EVALUATION OF THYMOQUINONE FOR CYTOTOXIC ACTIVITY AGAINST HUMAN BREAST CANCER CELL LINES AND TUMOR XENOGRAFT IN NUDE MICE

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Declaration

I hereby declare that the thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.

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(Woo Chern Chiuh)

12 Aug 2013

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Summary

Thymoquinone (TQ) is a natural compound isolated from the seed oil of *Nigella sativa*, a traditional herb native to Southwest Asia. Many types of carcinoma, for example lung, colon, liver and prostate, were found to be inhibited by TQ. However, the mechanism of the inhibitory effect of TQ on breast cancer is unclear. As such, in the present study, the effects of TQ on breast carcinoma were investigated both *in vitro* and *in vivo*. TQ was found to inhibit the growth of MCF-7, MDA-MB-231 and BT-474 breast cancer cells in a dose- and time-dependent manner. This growth inhibition could be further enhanced by combining TQ with known chemotherapeutic drugs, such as doxorubicin and 5-fluorouracil. No cell cycle arrest was observed after TQ treatment, however, subG1 accumulation was detected indicating apoptosis induction. Indeed, increased percentage of annexin V positive cells and increased PARP protein cleavage were observed after TQ treatment. In addition to apoptosis induction, TQ was able to inhibit breast cancer cell migration and invasion.

TQ was found to induce PPAR-γ activity in a dose- and time-dependent manner. Pre-treatment with GW9662, a PPAR-γ specific inhibitor, could abrogate TQ-induced PPAR-γ activity and TQ-induced apoptosis. Moreover, treatment with GW9662 and PPAR-γ dominant negative could reverse the decrease of survivin mRNA and protein levels induced by TQ. These results suggest that TQ suppressed survivin expression via PPAR-γ induction.

We found that TQ was able to induce ROS production in breast cancer cells in a time-dependent manner. This induction could be reversed by pre-treatment with N-acetylcysteine (NAC), a strong antioxidant. The growth inhibition and pro-apoptotic effects of TQ could also be abrogated by NAC. Moreover, the decrease of anti-apoptotic proteins, such as survivin, XIAP, Bcl-2 and Bcl-xL, induced by TQ could also be reversed by NAC. We also found that PPAR-γ could be the downstream effector of ROS in the mechanism of action of TQ.

TQ was found to increase p38 phosphorylation, whereby this induction could be reversed by pre-treatment with SB203580, a p38-specific inhibitor. Moreover, the growth inhibition and pro-apoptotic effects of TQ in breast cancer cells could also be abrogated by SB203580. The pro-apoptotic role of TQ-induced p38 activation was also confirmed by p38 siRNA gene silencing. We found that TQ-induced ROS production was able to affect p38 phosphorylation but not *vice versa*. In MCF-7 cells, PPAR-γ and p38 appeared to antagonize each other in the mechanism of action of TQ.

In addition, TQ was able to suppress breast tumor growth in nude mice and combined with doxorubicin to produce greater suppression. Reduced cell proliferation and increased apoptosis were found in the tumor tissues of TQ-treated mice. Moreover, TQ increased the hepatic level of anti-oxidant enzymes/molecules (catalase, superoxide dismutase and glutathione) in these mice.

Taken together, the present study demonstrates the potential anticancer activities of TQ in human breast carcinoma. ROS, PPAR-γ and p38 pathways are possibly involved in the antitumor action of TQ.

List of Publications

Journals

Woo CC, Hsu Annie, Kumar AP, Sethi G, Tan BKH. Thymoquinone inhibits tumor growth and induces apoptosis in a breast cancer xenograft mouse model: the role of p38 MAPK and ROS. PLoS One. 2013 Oct 2;8(10):e75356.

Wong FC, Woo CC, Hsu A, Tan BKH. The anti-cancer activities of Vernonia amygdalina extract in human breast cancer cell lines are mediated through caspase-dependent and p53-independent pathways. PLoS One. 2013 Oct 24;8(10):e78021.

Woo CC, Kumar AP, Sethi G, Tan BKH. Thymoquinone: potential cure for inflammatory disorders and cancer. Biochem Pharmacol. 2012 Feb 15;83(4):443-51.

Woo CC, Loo SY, Gee V, Yap CW, Sethi G, Kumar AP, Tan BKH. Anticancer activity of thymoquinone in breast cancer cells: possible involvement of PPAR-γ pathway. Biochem Pharmacol. 2011 Sep 1;82(5):464-75.

Conferences (poster presentation)

Woo CC, Kumar AP, Sethi G, Tan BKH. Thymoquinone inhibits proliferation and induces apoptosis by ROS mediated p38 MAP Kinase activation in breast cancer cells. National Cancer Research Institute (NCRI) Cancer Conference 4-7 Nov 2012, BT Convention Centre, Liverpool, UK.

Woo CC, Kumar AP, Sethi G, Tan BKH. Cytotoxicity of thymoquinone: possible involvement of the PPAR-γ pathway. Frontier in Cancer Sciences 8-10 Nov 2010, NUHS Auditorium, Singapore.

Woo CC, Sethi G, Tan BKH. Thymoquinone induces apoptosis and down-regulate Bcl-2 protein in breast cancer cell lines. International Anatomical Sciences and Cell Biology Conference 26-29 May 2010, NUS UCC, Singapore.

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List of Abbreviations

AP-1	Activator protein 1
BMI	Body mass index
BRCA1	Breast cancer type 1 susceptibility protein
BRCA2	Breast cancer type 2 susceptibility protein
Cdk-4	Cyclin-dependent kinase 4
C/EBPβ	CCAAT/enhancer-binding protein beta
CML	Chronic myelogenous leukemia
COX-2	Cyclooxygenase 2
DAPI	4',6-diamidino-2-phenylindole
DMP1	Dentin matrix acidic phosphoprotein 1
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ER	Estrogen receptor
ERK1/2	Extracellular signal-regulated kinases 1/2
FDA	U.S. Food and Drug Administration
FOXO3a	Forkhead box O3
HBP1	HMG-box transcription factor 1
HER-2	Human epidermal growth factor receptor 2
HIF-α	Hypoxia-inducible factors α
HRP	Horseradish peroxidase
IL-2	Interleukin 2
IL-10	Interleukin 10
JNK	c-Jun N-terminal kinases
MAPK	Mitogen-activated protein kinase
MAPKKK	Mitogen-activated protein kinase kinase kinase

MKK3	Mitogen-activated protein kinase kinase 3
MKK6	Mitogen-activated protein kinase kinase 6
NAC	N-acetylcysteine
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NOXA	Phorbol-12-myristate-13-acetate-induced protein 1
PAGE	Polyacrylamide gel electrophoresis
PPAR-α	Peroxisome proliferator-activated receptor α
PPAR-β/δ	Peroxisome proliferator-activated receptor β/δ
PPAR-γ	Peroxisome proliferator-activated receptor γ
PPRE	Peroxisome proliferators response element
PUMA	p53 upregulated modulator of apoptosis
ROS	Reactive oxygen species
RXR	Retinoid X receptor
SDS	Sodium dodecyl sulfate
SiRNA	Small interfering RNA
SOD	Superoxide dismutase
STAT-3	Signal transducers and activators of transcription 3
TBST	Tris-Buffered Saline and Tween 20
TNF-α	Tumor necrosis factor α
TQ	Thymoquinone
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling

1 INTRODUCTION

1.1 Breast cancer: epidemiology and risk factors

Breast cancer is a type of cancer occurring at breast tissue, and this type of cancer is more common in female population than male. There are two types of breast cancer namely ductal carcinoma and lobular carcinoma. Ductal carcinoma is originating from breast ducts, which are tubes that move milk from the breast to nipple. Lobular carcinoma is originating from lobules, the parts of the breast that produce milk. Classification of breast cancer is based on several aspects such as histopathology, grade, stage, DNA classification (gene mutation such as BRCA1/2 and p53) and receptor status (estrogen receptor (ER), progresterone receptor (PR) and human epidermal growth factor receptor 2 (HER2)). Classification of breast cancer is important for physicians to design appropriate regimen to treat breast tumor. Triple-negative breast cancer refers to breast cancer that demonstrated the absence of ER and PR, as well as the lack of HER2 over-expression. This type of breast cancer accounts for 10-20% of invasive breast cancer cases (Boyle, 2012). Luminal-A breast cancers represent ER-positive and/or PR-positive but HER2-negative, while luminal-B breast cancers exhibit ER-positive and/or PR-positive as well as HER2-positive. HER2 over-expressing breast cancers are ER-negative and PR-negative but HER2-positive. “Basal-like” breast cancers are defined as ER-negative, PR-negative, HER2 negative, cytokeratin 5/6 positive and/or epidermal growth factor receptor positive (Boyle, 2012).

