCF-7 cells. Similarly, TQ was also found to activate caspases 8, 9 and 3 in HL-60 cells [10]. However, p53 protein level was unchanged after TQ treatment, suggesting that the apoptotic effect of TQ was independent of p53. A recent study showed that TQ was able to induce PTEN expression in which responsible in apoptosis induction in doxorubicin-resistant human breast cancer cells [52]. We also found that Bcl-2 protein was down-regulated after TQ treatment. The increase in total Bax/Bcl-2 ratio suggests that this might be one of the mechanisms of TQ-induced apoptosis in MCF-7 cells. The increase in Bax/Bcl-2 ratio by TQ was also reported in other cancer cells, including HL-60 [10] and HCT116 cancer cells [11].

Metastasis has been suggested as one of the characteristics of cancer [53]. It is a complex process involving cell adhesion, cell migration and cell invasion [41]. The process of invasion requires the tumor cells to penetrate the basement membrane before entering the blood circulation. Cell migration is also an important property for tumor cells to migrate from the primary site to a

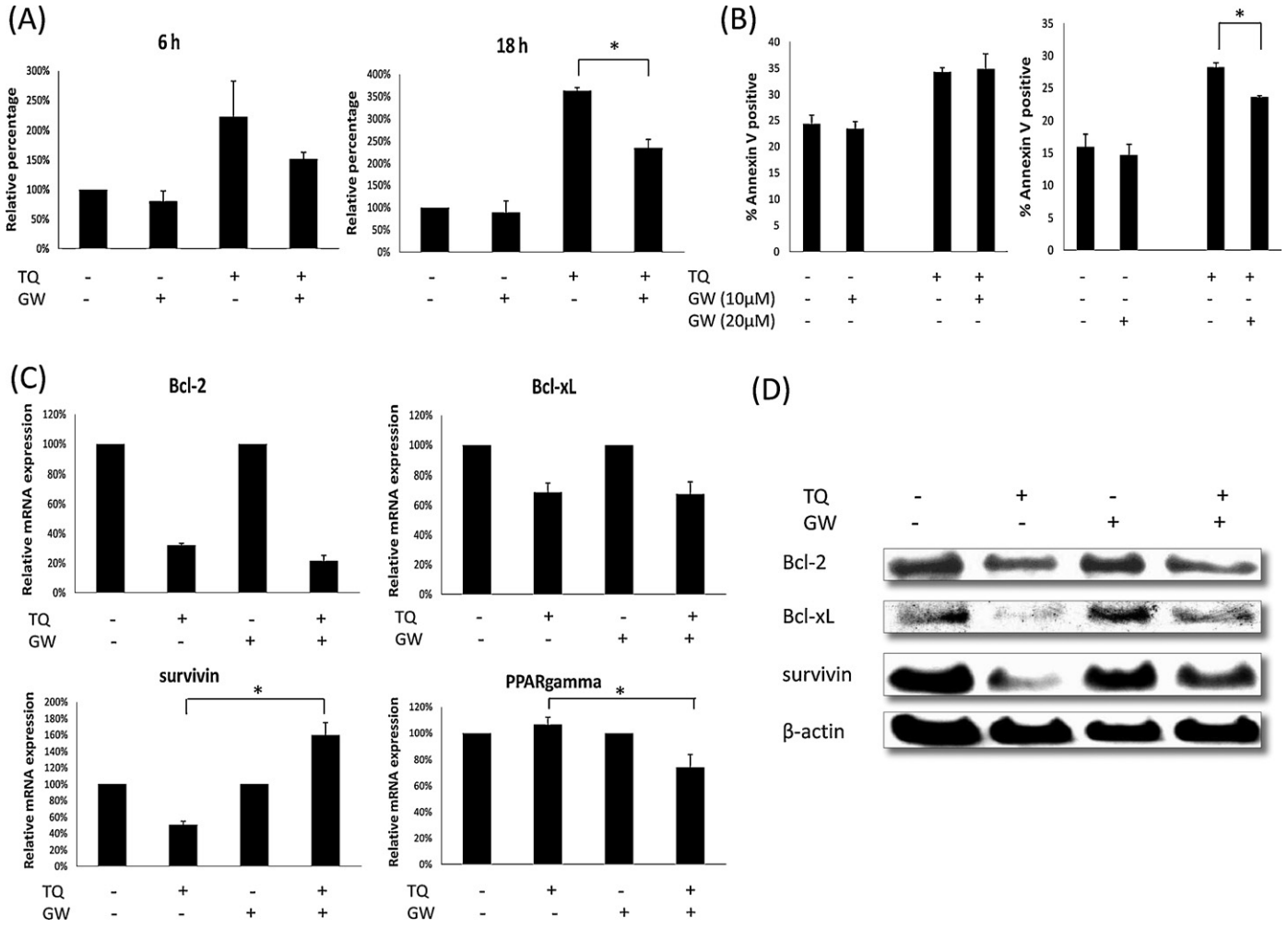

Fig. 6. GW9662 reverses the effect of TQ on cell apoptosis and survivin expression level in MCF-7 breast cancer cells. (A) GW9662 reverses TQ-induced PPAR-γ activity. Cells were transfected with pPPRE-tk-Luc and Renilla plasmids for 12–14 h. The cells were pre-treated with 10 μM of GW9662 for 2 h before treatment with 40 μM of TQ for 6 and 18 h. The data are expressed as percentages of the PPAR-γ activity relative to the control. Values are means ± S.E.M. of two or three independent experiments. $^*p < 0.05$. (B) GW9662 partially reverses TQ-induced apoptosis in MCF-7 cells. Cells were pre-treated with 10 or 20 μM of GW9662 for 2 h before treatment with 50 μM of TQ for 12 h. The cells were then harvested and stained with annexin V-propidium iodide as described in Section 2. Values are means ± S.E.M. of three independent experiments. $^*p < 0.05$. (C) The mRNA expression of various targets with or without GW9662 treatment in TQ-treated cells. Cells were pre-treated with 10 μM of GW9662 for 2 h before treatment with 40 μM of TQ for 8 h. Total RNA was isolated and analyzed by real time RT-PCR as described in Section 2. The data are expressed as the percentage of mRNA expression relative to their respective control. Values are means ± S.E.M. of two or three independent experiments. $^*p < 0.05$. (D) The protein expression of various targets with or without GW9662 treatment in TQ-treated cells. Cells were pre-treated with 10 μM of GW9662 before treatment with 40 μM of TQ for 24 h. Whole-cell extracts were resolved on SDS-PAGE gel and probed with the indicated antibodies. The data are representative of at least three independent experiments.