According to the global cancer statistic by Jemal et al., breast cancer showed the highest number of estimated new cases (23%) and estimated deaths (14%) than other types of cancer in female population worldwide (Jemal et al., 2008). The incidence of breast cancer is relatively higher in developed countries/regions including Western and Northern Europe, North America, Australia and New Zealand (Jemal et al., 2008). In Singapore, breast cancer is the commonest cancer in females follow by colo-rectum and lung cancer (Teo and Soo, 2013). The age-standardized rate of breast cancer in Singapore is 60/100,000 at recent years (Teo and Soo, 2013), increased dramatically from 20/100,000 in 1968-1972 (Singapore Cancer Registry). The age-standardized

mortality rate of breast cancer is 14.1/100,000, which is the highest among other cancers in Singapore females (Teo and Soo, 2013). In a life time, 1 in 16 of Singaporean women will be diagnosed with breast cancer, compared to 1 in 8 women in Western countries.

There are many risk factors in breast cancer such as age and gender, gene mutations, family history, early menarche, late menopause and alcohol intake (Key et al., 2001; Higa, 2009). From epidemiological studies, obesity has been associated with increased risk of cancer including breast, kidney, pancreas and liver cancer (World Cancer Research Fund/American Institute for Cancer Research 2007). Adipose tissues express sex-steroid metabolizing enzyme such as aromatase that can increase the formation of estrogens from androgenic precursors (Renehan et al., 2006). Higher level of pro-inflammatory cytokines, such as TNF-α, IL-2 and IL-10, are associated with body adiposity (Vucenik and Stains, 2012). The conclusion that obesity leads to poorer prognosis can be explained by a meta-analysis study reported that breast cancer patients who were obese at the time of diagnosis had 33% higher rate of cancer-specific and overall mortality compared to non-obese patients (Protani et al., 2010). Indeed, patients with triple-negative breast cancer were more likely being overweight (Kwan et al., 2009). Furthermore, physically active women showed 25% lower in breast cancer risk compared to the least active women (Lynch et al., 2011). A large scale case-referent study in Japan had reported that women who exercised for healthy life twice or more per week had reduced risk of breast cancer, and this protection was greater in high BMI women (OR=0.57) than medium BMI women (OR=0.71) (Hirose et al., 2003).

In addition, childbirth is able to reduce the risk of breast cancer, with greater protection for early first birth and large number of childbearing (Key et al., 2001). A re-analysis of 47 epidemiological studies in 30 countries showed that the relative risk of breast cancer could be reduced by 4.3% with every 12 months of breastfeeding, and 7% for each childbirth (Collaborative Group on Hormonal Factors in Breast Cancer 2002). This report also suggested that the shorter duration of breastfeeding in developed countries might partly responsible for the high incidence rate of breast cancer in these countries.

Smoking is a well-known risk factor for several types of malignancy namely lung, breast and head and neck cancer (Ligibel, 2012). It had been reported that the relative risk of breast cancer of current smokers versus never smokers was 1.7 in Japan population (Nagata et al., 2006). A recent study of 300,000 Norwegian women reported that ever smokers had 15% increased risk of breast cancer compared to never smokers (Bjerkaas et al., 2013). Interestingly, smokers who started to smoke after the first childbirth did not showed significant difference in breast cancer risk compared to never smokers (Bjerkaas et al., 2013).

Migrant and other ecological studies showed that people migrated from areas of low breast cancer incidence to areas of high breast cancer incidence would acquire the risk of the indigenous population in one or two generations (Nelson, 2006). These studies suggested that the environmental factors, such as diet and life-styles, were able to affect the risk of breast cancer because the genetic pool of a population won't deviate much in one or two generations (Vera-Ramirez et al., 2013). Indeed, high consumption of meat, particularly red meat, has been associated with increased risk of breast cancer (Zheng and Lee, 2009), with Indonesia OR=8.47, Taiwan OR=5.1 and China OR=2.9 (Park et al., 2008). This could be due to the excessive exposure to sex hormone through the consumption of meat derived from animals treated with sex hormones. In addition, the intake of animal fat was found to increase hormone level, which in turn, increased the risk of breast cancer (Vera-Ramirez et al., 2013). The study of 15,351 female subjects showed that women in high consumption of processed meat, butter, fish and other animal fats, as well as low consumption of bread and fruit juices, exhibited 2-fold higher risk of breast cancer (Schulz et al., 2008). Moreover, alcohol intake is also associated with increased incidence of breast cancer, particularly ER-positive, which could be due to the increase in the level of serum sex hormones (Zhang et al., 2007).

Cyclin D1 plays an important role in cell cycle progression, particularly G1 to S phase, by forming active enzyme complexes with cyclin-dependent kinases 4/6 (Matsushime et al., 1994). It was found that cyclin D1 was overexpressed in 50% of breast tumors (Velasco-Velázquez et al., 2011). This protein

negatively correlated with overall survival and relapse-free survival in breast cancer patients (Umekita et al., 2002). Interestingly, breast tumors with cyclin D1 overexpression were mostly estrogen receptor positive (Kenny et al., 1999). Breast cancer patients expressing low/moderate level of cyclin D1 showed higher response and better survival rate in tamoxifen therapy (Stendahl et al., 2004; Jirstrom et al., 2005). This might explain the failure of anti-estrogen therapy in some tumors despite they were ER-positive. In addition of its role in cell cycle progression, cyclin D1 can act as transcription regulator (Roy and Thompson, 2006). For example, cyclin D1 was shown to activate estrogen receptor signaling via ligand-independent fashion (Zwijsen et al., 1997). Moreover, cyclin D1 is able to interact with different transcription factors such as androgen receptor, DMP1 and C/EBPβ (Coqueret, 2002).

Mutations of genes which involved in DNA damage repair, chromosome remodeling and cell cycle progression, such as BRCA1 and BRCA2, are associated to about 10% breast cancer cases (Bayraktar and Glück, 2012). Women with BRCA1/2 mutations showed three times higher risk in developing breast cancer compared to general population (Liebens et al., 2007). BRCA1 is located at chromosome 17q21, while BRCA2 is 13q12.3. Point mutation in these genes can cause frameshift, nonsense and missense mutations (Cao et al., 2013). BRCA1 protein plays a role in repairing gene mutations, while BRCA2 protein is involved in the repair of chromosomal damage. Lifetime risk of developing breast cancer for women with BRCA1 mutation is 60-70%, while BRCA2 mutation is 40-60% (King et al., 2003; Antoniou et al., 2003). BRCA1 mutation carriers showed lower short-term and long-term overall survival rates than non-carriers (Lee et al., 2010). Among carriers with BRCA1 mutation, one-third were triple-negative breast cancer (Peshkin et al., 2010). Tumors with BRCA2 mutation usually expressed estrogen receptor and progresterone receptor, unlike BRCA1 mutation (Lakhani et al., 1998). Loss of BRCA1/2 functions could lead to chromosomal instability, which in turn, promoting tumorigenesis (Dhillon et al., 2011). BRCA1/2 mutation cancer cells were unable to repair DNA damages through homologous recombination (Moynahan et al., 1999), thus they were sensitive to inter-strand DNA cross-linking agents such as cisplatin. However, there are

studies reported that these cancer cells could acquire resistance to such chemotherapy agents by restoring BRCA1/2 functions via secondary BRCA1/2 mutations (Dhillon et al., 2011). Therefore, BRCA1/2 mutation carriers are recommended to undertake a particular surveillance protocol starting age of 30 to detect the onset of breast cancer (Apostolou and Fostira, 2013). Recently, there are increasing studies reporting the efficacy of Poly(ADP-ribose)polymerase inhibitors in patients with BRCA1/2 mutation, and several potential drug candidates of this class are currently in phase I/II clinical investigation (Lee et al., 2014).

TP53 gene, which encodes p53 protein, is an important gene that regulates cell cycle progression, DNA repair, cell senescence and apoptosis. This gene has been described as "guardian of the genome" for its role in conserving stability by preventing genome mutation. It has been reported that more than 50% of human tumors contained a mutation or deletion of TP53 gene (Hollstein et al., 1991). TP53 mutations are mostly missense point mutations that located in the central region encoding the DNA binding domain (Varna et al., 2011; Soussi et al., 2006). Some of the molecular targets of p53 are tsp1, p21, GADD45, Puma and Noxa. In addition to p53, there are another two members of p53 family, namely p63 and p73, which share the same functional domains of p53 (Lai et al., 2012). p53 plays an important role in regulating cellular redox status. Under normal physiological condition, low level of p53 suppresses ROS, while high level of p53 induces ROS accumulation in response to cellular stress (Vurusaner et al., 2012). Increased ROS will promote apoptotic cell death. Mutation to TP53 gene will cause a disease known as Li-Fraumeni syndrome. These patients are at high risk to develop cancer, which is 50% at age of 40 and up to 90% at age of 60 (Birch et al., 1998). Females with TP53 mutation are at high risk to develop breast cancer and 5% of these cases were occurred before the age of 30 (Gonzalez et al., 2009). It was shown that triple-negative breast cancer has increased frequency of TP53 mutation (Chae et al., 2009).

PTEN is a tumor suppressor gene because it can regulate PI3K/Akt pathway that is frequently involved in cancer survival and cell proliferation. Mutation of PTEN gene, which will cause Cowden syndrome, predisposed carriers to

several types of cancer including breast cancer, endometrial cancer and thyroid carcinoma (Li et al., 1997). Indeed, females with PTEN mutation have 50% lifetime risk to develop breast cancer (Apostolou and Fostira, 2013). In addition to BRCA1/2, TP53 and PTEN, there are other high-penetrant genes such as STK11 (serine/threonine kinase 11) and CDH1 (cadherin 1). Mutation of STK11 gene increased the risk of developing cancer to up to 85% (Hearle et al., 2006). Women with CDH1 mutation displayed 40–54% lifetime risk of developing lobular breast cancer (Kaurah et al., 2007).

In addition of loss-of-function in tumor suppressor genes, gain-of-function in oncogenes can also lead to tumorigenesis. Oncogenes such as ErbB2, PI3KCA, Myc and CCND1 are often deregulated in breast cancer (Lee and Muller, 2010). 20-30% of breast cancer cases exhibited increased level of HER2 due to gene amplification of ErbB2 (Slamon et al., 1989). Overexpression of ErbB2 often leads to aggressive tumor type (Lee and Muller, 2010). Moreover, there are studies showed that miR-155 was oncogenic in many types of tumor including breast (Wang and Wu et al., 2012).

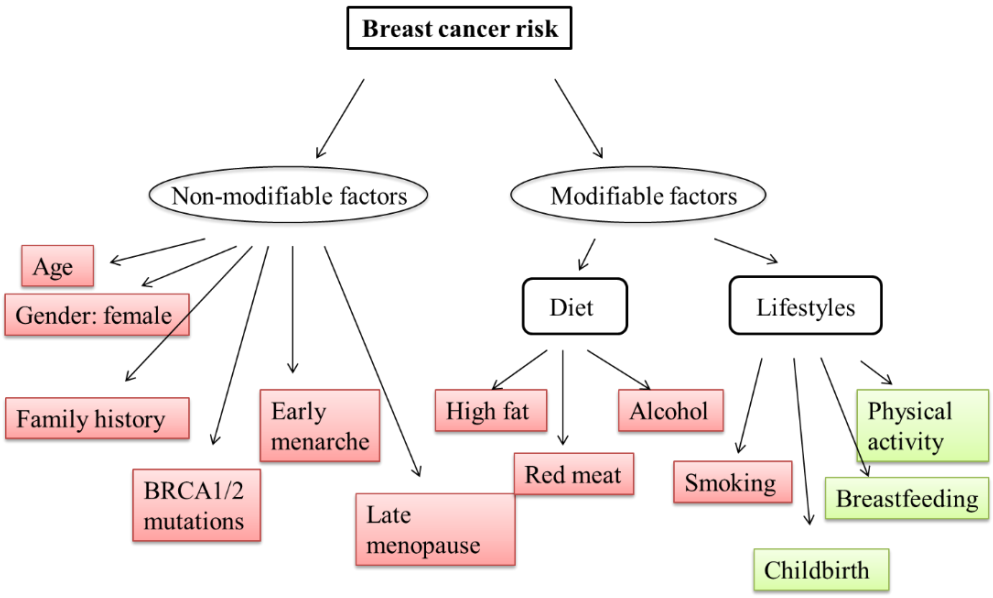


Figure 1.1: Factors that influence the risk of development of breast cancer.

Red boxes indicate increased risk, while green boxes indicate reduced risk.

1.2 Breast cancer: chemoprevention and treatment

Early signs of breast cancer include lump in breast area, change in nipple appearance, fluid leaking from the nipple and skin dimpling. After detection of signs and symptoms of breast cancer, imaging tests such as mammogram and magnetic resonance imaging scan will be used to further examine for breast disease. If these exams suggest the possibility of breast cancer, biopsy will eventually be used to confirm the disease. The tissues removed for biopsy will be analyzed by pathologist to classify breast cancer on several aspects such as type, grade and receptor status. This information will allow physicians to design appropriate treatment regimen to target the disease. For example, triple-negative breast cancers that usually led to poorer prognosis and higher risk of recurrence would receive greater attention from the physicians (Boyle, 2012).

A number of studies showed that breast screening could effectively lead to early detection of the disease, which in turn, resulting in an increase in breast cancer survival (Wang et al., 2011b). In the present, the treatments for breast cancer include surgery, radiation therapy, chemotherapy, hormone therapy and targeted therapy (Higa, 2009). Surgery is to physically remove tumor tissue, either lumpectomy or mastectomy. Lumpectomy removes the breast tumor with a margin of surrounding normal tissue, while mastectomy removes the entire breast and possibly nearby tissue.

Radiation therapy is to apply ionizing radiation on tumor area to control or kill cancerous cells. This type of therapy is normally given to the whole breast, while in certain cases it is also given to areas of lymph node close to the breast. Radiation therapy may be given before surgery to shrink the tumor or after surgery to kill any remaining cancer cells. Radiation therapy achieved high cure rate if distant metastasis has not occurred (Langlands et al., 2013).

Chemotherapy is to use chemotherapeutic drugs to kill highly replicating malignant cells, and it is normally given for patients with invasive and metastatic breast cancer. This type of therapy usually runs for 3-6 months and it is relatively well-tolerated in most women (Thomson et al., 2012). Neoadjuvant chemotherapy is given prior to surgery or radiation therapy for the purpose of tumor shrinking, which allows the later operation more feasible

and less destructive. Adjuvant chemotherapy is given after surgery to destroy remaining cancerous cells and prevent recurrence. Some of the examples of chemotherapeutic agent are doxorubicin, cyclophosphamide, paclitaxel and 5-fluorouracil (Lai et al., 2012). Doxorubicin is a DNA intercalating agent that is used in many different types of cancer including breast, lung, stomach and leukemia. It can stabilize topoisomerase II after this enzyme has broken the DNA chain for replication, whereby this will prevent DNA strands from being resealed and thus stopping DNA replication (Pommier et al., 2012). Cyclophosphamide is a nitrogen mustard alkylating agent that is first converted in liver to form active metabolites for its chemotherapeutic effect. The active metabolite, phosphoramide mustard, can form irreversible DNA crosslinks between and within DNA strands, which in turn, resulting in apoptotic cell death. Paclitaxel is a mitotic inhibitor used in cancer therapy for breast, ovarian and lung carcinoma. This drug can stabilize microtubules by interfering with the breakdown of microtubules during cell division (Bharadwaj and Yu, 2004). 5-fluorouracil is a pyrimidine analog that acts as a thymidylate synthase inhibitor. After administration, this drug can incorporate into DNA to prevent DNA synthesis, which in turn, resulting in cell cycle arrest and apoptosis. Tumors with TP53 mutation has been associated with poor response to chemotherapy (Varna et al., 2011).