secondary organ [41]. The present study is the first to demonstrate the ability of TQ to inhibit the metastatic process of MDA-MB-231 breast cancer cells through the reduction of cell migration and invasion. Similarly, it has been recently reported that TQ was able to decrease the migration of FG/COLO357 cells (pancreatic cancer) in a dose-dependent manner [54].

While age and gender are two important factors in the development of breast cancer, studies have shown that diet and obesity are also correlated with increased breast cancer risk [55]. Since PPAR-γ is the main regulator of adipogenesis and lipid metabolism, many researchers have investigated the relationship of this gene to breast carcinoma. PPAR-γ mRNA level of breast cancer tissues was found to be lower than normal tissues [56]. Moreover, PPAR-γ activity has been shown to induce apoptosis in different cancer cell lines including breast cancer [21,27,28]. This is the first report to suggest that TQ could activate PPAR-γ followed by down-regulation of its subsequent anti-apoptotic genes. TQ was found to bind to amino acid residues inside PPAR-γ, as shown by molecular docking analysis, that are important for the binding and activity. TQ-induced apoptosis could be inhibited by PPAR-γ antagonist, GW9662. We also found that TQ could down-regulate PPAR-γ-related genes, including Bcl-2, Bcl-xL and survivin at both mRNA and protein expression levels, in a time-dependent manner. These genes are commonly associated with the increase in resistance to apoptosis in human cancer cells [57]. The activation of PPAR-γ by TQ was reduced in the presence of GW9662 together with a reversal in decreased survivin levels. In addition, the results in PPAR-γ dominant negative transfected cells confirmed the role of the activation of PPAR-γ in decreasing survivin levels. Together, these results suggest that TQ can be considered as a PPAR-γ agonist, and the decrease in survivin was due to the activation of the PPAR-γ pathway by TQ. It has been previously demonstrated that PPAR-γ agonists, such as troglitazone, could suppress tumor growth *in vitro* and *in vivo* [23,24]. A recent phase II study showed that pioglitazone, also a PPAR-γ agonist, in combination with the COX-2 inhibitor, rofecoxib, was well tolerated in patients with high-grade gliomas [58]. The increase in PPAR-β/δ activity by TQ treatment was also found in this study, with no significant