The main idea of hormone therapy is to block estrogen hormone from supporting tumor growth, and this therapy is normally given for patients with ER-positive breast cancer. For example, tamoxifen can block ER from its ligand, while anastrozole (aromatase inhibitor) can block estrogen production (Cazzaniga and Bonanni, 2012). Tamoxifen is a prodrug, whereby it is metabolized in the liver by cytochrome P450 isoforms CYP2D6 and CYP3A4 to produce active metabolite 4-hydroxytamoxifen (Desta et al., 2004). 4-hydroxytamoxifen binds to ER to prevent the transcription of estrogen-responsive genes. Anastrozole can inhibit aromatase, which is the enzyme that involved in the conversion of androgen to estrogen. About 80% of breast cancers rely on hormone estrogen to grow, thus the inhibition of estrogen synthesis serves a good treatment strategy.

Targeted therapy, also known as biologic therapy, is to use special designed drug to target protein molecules that are involved in tumorigenesis. There are two main types of targeted therapy namely monoclonal antibody (e.g. trastuzumab and pertuzumab) and tyrosine kinase inhibitor (e.g. lapatinib) (Cazzaniga and Bonanni, 2012). HER2 over-expression can promote cell proliferation via PI3K/Akt pathway. Trastuzumab can bind to the domain IV of HER2 causing HER2 down-regulation. This will in turn interfere with Akt signaling (Kute et al., 2004). Slamon et al. reported that the combination of trastuzumab and standard chemotherapy produced greater clinical benefits including longer time to disease progression, higher response rate, longer survival and 20% reduction in death risk (Slamon et al., 2001). On the other hand, lapatinib can bind to the ATP-binding pocket of EGFR/HER2 protein kinase domain preventing self-phosphorylation, which in turn, resulting in the inhibition of subsequent downstream signaling (Nelson and Dolder, 2006).

In addition of treatment, more and more studies searched for effective ways to prevent or reduce breast cancer cases. Chemoprevention is defined as the use of natural, synthetic or biochemical agents to prevent, reverse or suppress carcinogenic process from developing into neoplastic disease (Cazzaniga and Bonanni, 2012). Since long term administration of a drug may cause side effect to the human body, chemoprevention is recommended to people with high risk to develop cancer. For carriers with BRCA1/2 mutations, yearly mammography and bilateral breast MRI screening are recommended starting at age of 25-30 (Kriege et al., 2004). Generally, breast cancer chemoprevention can be divided into two categories, namely ER-positive and ER-negative. For ER-positive breast cancer prevention, two major classes of agent are selective estrogen receptor modulators (e.g. tamoxifen and raloxifene) and aromatase inhibitors (e.g. anastrozole and exemestane). The first chemoprevention drug to receive FDA approval is tamoxifen, of which it can reduce the risk of breast cancer as much as one-half. Although the therapeutic efficacy of raloxifene is lower than tamoxifen, it is less toxic and does not increase the risk of endometrial cancer (Umar et al., 2012). The data from adjuvant trials suggested that aromatase inhibitors decreased the incidence of breast cancer by 40-50% (Cuzick, 2005). In contrast of ER-

positive breast cancer prevention, the main idea of ER-negative breast cancer prevention is to target certain cellular signaling pathways involved in carcinogenesis. Several classes of agent are used such as nuclear receptors (e.g. PPAR-γ), anti-inflammatory and antioxidant (e.g. COX-2), and membrane receptors and signal transduction (e.g. HER-2 and tyrosine kinase) (Cazzaniga and Bonanni, 2012). Nevertheless, there are studies looking at immunotherapy for cancer prevention, for example vaccines are used to target tumor-associated or tumor-specific antigens (Umar et al., 2012).

1.3 Breast cancer: limitations of current cancer treatment

Despite the effectiveness of anticancer agents in treating breast cancer, their applications are often limited by the toxicities caused by these agents. These toxicities may result in organ damage and even fatal in extreme cases.

Even though surgery is a good way to remove a solid tumor, however, post-mastectomy pain syndrome might occur in 20-30% of patients who had mastectomies. Post-mastectomy pain syndrome is thought to be linked with the damage of nerves in the chest and armpit after surgery. On the other hand, the main purpose of chemotherapy is to kill rapidly replicating malignant cells, however, it can also affect other highly dividing normal cells in human body. Gastrointestinal distresses such as nausea and vomiting are common in patients who had received chemotherapy. Also, these patients had suppressed immune system which might lead to viral infection such as herpes simplex virus (Elad et al., 2010). Moreover, paclitaxel was found to cause neuropathy in human body (Cliffer et al., 1998). Doxorubicin and trastuzumab have been shown to be associated with cardiotoxicity (Morris and Hudis, 2010) such as left ventricular dysfunction (Schmitz et al, 2012). Furthermore, tamoxifen has been reported to cause increased occurrence of deep vein thrombosis and possibly endometrial cancer (Brown, 2009). The combination of pertuzumab and trastuzumab was reported to cause cardiac toxicity in certain breast cancer patients (Portera et al., 2008). In addition to nausea and vomiting, 5-

fluorouracil can also damage cognitive function in rare cases (Wigmore et al., 2010). Furthermore, even though the external beam of radiation therapy is safe, some side effects might possibly occur such as fatigue, skin erythema and mild swelling, which could affect up to 100% patients (Whelan et al., 2000). Besides that, late side effects might possibly occur after radiation therapy, for example telangectasia and impaired cosmesis with fibrosis, as well as long term side effects such as arm lymphoedema and shoulder stiffness (Langlands et al., 2013). Nevertheless, patients who received hormone therapy commonly experienced hot flushes, painful joints and mood swings.

In addition to drug toxicity, cancer treatment is also plagued by the development of drug resistance. For example, cancer cells can increase the expression of drug efflux pump (e.g. P-glycoprotein, breast cancer resistance protein (ABCG2)) to transport drug molecules out from the cells, thus preventing therapeutic actions (Szakács et al., 2004). Moreover, certain cancer cells were found to amplify survival pathway to overcome drug action, for example, PI3K/Akt pathway was amplified as a mechanism to overcome trastuzumab (Puglisi et al., 2012). Furthermore, the effect of lapatinib, such as inhibition of Akt pathway, could be reversed by derepression of FOXO3a which resulting in the increase of estrogen receptor transcription and estrogen receptor signaling (Guo and Sonenshein, 2004). Cancer stem cells can up-regulate FOXO genes to increase the level of antioxidant enzymes, such as SOD (superoxide dismutase) and catalase, to maintain the redox status in the cells; hence, this will reduce the oxidative stress caused by radiation therapy and chemotherapy with redox-cycling agents (Tothova and Gilliland, 2007). Drug resistance was also found in the treatment of BRCA1/2 mutation cancers using platinum agents and PARP inhibitors. This might be due to the development of secondary or ‘reversion’ mutations that restored the activities of BRCA to certain degree (Maxwell and Domchek, 2012).

Due to drug toxicity and drug resistance problems, there are increasing studies searching for potential drugs that can replace or complement conventional medicine for greater treatment efficacy. Natural products or traditional medicine serve as a large reserve pool for such purpose.