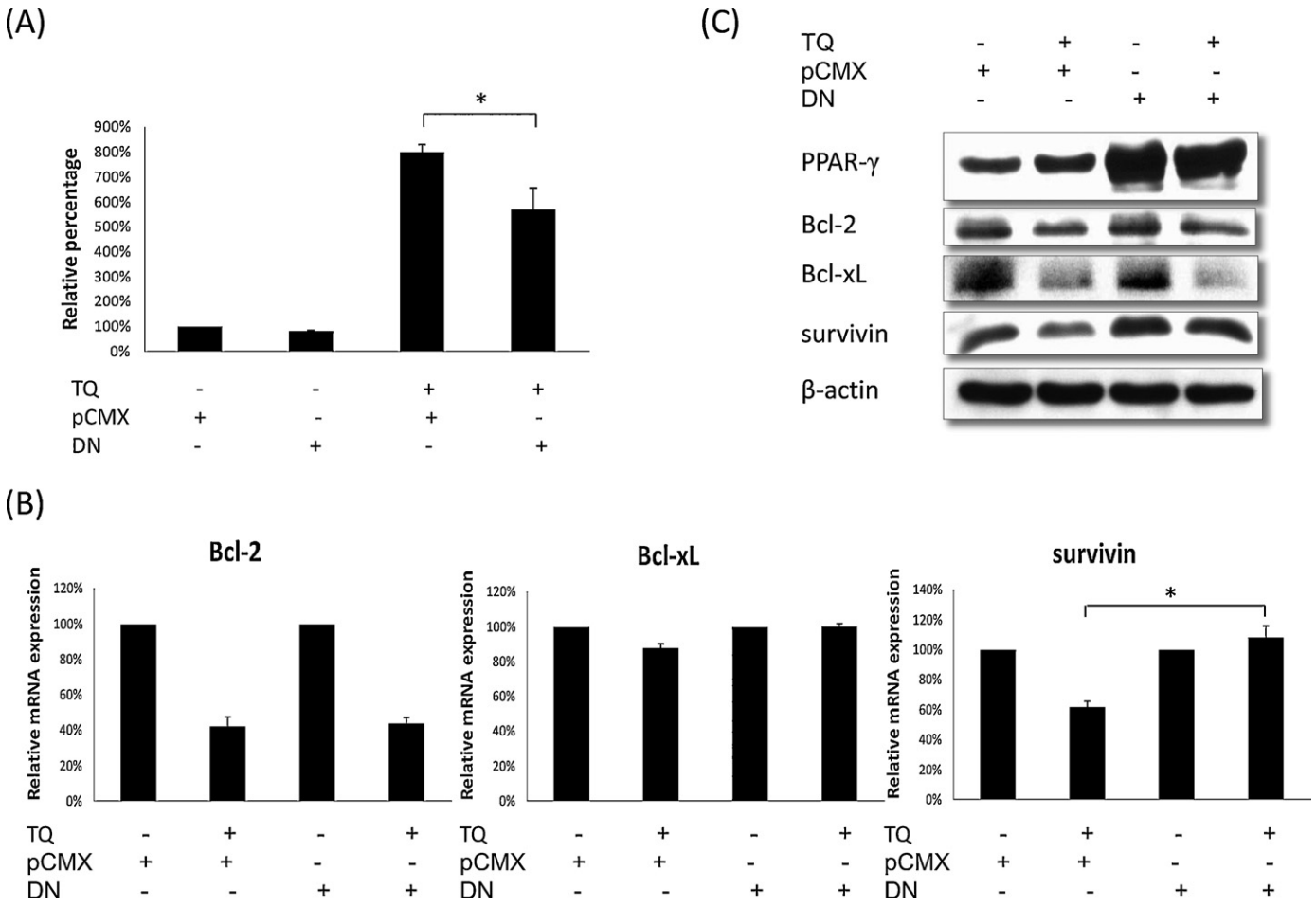

Fig. 7. PPAR-γ dominant negative reverses the effect of TQ on PPAR-γ activity and survivin expression level in MCF-7 breast cancer cells. (A) PPAR-γ dominant negative reverses TQ-induced PPAR-γ activity. Cells were transfected with PPAR-γDN or pCMX plasmids, together with pPPRE-tk-Luc and Renilla plasmids for 12–14 h before treatment with 40 μM of TQ for 18 h. The data are expressed as percentages of the PPAR-γ activity relative to the control. Values are means ± S.E.M. of two or three independent experiments. $^*p < 0.05$. (B) The mRNA expression of various targets with or without PPAR-γ dominant negative transfection in TQ-treated cells. Cells were transfected with PPAR-γDN or pCMX plasmids for 12–14 h before treatment with 40 μM of TQ for 8 h. Total RNA was isolated and analyzed by real time RT-PCR as described in Section 2. The data are expressed as the percentages of mRNA expression relative to their respective control. Values are means ± S.E.M. of two or three independent experiments. $^*p < 0.05$. (C) The protein expression of various targets with or without PPAR-γ dominant negative transfection in TQ-treated cells. Cells were transfected with PPAR-γDN or pCMX plasmids for 12–14 h before treatment with 40 μM of TQ for 24 h. Whole-cell extracts were resolved on SDS-PAGE gel and probed with the indicated antibodies. The data are representative of at least three independent experiments.

reduction after antagonist treatment suggesting that the increase was not specific. Some studies had described the involvement of PPAR-β/δ activity with tumorigenesis. For example, PPAR-β/δ was found to be accumulated in highly malignant cancer cells; however this was not correlated with Ki-67 proliferation marker in immunohistochemistry of colorectal cancer samples [59]. The use of PPAR-β/δ ligand, GW501516, was found to promote human hepatocellular cell growth [60]. However, there was study showing that PPAR-β/δ ligands, GW0742 and GW501516, were able to reduce the cell growth of both MCF-7 and UACC903 cell lines [61]. The role of PPAR-β/δ in cancer therapeutic still requires further comprehensive research, and the relationship of TQ and PPAR-β/δ may serve as an alternative platform to study PPAR-β/δ in cancer pathways.

In conclusion, this study showed that TQ inhibited breast cancer cell proliferation efficiently, revealing it to be a potentially effective suppressor of tumor cell growth, invasion and migration. It also highlighted for the first time that the anticancer effects of TQ involve the activation of the PPAR-γ pathway via binding to the receptor, and affecting its regulated gene products. Further *in vivo* studies are needed to better understand the role of TQ in breast cancer prevention and treatment.

Acknowledgements

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References

[1] Salem ML. Immunomodulatory and therapeutic properties of the *Nigella sativa* L. seed. Int Immunopharmacol 2005;5:1749–70.
[2] Burits M, Bucar F. Antioxidant activity of *Nigella sativa* essential oil. Phytother Res 2000;14:323–8.
[3] Hajhashemi V, Ghannadi A, Jafarabadi H. Black cumin seed essential oil, as a potent analgestic and anti-inflammatory drug. Phytother Res 2004;18:195–9.
[4] Badary OA, Taha RA, Gamal el-Din AM, Abdel-Wahab MH. Thymoquinone is a potent superoxide anion scavenger. Drug Chem Toxicol 2003;26:87–98.
[5] El Gazzar M, El Mezayen R, Marecki JC, Nicolls MR, Canastar A, Dreskin SC. Anti-inflammatory effect of thymoquinone in a mouse model of allergic lung inflammation. Int Immunopharmacol 2006;6:1135–42.