1.4 Thymoquinone: a potential anticancer drug from natural products

Cancer is multi-factorial in origin and is affected by both genetic and environmental risk factors. The current trend of drug development is to develop a drug that can specifically target the signaling pathway that causes tumorigenesis. However, this kind of drug often leads to adverse effects and tumor resistance (Aggarwal et al., 2007). Thus, people start questioning whether this "one-target, one-drug approach" is capable to favorably solve the disease. Can a single-target drug treat a multi-factorial disease like cancer? It is well-known that drug combinations are more effective in treating complex diseases and are also less prone to acquired resistance (Keith et al., 2005). As such, it is possible that a compound that can target multiple signaling pathways is more effective because the disease system is less able to counter two or more interferences simultaneously (Aggarwal et al., 2007).

Cancer is a complex disease arising from multiple alterations in DNA such as mutations, deletions and rearrangements. Thus, the assumption of "one drug for one target" may not adequate to address complex disease like cancer, which has deregulation of multiple signaling pathways and possible development of drug resistance (Fimognari et al., 2012). As compared to specific inhibitors, natural products generally target multiple signaling pathways which might be more effective in inhibiting tumor growth. Since natural products are derived mostly from edible vegetables, fruits and tea, they are likely to be safe as a source of pharmacological chemicals.

48 of 65 drugs approved for cancer treatment over the period 1981-2002 were natural products or natural products-related (Newman et al., 2003). This suggests that natural products are great potential sources for new drug discovery. Numerous reports suggested that cancer signaling pathways could be inhibited by spice-derived nutraceuticals, for example capsaicinoids from red chili, curcumin from turmeric, and ursolic acid from rosemary (Sung, 2012). A case-control study of gastric cancer in Italy reported that the increased intake of fresh vegetables, fresh fruits, spices and garlic could reduce the risk of this disease (Buiatti et al., 1989). Moreover, it has been suggested that the lower colon cancer incidence in India compared to most

Western countries could be attributed to the consumption of spice (Kaefer and Milner, 2008). Indeed, high consumption of dark yellow-orange or green vegetables and fruits was found to reduce the risk of breast cancer (Park et al., 2008). A case-control study in Japan suggested that the consumption of soybean products, such as soy milk and tofu, could reduce the risk of breast cancer (OR=0.44) particularly in premenopausal women (Hirose et al., 2005). With extensive research, many natural products have great potential to be developed into anticancer agents.

Thymoquinone (TQ) is a phytochemical found in the traditional Ayurvedic herb, *Nigella sativa*, which is native to South and Southwest Asia. TQ was first extracted in 1963 by El-Dakhakhany (El-Dakhakhany, 1963). Since its identification, TQ has been investigated extensively for its therapeutic effects in different types of disease such as cancer, inflammation, atherosclerosis, sepsis and diabetes (Woo et al., 2012). The results from a double-blind crossover clinical trial showed that TQ was able to produce antiepileptic effect in children with epilepsy (Akhondian et al., 2011). It was also found that the human body could tolerate a dose of TQ up to 2600 mg/day without any significant adverse effect (Al-Amri et al., 2009), suggesting the safe use of TQ in humans.

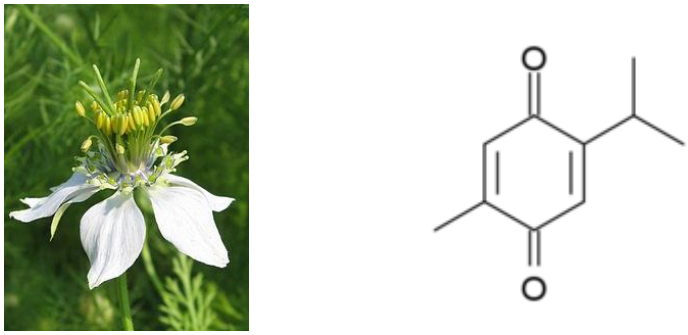


Figure 1.2: Flower of *Nigella sativa* (left panel) and the molecular structure of thymoquinone (right panel).

Both pictures are adapted from internet Wikipedia.

It was reported in many studies that TQ was able to suppress various types of cancer cells, including leukemia (HL-60 and Jurkat) (El-Mahdy et al., 2005; Alhosin et al., 2010), glioma/glioblastoma (U87 MG and T98G, M059K and M059J) (Cecarini et al., 2010; Gurung et al., 2010), pancreatic cancer (MIA PaCa-2, HPAC and BxPC-3) (Banerjee et al., 2009; Rooney et al., 2010), colorectal carcinoma (HT-29, HCT-116, DLD-1, Lovo and Caco-2) (Rooney et al., 2010; El Najjar et al., 2010), osteosarcoma (MG63 and MNNG/HOS) (Roepke et al., 2007) and prostate cancer (LNCaP, C4-2B, DU145 and PC-3) (Richards et al., 2006; Kaseb et al., 2007; Koka et al., 2010). In addition, the combination of TQ with conventional medicine produced greater cytotoxic effect, for example combined with cisplatin in NCI-H460 non-small cell lung cancer cells (Jafri et al., 2010), paclitaxel or doxorubicin in KBM-5 human myeloid cells (Sethi et al., 2008), and gemcitabine or oxaliplatin in HPAC human pancreatic cancer cells (Banerjee et al., 2009).

The antitumor effects of TQ were also reported in animal models with different types of carcinoma. TQ was found to reduce the number and size of aberrant crypt foci in 1,2-dimethyl hydrazine-induced colon cancer in mice (Gali-Muhtasib et al., 2008b). Oral administration of TQ inhibited forestomach tumor incidence and multiplicity in benzo(a)pyrene-induced forestomach tumor mouse model (Badary et al., 1999). Moreover, TQ was found to inhibit the growth of various tumor xenograft models, including HCT-116 cell-induced colon tumor xenograft (Gali-Muhtasib et al., 2008b), C4-2B cell-induced prostate tumor xenograft (Kaseb et al., 2007), HPAC cell-induced pancreatic tumor xenograft (Banerjee et al., 2009) and NCI-H460 cell-induced lung tumor xenograft (Jafri et al., 2010).

In addition, TQ showed potential inhibitory effects in cancer metastasis and angiogenesis. For example, TQ was found to inhibit human umbilical vein endothelial cell migration, invasion, and tube formation (Yi et al., 2008). TQ suppressed the migration of FG/COLO357 pancreatic cancer cells in a dose-dependent manner (Torres et al., 2010), and inhibited the invasion of NCI-H460 cells (Jafri et al., 2010). Moreover, TQ was found to significantly reduce the number of blood vessels in the tumors of PC-3 cell-induced prostate tumor

xenograft mouse model, indicating its inhibitory role in angiogenesis (Yi et al., 2008).

Taken together, TQ has promise as a potential anticancer agent. However, the anticancer effect of TQ in breast carcinoma was not well explained. As such, we were interested to investigate the detailed molecular mechanism(s) of action of TQ in breast carcinoma.

Table 1.1: The anticancer effects of thymoquinone and its molecular targets.

Anticancer effects	Molecular Targets	References
Growth inhibition	p53	Gali-Muhtasib et al. (2004a)
	p73	Alhosin et al. (2010)
	ROS	El-Najjar et al. (2010)
Pro-apoptotic	p53	Gali-Muhtasib et al. (2008a)
	p73	Alhosin et al. (2010)
	PTEN	Arafa et al. (2011)
	ROS	El-Najjar et al. (2010)
	Mucin-4	Torres et al. (2010)
Anti-inflammatory	STAT-3	Li et al. (2010)
	NF-κB	Sethi et al. (2008)
Anti-metastasis	MMP-9	Sethi et al. (2008)
Anti-angiogenesis	VEGF	Sethi et al. (2008)

1.5 Reactive oxygen species (ROS): role in tumorigenesis

Reactive oxygen species (ROS) are oxygen-containing reactive molecules or ions, which are formed via incomplete one electron reduction of oxygen (Pan et al., 2009).