[6] Gali-Muhtasib H, Ocker M, Kuester D, Krueger S, El-Hajj Z, Diestel A, et al. Thymoquinone reduces mouse colon tumor cell invasion and inhibits tumor growth in murine colon cancer models. J Cell Mol Med 2008;12:330–42.
[7] Kaseb AO, Chinnakannu K, Chen D, Sivanandam A, Tejwani S, Menon M, et al. Androgen receptor- and E2F-1-targeted thymoquinone therapy for hormone-refractory prostate cancer. Cancer Res 2007;67:7782–8.
[8] Roepke M, Diestel A, Bajbouj K, Walluscheck D, Schonfeld P, Roessner A, et al. Lack of p53 augments thymoquinone-induced apoptosis and caspase activation in human osteosarcoma cells. Cancer Biol Ther 2007;6:160–9.
[9] Ivankovic S, Stojkovic R, Jukic M, Milos M, Milos M, Jurin M. The antitumor activity of thymoquinone and thymohydroquinone in vitro and in vivo. Exp Oncol 2006;28:220–4.
[10] El-Mahdy MA, Zhu Q, Wang QE, Wani G, Wani AA. Thymoquinone induces apoptosis through activation of caspase-8 and mitochondrial events in p53-null myoloblastic leukemia HL-60 cells. Int J Cancer 2005;117:409–17.
[11] Gali-Muhtasib H, Diab-Assaf M, Boltze C, Al-Hmaira J, Hartig R, Roessner A, et al. Thymoquinone extracted from black seed triggers apoptotic cell death in human colorectal cancer cells via a p53-dependent mechanism. Int J Oncol 2004;25:857–66.
[12] El-Najjar N, Chatila M, Moukadem H, Vuorela H, Ocker M, Gandesiri M, et al. Reactive oxygen species mediate thymoquinone-induced apoptosis and activate ERK and JNK signaling. Apoptosis 2010;15:183–95.
[13] Yi T, Cho SG, Yi Z, Pang X, Rodriguez M, Wang Y, et al. Thymoquinone inhibits tumor angiogenesis and tumor growth through suppressing Akt and extracellular signal-regulated kinase signaling pathways. Mol Cancer Ther 2008;7:1789–96.
[14] Gali-Muhtasib H, Kuester D, Mawrin C, Bajbouj K, Diestel A, Ocker M, et al. Thymoquinone triggers inactivation of the stress response pathway sensor CHEK1 and contributes to apoptosis in colorectal cancer cells. Cancer Res 2008;68:5609–18.
[15] Li F, Rajendran P, Sethi G. Thymoquinone inhibits proliferation, induces apoptosis and chemosensitizes human multiple myeloma cells through suppression of signal transducer and activator of transcription 3 activation pathway. Br J Pharmacol 2010;161:541–54.
[16] Feige JN, Gelman L, Michalik L, Desvergne B, Wahli W. From molecular action to physiological outputs: PPAR are nuclear receptors at the crossroads of key cellular functions. Prog Lipid Res 2006;45:120–59.
[17] Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schütz G, Umesono K, et al. The nuclear receptor superfamily: the second decade. Cell 1995;83:835–9.
[18] Berger J, Moller DE. The mechanism of action of PPARs. Annu Rev Med 2002;53:409–35.
[19] Sertznig P, Seifert M, Tilgen W, Reichrath J. Present concepts and future outlook: function of peroxisome proliferator-activated receptors (PPARs) for pathogenesis, progression, and therapy of cancer. J Cell Physiol 2007;212:1–12.
[20] Yin F, Wakino S, Liu Z, Kim S, Hsueh WA, Collins AR, et al. Troglitazone inhibits growth of MCF-7 breast carcinoma cells by targeting G1 cell cycle regulators. Biochem Biophys Res Commun 2001;286:916–22.
[21] Kumar AP, Quake AL, Chang MK, Zhou T, Lim KS, Singh R, et al. Repression of NHE1 expression by PPARgamma activation is a potential new approach for specific inhibition of the growth of tumor cells in vitro and in vivo. Cancer Res 2009;69:8636–44.
[22] Venkatachalam G, Kumar AP, Yue LS, Pervaiz S, Clement MV, Sakharkar MK. Computational identification and experimental validation of PPRE motifs in NHE1 and MnSOD genes of human. BMC Genomics 2009;10:S5.
[23] Sarraf P, Mueller E, Jones D, King FJ, DeAngelo DJ, Partridge JB, et al. Differentiation and reversal of malignant changes in colon cancer through PPARgamma. Nat Med 1998;4:1046–52.
[24] Kubota T, Koshizuka K, Williamson EA, Asou H, Said JW, Holden S, et al. Ligand for peroxisome proliferator-activated receptor gamma (troglitazone) has potent antitumor effect against human prostate cancer both in vitro and in vivo. Cancer Res 1998;58:3344–52.
[25] Chang TH, Szabo E. Induction of differentiation and apoptosis by ligands of peroxisome proliferator-activated receptor gamma in non-small cell lung cancer. Cancer Res 2000;60:1129–38.
[26] Motomura W, Okumura T, Takahashi N, Obara T, Kohgo Y. Activation of peroxisome proliferator-activated receptor gamma by troglitazone inhibits cell growth through the increase of p27KiP1 in human pancreatic carcinoma cells. Cancer Res 2000;60:5558–64.
[27] Elstner E, Müller C, Koshizuka K, Williamson EA, Park D, Asou H, et al. Ligands for peroxisome proliferator-activated receptorgamma and retinoic acid receptor inhibit growth and induce apoptosis of human breast cancer cells in vitro and in BNX mice. Proc Natl Acad Sci USA 1998;95:8806–11.
[28] Kim KY, Kim SS, Cheon HG. Differential anti-proliferative actions of peroxisome proliferator-activated receptor-γ agonists in MCF-7 breast cancer cells. Biochem Pharmacol 2006;72:530–40.
[29] Liu H, Zang C, Fenner MH, Possinger K, Elstner E. PPARgamma ligands and ATRA inhibit the invasion of human breast cancer cells in vitro. Breast Cancer Res Treat 2003;79:63–74.
[30] Panigrahy D, Singer S, Shen LQ, Butterfield CE, Freedman DA, Chen EJ, et al. PPARgamma ligands inhibit primary tumor growth and metastasis by inhibiting angiogenesis. J Clin Invest 2002;110:923–32.
[31] Han S, Roman J. Peroxisome proliferator-activated receptor gamma: a novel target for cancer therapeutics? Anticancer Drugs 2007;18:237–44.
[32] Abe A, Kiriyama Y, Hirano M, Miura T, Kamiya H, Harashima H, et al. Troglitazone suppresses cell growth of KU812 cells independently of PPARgamma. Eur J Pharmacol 2002;436:7–13.
[33] Lecomte J, Flament S, Salamone S, Boisbrun M, Mazerbourg S, Chapleur Y, et al. Disruption of ERalpha signalling pathway by PPARgamma agonists: evidences of PPARgamma-independent events in two hormone-dependent breast cancer cell lines. Breast Cancer Res Treat 2008;112:437–51.
[34] Colin C, Salamone S, Grillier-Vuissoz I, Boisbrun M, Kuntz S, Lecomte J, et al. New troglitazone derivatives devoid of PPARgamma agonist activity display an increased antiproliferative effect in both hormone-dependent and hormone-independent breast cancer cell lines. Breast Cancer Res Treat 2010;124:101–10.
[35] Zaytseva YY, Wang X, Southard RC, Wallis NK, Kilgore MW. Down-regulation of PPARgamma1 suppresses cell growth and induces apoptosis in MCF-7 breast cancer cells. Mol Cancer 2008;7:90.
[36] Lefebvre AM, Chen I, Desreumaux P, Najib J, Fruchart JC, Geboes K, et al. Activation of the peroxisome proliferator-activated receptor gamma promotes the development of colon tumors in C57BL/6J-APCMin/+ mice. Nat Med 1998;4:1053–7.
[37] Lacroix M, Leclercq G. Relevance of breast cancer cell lines as models for breast tumours: an update. Breast Cancer Res Treat 2004;83:249–89.
[38] Zaytseva YY, Wallis NK, Southard RC, Kilgore MW. The PPAR{gamma} antagonist T0070907 suppresses breast cancer cell proliferation and motility via both PPAR{gamma}-dependent and -independent mechanisms. Anticancer Res 2011;31:813–23.
[39] Kourtidis A, Srinivasaiah R, Carkner RD, Brosnan MJ, Conklin DS. Peroxisome proliferator-activated receptor-gamma protects ERBB2-positive breast cancer cells from palmitate toxicity. Breast Cancer Res 2009;11:R16.
[40] Li F, Fernandez PP, Rajendran P, Hui KM, Sethi G. Diosgenin, a steroidal saponin, inhibits STAT3 signaling pathway leading to suppression of proliferation and chemosensitization of human hepatocellular carcinoma cells. Cancer Lett 2010;292:197–207.
[41] Chua AW, Hay HS, Rajendran P, Shanmugam MK, Li F, Bist P, et al. Butein downregulates chemokine receptor CXCR4 expression and function through suppression of NF-κB activation in breast and pancreatic tumor cells. Biochem Pharmacol 2010;80:1553–62.