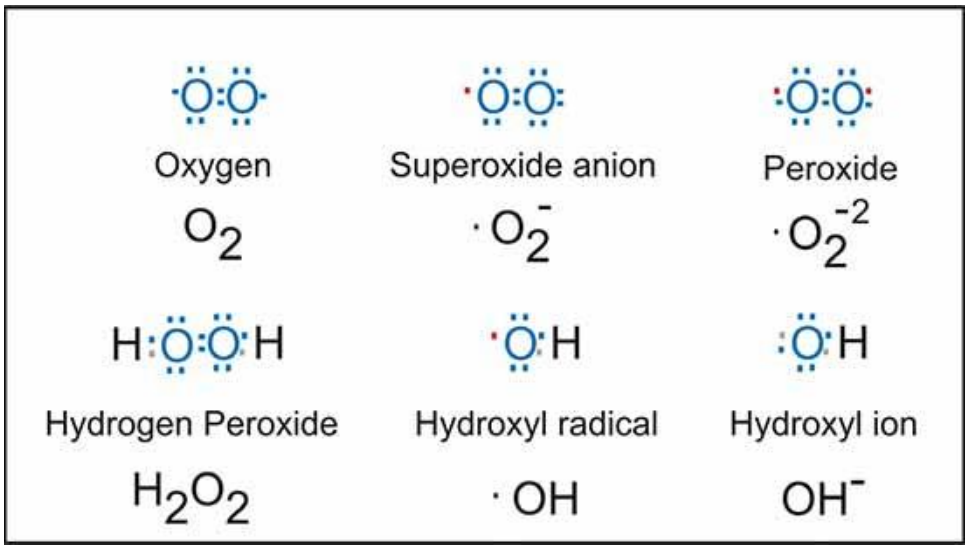


Figure 1.3: Electron structure of common reactive oxygen species.

Diagram adapted from http://www.biotek.com/resources/articles/reactive-oxygen-species.html

ROS can be generated from exogenous or endogenous sources of which some of them are carcinogenic (Cui, 2012). Mitochondria are significant sources of ROS due to their function as the power producer of a cell which consume oxygen molecules. ROS play important functions in cellular system including apoptosis, innate immunity, biosynthetic processes and cell signaling cascades (Rada and Leto, 2008; Brieger et al., 2012). Under normal physiological system, cells regulate the level of ROS by balancing ROS production and their scavenging system. However, the accumulation of ROS will damage proteins, lipids, membranes and even DNA. Some of the examples of DNA damage are single and double-stranded DNA breaks, DNA-protein crosslinks, depurination and depyrimidination (Barzilai and Yamamoto, 2004). Cellular

defense systems against ROS accumulation comprise of many detoxification enzymes including glutathione peroxidase, glutathione-S-transferase, catalase, superoxide dismutase and epoxide hydrolase (Acharya et al., 2010). Moreover, small molecules such as glutathione, ascorbic acid (vitamin C) and tocophenol (vitamin E) are also involved in cellular antioxidant processes. Excessive oxidative stress will lead to the development of human diseases such as neurodegenerative, metabolic and inflammatory diseases as well as cancer (Vurusaner et al., 2012).

Increasing studies reported the idea of ROS which they play a double edge "sword" in carcinogenesis. The DNA damage caused by ROS can lead to the initiation and progression of carcinogenesis (Brieger et al., 2012). Gene mutations, particularly TP53, induced by ROS are mainly due to the modification of guanine, causing G to T transversion (Hollstein et al., 2001; Lunec et al., 2002). Low level of ROS promotes cancer cell growth partly due to its role as a mediator to Ras-induced cell cycle progression (Irani et al., 1997). ROS were found to promote tumorigenesis by activating ERK1/2 via Ras, whereby ERK1/2 involved in survival pathways such as cell growth and apoptosis prevention (Abe et al., 2000; Aikawa et al., 1997). Oxidized DNA bases, such as 8-oxo-deoxyguanosine and thymine glycol, can lead to the initiation of oncogenes as well as the suppression of tumor suppressor genes (Kang, 2002). In addition, ROS were found to activate tumor promoting transcription factors such as NF-κB, AP-1, STAT3 and HIF-1α (Gupta et al., 2012). NF-κB-regulated genes, such as Bcl-2, Bcl-xL and SOD, can promote tumor cell survival by inhibiting apoptosis. HIF-1α plays an important role in tumor angiogenesis by stimulating blood vessel formation. It was reported that the progression of human breast cancer to metastatic state was correlated to hydroxyl radical-induced DNA damage (Malins et al., 1996). Chronic hepatitis infections by Hepatitis B and Hepatitis C viruses increased oxidative stress (activation of NF-κB and STAT3) in liver tissues promoting hepatocellular carcinoma (Waris and Siddiqui, 2003).

With low to modest levels of ROS promote tumor cell proliferation and metastasis, high level of ROS can suppress tumor growth by activating apoptosis (Gupta et al., 2012). Both extrinsic and intrinsic pathways of

apoptosis involve ROS (Ozben, 2007). ROS was shown to activate p38 MAPK for apoptotic cell death in human cervical cancer cells (Kang and Lee, 2008). Moreover, p38α can be activated via p53-mediated ROS production, whereby this p53/ROS/p38α cascade involved in cisplatin-induced apoptosis in HCT116 colorectal cancer cells (Bragado et al., 2007). Furthermore, p53 was found to increase cellular ROS level for cell death mechanism by initiating the transcription of pro-oxidant genes such as PIG3 and PIG6 (Polyak et al., 1997). High level of ROS suppressed tumor growth by activating cell cycle inhibitor such as LATS1 (Large tumor suppressor kinase 1) (Takahashi et al., 2006). Interestingly, Dolado et al. suggested that ROS promote tumorigenesis, and p38 MAPK-induced apoptosis is initiated in response to ROS accumulation; this response plays an important role in inhibiting tumor initiation during oxidative stress (Dolado et al., 2007). Exogenous administration of hydrogen peroxide activated caspase 3 for apoptotic cell death in lymphoma cells (Hampton and Orrenius, 1997). Bortezomib, a selective inhibitor of the proteasome, suppressed gastric cancer cells by NF-κB inhibition, as well as ROS induction and JNK activation (Nakata et al., 2011). Moreover, increased level of hydrogen peroxide by piperlongumine treatment was able to suppress various types of tumor xenograft mouse model, with no apparent toxicity in normal mice (Raj et al., 2011). Thus, agents that can modulate ROS level have the potential to suppress cancer cells.

There are numerous natural products that can modulate cellular redox status to inhibit cancer cell growth, for example curcumin (Sandur et al., 2007), gambogic acid (Nie et al., 2009) and promegranate extract (Weisburg et al., 2010). Beta-sitosterol, a type of phytosterols, was found to induce apoptosis in U266 multiple myeloma cells through ROS-mediated AMPK and JNK activations (Sook et al., 2013). Hirsutanol A, a compound isolated from the fungus, *Chondrostereum* sp., inhibited SW620 human colon cancer cells via mitochondrial-independent ROS production (Yang et al., 2013). In contrast, genistein, an isoflavone found in soybean enriched foods, was able to protect cells from oxidative stress by acting as a ROS scavenger. Also, this compound is a strong inhibitor of NF-κB, Akt and PTK signaling pathways (Banerjee et

al., 2008). Indole-3-carbinol, a phytochemical found in vegetables, was found to induce BRCA1 for cell protection against oxidative stress from hydrogen peroxide and γ-radiation (Fan et al., 2009). Moreover, garlic extract was able to inhibit the oxidative modification of lipids from 7,12-dimethylbenz(a)anthracene (Das and Saha, 2009).

It has been shown in several reports that TQ was able to induce ROS production as a mechanism to kill cancer cells (Woo et al., 2012). TQ showed anti-oxidative activity at lower concentration (Mansour et al., 2002), but acted as a pro-oxidant at higher concentration (El-Najjar et al., 2010; Koka et al., 2010). It was reported that TQ induced ROS production for apoptotic cell death and Akt inhibition in primary effusion lymphoma cells (Hussain et al., 2011). Pre-treatment with N-acetylcysteine, a strong antioxidant, could reverse TQ-induced apoptosis in primary effusion lymphoma cells (Hussain et al., 2011), DLD-1 human colon cancer cells (El-Najjar et al., 2010) and C4-2B prostate cancer cells (Koka et al., 2010).

The effect of TQ on ROS production in breast cancer cells was not explained. Therefore, we were interested in investigating the role that ROS might play in the anticancer effects of TQ. The relationship of ROS with PPAR-γ and p38 in the action of TQ were also examined in this study.

1.6 Peroxisome proliferator-activated receptor gamma (PPAR-γ): role in cancer suppression

PPARs are nuclear receptor that have three main isoforms namely PPAR-α, PPAR-β/δ and PPAR-γ. They are ligand-activated transcription factors, whereby upon binding to agonist will increase the rate of transcription initiation (Berger and Moller, 2002). Once binding to ligand, PPAR-γ will hetero-dimerize with retinoid X receptor, whereby this complex moves into nucleus to bind to PPRE (Peroxisome Proliferators Response Element) sequence in the regulated promoter region to initiate transcription. PPARs play a critical role in lipid metabolism, and many of the natural fatty acids are

ligands to these receptors. For example, linoleic acid and arachidonic acid was found to bind to PPAR-α in micromolar range (Göttlicher et al., 1992). Palmitic acid and its analogue, 2-bromopalmitic acid, are known as PPAR-β/δ agonists (Amri et al., 1995). PPAR-γ regulates fatty acid storage and glucose metabolism, and it was shown that adipose-specific PPAR-γ knockout mice failed to develop adipose tissue after fed with high fat diet (Jones et al., 2005).

Thiazolidinedione is a class of medicine used for type 2 diabetes. Its members, including rosiglitazone, pioglitazone and ciglitazone, can bind to PPAR-γ for gene transcription to regulate adipocyte differentiation, lipid and glucose metabolism, and energy homeostasis (Berger and Moller, 2002). However, increasing studies reported that PPAR-γ might play an important role in cell proliferation, differentiation and apoptosis (Sertznig et al., 2007). Through non-genomic targets, PPAR-γ was shown to inhibit β-catenin pathway, STAT3/NF-κB signaling and androgen receptor (Robbins and Nie, 2012). PPAR-γ showed positive correlation to patients' survivor in breast cancer (Jiang et al., 2009), but opposite fashion in pancreatic cancer (Giaginis et al., 2009). Moreover, transgenic mice with lung-specific PPAR-γ over-expression showed reduced tumor formation after ethyl carbamate induction (Nemenoff et al., 2008). The administration of diet rich in conjugated linoleic acid (a PPAR-γ ligand) was able to protect against tumor formation in azoxymethane-induced mice (Evans et al., 2010).

Ligand activation of PPAR-γ was able to induce apoptosis in breast cancer (Kumar et al., 2009) and non-small cell lung cancer cells (Chang et al., 2000). Moreover, the invasion and metastasis of breast cancer cells could be inhibited by ligand activation of PPAR-γ (Liu et al., 2003; Panigrahy et al., 2002). It has been shown that PPAR-γ was able to induce G1/S arrest by up-regulating $p21^{WAF1/Cip1}$ (Chang et al., 2000) or $p27^{Kip1}$ (Motomura et al., 2000), and down-regulating cyclin D1 (Yin et al., 2001). In addition, PPAR-γ activation by PPAR-γ ligands, 15d-PGJ_2 and troglitazone, was able to suppress MCF-7 and MDA-MB-231 cell growth possibly by inhibition of cell cycle progression (Clay et al., 1999). Moreover, PPAR-γ activation by 15d-PGJ_2 suppressed gastric cancer cells by G1 cell cycle arrest, and this inhibitory effect was greater when combined with 9-cis retinoic acid, a ligand of RXRα (Sato et al.,

2000). It was shown that troglitazone-induced apoptosis in NCI-H23 human non-small lung cancer cells was mediated via PPAR-γ and ERK1/2, whereby the knock-down of PPAR-γ and treatment of EKR1/2-specific inhibitor were able to prevent troglitazone-induced apoptosis (Li et al., 2006). In addition, activation of PPAR-γ was found to inhibit hepatocellular carcinoma metastases both *in vitro* and *in vivo* (Shen et al., 2012).

Synergism was observed in the combination of rosiglitazone and platinum-based drugs in the treatment of several types of cancer, whereby this could be due to the PPAR-γ-mediated down-regulation of metallothioneins, a protein responsible for resistance in platinum-based therapy (Girnun et al., 2007). This is further explained by another study showing that rosiglitazone combined with cisplatin for enhanced anticancer effects in 7,12-dimethylbenz(a)anthracene-induced breast cancer rats, and this combination could also reduce the nephrotoxicity induced by cisplatin (Tikoo et al., 2009). Combined treatment of troglitazone and TRAIL synergistically induced apoptosis in DLD-1 human colon cancer cells through DR5 (Death Receptor-5) up-regulation (Koyama et al., 2014).

However, there are studies revealed the contradiction for the inhibitory role of PPAR-γ activation in tumorigenesis. For example, 15d-PGJ_2 was found to up-regulate VEGF via heme oxygenase-1 and ERK1/2 in MCF-7 cells (Kim et al., 2006). Moreover, there are studies reported that the anticancer activities of PPAR-γ ligands might be mediated via PPAR-γ-independent pathway. For example, troglitazone suppressed KU812 leukemia cells with undetectable level of PPAR-γ mRNA (Abe et al., 2002). This is further explained by another study showing that delta2-troglitazone, which devoid of PPAR-γ agonist activity, was able to suppress MCF-7 and MDA-MB-231 breast cancer cells (Colin et al., 2010). In addition, the cytotoxicity of 15d-PGJ_2 in 786-O, Caki-2 and ACHN human renal cancer cell lines was mediated in caspase-dependent and PPAR-γ-independent manners (Fujita et al., 2012). Moreover, troglitazone-induced cytotoxicity in 786-O, Caki-2 and ACHN human renal cancer cells was mediated via a PPAR-γ-independent pathway and p38 MAPK pathway (Fujita et al., 2011). It was suggested that the PPAR-γ-independent effects of PPAR-γ ligands could be due to the binding of these ligands to other

proteins such as p50 (Kulkarni et al., 2012). Thus, these effects did not require PPAR-γ-dependent transcriptional activation and could occur even PPAR-γ is functionally inactivated or deleted.

It was reported that many anticancer natural products could modulate PPAR-γ activity for anticancer action. For example, white tea extract induced apoptosis in A549 and H520 non-small cell lung cancer cells by up-regulating PPAR-γ activity (Mao et al., 2010). In addition, bitter gourd seed oil was able to induce apoptosis in Caco-2 colon cancer cells by up-regulating PPAR-γ, GADD45 and p53 (Yasui et al., 2005). Thus, agents that can modulate PPAR-γ could be potential drugs for cancer treatment, and natural products serve as a huge source for this purpose. There was no study reporting the effect of TQ on the PPAR-γ pathway in breast cancer cells. Therefore, we were interested to examine the role that PPAR-γ might play in TQ's anticancer activities.

1.7 The p38 MAPK pathway: role in tumor suppression

In order to survive and perform physiological functions, cells respond to a number of extracellular stimuli such as hormones, mitogens and biological ligands, follow by conversion of these signals into a wide range of intracellular responses. MAPKs (Mitogen-activated protein kinases) are protein Ser/Thr kinases that can initiate signaling cascades in response to extracellular stimuli, and this is often mediated through the activation of transcription factors. Nearly all eukaryotic cells utilize multiple MAPK pathways to regulate a number of cellular functions including gene expression, mitosis, metabolism, survival and apoptosis (Cargnello and Roux, 2011). By far, three most extensively studied groups of MAPKs are the extracellular signal-regulated kinases 1/2 (ERK1/2), c-Jun amino (N)-terminal kinases 1/2/3 (JNK1/2/3) and p38 isoforms (α, β, γ and δ). Usually, ERK1/2 is involved in cell proliferation and differentiation, while JNK and p38 cascades are activated by cellular stresses. However, depending on cell lines and types of stimulation, these cascades may response differently and even opposing

function (Keshet and Seger, 2010). Under rare condition, ERK1/2 may play a role in response to stress and apoptosis (Bacus et al., 2001); while JNK can mediate cell proliferation in certain conditions. Deregulation of these cascades often leads to diseases such as diabetes (Zick, 2005) and cancer (Dhillon et al., 2007).