[42] Effenberger-Neidnicht K, Schobert R. Combinatorial effects of thymoquinone on the anti-cancer activity of doxorubicin. Cancer Chemother Pharmacol 2011;67:867–74.
[43] Nolte RT, Wisely GB, Westin S, Cobb JE, Lambert MH, Kurokawa R, et al. Ligand-binding and coactivator assembly of the peroxisome proliferator-activated receptor-gamma. Nature 1998;395:137–43.
[44] Gampe Jr RT, Montana VG, Lambert MH, Miller AB, Bledsoe RK, Milburn MV, et al. Asymmetry in the PPARgamma/RXRalpha crystal structure reveals the molecular basis of heterodimerisation among nuclear receptors. Mol Cell 2000;5:545–55.
[45] Salam NK, Huang TH, Kota BP, Kim MS, Li Y, Hibbs DE. Novel PPARg agonists identified from a natural product library: a virtual screening, induced-fit docking and biological assay study. Chem Biol Drug Des 2008;71:57–70.
[46] Parkin DM. Global cancer statistics in the year 2000. Lancet Oncol 2001;2:533–43.
[47] Bristow MR, Billingham ME, Mason JW, Daniels JR. Clinical spectrum of anthracycline cardiotoxicity. Cancer Treat Rep 1978;62:873–9.
[48] Sethi G, Ahn KS, Aggarwal BB. Targeting NF-κB activation pathway by thymoquinone: role in suppression of antiapoptotic gene products and enhancement of apoptosis. Mol Cancer Res 2008;6:1059–70.
[49] Jafri SH, Glass J, Shi R, Zhang S, Prince M, Kleiner-Hancock H. Thymoquinone and cisplatin as a therapeutic combination in lung cancer: in vitro and in vivo. J Exp Clin Cancer Res 2010;29:87.
[50] Badary OA, Nagi MN, Al-Shabanah OA, Al-Sawaf HA, Al-Sohaibani MO, Al-Bekairi AM. Thymoquinone ameliorates the nephrotoxicity induced by cisplatin in rodents and potentiates its antitumor activity. Can J Physiol Pharmacol 1997;75:1356–61.
[51] Al-Shabanah OA, Badary OA, Nagi MN, Al-Gharably NM, Al-Rikabi AC, Al-Bekairi AM. Thymoquinone protects against doxorubicin-induced cardiotoxicity without compromising its antitumor activity. J Exp Clin Cancer Res 1998;17:193–8.
[52] Arafa ES, Zhu Q, Shah ZI, Wani G, Barakat BM, Racoma I, et al. Thymoquinone up-regulates PTEN expression and induces apoptosis in doxorubicin-resistant human breast cancer cells. Mutat Res 2011;706:28–35.
[53] Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000;100:57–70.
[54] Torres MP, Ponnusamy MP, Chakraborty S, Smith LM, Das S, Arafat HA, et al. Effects of thymoquinone in the expression of mucin 4 in pancreatic cancer cells: implications for the development of novel cancer therapies. Mol Cancer Ther 2010;9:1419–31.
[55] Carmichael AR. Obesity as a risk factor for development and poor prognosis of breast cancer. Br J Obstet Gynaecol 2006;113:1160–6.
[56] Jiang WG, Douglas-Jones A, Mansel RE. Expression of peroxisome-proliferator activated receptor-gamma (PPARgamma) and the PPARgamma co-activator PGC-1, in human breast cancer correlates with clinical outcomes. Int J Cancer 2003;106:752–7.
[57] Johnstone RW, Ruefli AA, Lowe SW. Apoptosis: a link between cancer genetics and chemotherapy. Cell 2002;108:153–64.
[58] Hau P, Kunz-Schughart L, Bogdahn U, Baumgart U, Hirschmann B, Weimann E, et al. Low-dose chemotherapy in combination with COX-2 inhibitors and PPAR-gamma agonists in recurrent high-grade gliomas—a phase II study. Oncology 2007;73:21–5.

[59] Takayama O, Yamamoto H, Damdinsuren B, Sugita Y, Ngan CY, Xu X, et al. Expression of PPARdelta in multistage carcinogenesis of the colorectum: implications of malignant cancer morphology. Br J Cancer 2006;95: 889–95.
[60] Xu L, Han C, Lim K, Wu T. Cross-talk between peroxisome proliferator-activated receptor delta and cytosolic phospholipase A(2)alpha/cyclooxygenase-2/prostaglandin E(2) signaling pathways in human hepatocellular carcinoma cells. Cancer Res 2006;66:11859–68.
[61] Girroir EE, Hollingshead HE, Billin AN, Willson TM, Robertson GP, Sharma AK, et al. Peroxisome proliferator-activated receptor-beta/delta (PPARbeta/delta) ligands inhibit growth of UACC903 and MCF7 human cancer cell lines. Toxicology 2008;243:236–43.