In general, p38 isoform in mammals is activated by environmental stresses and inflammatory cytokines. These extracellular stimuli will activate MAPKKKs, which in turn, phosphorylate MKK3 and MKK6, the upstream protein kinases of p38. Activation of p38 is achieved by dual phosphorylation at the Thr-Gly-Tyr motif (New and Han, 1998). The p38 pathway plays a number of roles including regulation of apoptosis, cell cycle progression, growth and differentiation. Upon activation, p38 phosphorylates a number of proteins including Bax, Bcl-2, p53, ATF1/2/6 and MSK1/2 (Cuadrado and Nebreda, 2010). A number of diseases have been found to associate with p38 signaling, such as rheumatoid arthritis (Pargellis and Regan, 2003), cardiovascular disease (Behr et al., 2003) and Parkinson's disease (Wilms et al., 2003).

Many studies demonstrated that p38 pathway plays an important role in cancer therapy as a tumor suppressor gene. Molnar et al. reported that p38 could up-regulate p16 expression, which in turn, inhibiting cyclin D1/cdk4 activity (Molnar et al., 1997). p38 was shown to induce G1/S arrest by activating p53, which in turn, increased the level of p21 (Kim et al., 2002). Moreover, p38-mediated G2/M checkpoint could be initiated in response to DNA double strand breaks (Thornton and Rincon, 2009). It was shown that p38 could stabilize HBP1 protein by phosphorylating it (Xiu et al., 2003), whereby stabilized HBP1 could negatively regulate cell cycle genes such as N-myc and cyclin D1 (Sampson et al., 2001; Tevosian et al., 1997). In addition of cell cycle regulation, p38 also plays a role in apoptosis. p38 was found to increase the level of a new protein called p18 (Hamlet), of which p18 could interact with p53 for the transcription of pro-apoptotic genes such as Puma and Noxa (Cuadrado et al., 2007). It was shown that several chemotherapeutic agents, such as nocodazole, taxol, vincristine and vinblastine, required p38 activation for apoptotic cell death (Deacon et al., 2003). This was further explained by SB203580 and SB202190, whereby these p38-specific inhibitors were found

to reverse nocodazole-induced apoptosis (Deacon et al., 2003). Imatinib mesylate treatment (a specific inhibitor of BCR-ABL tyrosine kinase) was found to activate p38 pathway, whereby the treatment of p38-specific inhibitor could abrogate the anti-leukemic effects of imatinib mesylate in primary leukemic progenitors originated from patients with chronic myelogenous leukemia (Parmar et al., 2004). Inhibition of p38 was reported to be associated with the resistance to anoikis, thus allowing the circulating cancer cells to survive (Cheng et al., 2004). Interestingly, p38 could be activated by oncogenic stresses, whereas this activation involved in Ras-induced senescence in mouse embryo fibroblasts (Bulavin et al., 2003). Moreover, p-p38 was nearly undetectable in most solid tumors including lung, breast, gastric, liver, renal and ovarian cancers, while this protein was relatively higher expressed in normal organs (Liao and Hung, 2003).

In contrast to its antitumor activities, p38 was also found to play an important role in inflammation and cancer metastasis (Koul et al., 2013). The expression of several cytokines, such as IL-1, IL-6, IL-8 and TNF-α, is regulated by p38 pathway (Song et al., 2006). Moreover, p38 was found to regulate the induction of pro-inflammatory mediator cyclooxygenase 2 (COX-2) which involved in breast cancer progression (Timoshenko et al., 2006). The expression of MMP (matrix metalloproteinase) family enzymes, including MMP-1, MMP-2, MMP-9 and MMP-13, is regulated by p38 pathway (Song et al., 2006). Moreover, p38 was found to be involved in H-Ras-induced cell motility and invasive phenotype of MCF-10A human breast epithelial cells (Kim et al., 2003).

There are many natural products that modulate p38 pathway to produce anticancer activities. It was shown that platycodin D, a triterpenoid saponin isolated from the root of *Platycodon grandiflorum*, induced p38 activation for apoptosis in AGS human gastric cancer cells (Chun et al., 2013). In addition, acanthoic acid from *Acanthopanax koreanum* was able to induce growth inhibition and apoptosis in HL-60 leukemia cells, whereby these effects could be blocked by pre-treatment with SB203580 (Kim et al., 2012b). Moreover, neferine, a major alkaloid component in lotus embryos, was found to induce G1 arrest in human osteosarcoma cells through p38-mediated p21 stabilization

(Zhang et al., 2012). Pre-treatment with SB203580 could abrogate neferine-induced p21 up-regulation (Zhang et al., 2012).

There are few numbers of paper reported the effect of TQ on MAPKs pathway. TQ was found to increase the phosphorylation status of ERK and JNK, but not p38, in DLD-1 colon cancer cells (El-Najjar et al., 2010). Interestingly, the increased phosphorylation of ERK and JNK were found to protect against TQ-induced apoptosis, whereby pre-treatment with ERK (PD98059) and JNK (SP600125) specific inhibitors could sensitize the cells to the cytotoxicity of TQ (El-Najjar et al., 2010). On the other hand, TQ was shown to increase the activation of JNK and p38 in FG/COLO357 pancreatic cancer cells by down-regulating mucin-4 (Torres et al., 2010). Other than these studies, the role of p38 pathway in TQ's anticancer activities was not well explained. Thus, we aimed to study the effect of TQ on p38 pathway in breast cancer cells. The relationship of p38 with ROS and PPAR-γ in the effects of TQ was also elucidated.

1.8 Objectives and overview of study

1.8.1 Objectives of study

Despite numerous studies reporting the anticancer activities of TQ, the effect of TQ on breast carcinoma and its molecular mechanism(s) of action were still not well explained. Hence, we were interested to investigate the anticancer effects of TQ in breast carcinoma through *in vitro* and *in vivo* experiments, and also to elucidate the mechanism(s) of action of this compound via different assays.

Our first aim was to investigate the inhibitory effect of TQ in breast cancer cells by examining growth inhibition and apoptosis. The apoptotic pathway involved in TQ treatment was studied through the investigation on several apoptotic proteins. The effect of TQ on breast cancer cell migration and invasion was also examined. On the basis of evidence from our collaborator lab, we found that TQ was able to bind to PPAR-γ through interaction with several residues in the receptor (Woo et al., 2011). Therefore, we expanded our interest on PPAR-γ by investigating its role in TQ's anticancer effects. Next, we were interested to examine whether ROS would be the upstream regulator of PPAR-γ. We also investigated the role of ROS in TQ-induced apoptosis in breast cancer cells. ROS has been shown to regulate p38 MAPK for anticancer action of certain agents (Bragado et al., 2007; Kang and Lee, 2008); hence, we were interested to know whether this relationship was also involved in the mechanism of action of TQ. We studied the relationship between ROS and p38, and also, p38 and PPAR-γ. How p38 plays its role in TQ's anticancer effects was also one of our objectives. The later phase of the study was to translate *in vitro* data to *in vivo* by performing experiments on breast tumor xenograft mouse model. The protein expression of several genes of interest in the tumor tissues was examined and these findings were compared with those from cell line studies. The antitumor effect of the combination of TQ with doxorubicin, a chemotherapeutic agent, was also compared to the effect of either agent alone. We also measured the antioxidant enzymes/molecules in mouse liver tissues in an attempt to study the relationship between ROS and TQ.

These experiments would support our hypothesis that TQ is a potential anticancer agent that is able to suppress breast carcinoma through multiple signaling pathways, including ROS, PPAR-γ and p38 MAPK.

1.8.2 Overview of study

The mechanism of action of TQ was first investigated in breast cancer cells. MTT assay was used to examine the effect of TQ on cell viability. Several breast cancer cell lines (MCF-7, MDA-MB-231 and BT-474) were exposed to increasing concentrations of TQ for different incubation periods. This was to investigate the dose- and time-response effects of TQ on cell viability. In addition, the cytotoxicity of the combination of TQ and chemotherapeutic agents was also examined with MTT assay. This experiment was to investigate whether these combinations would produce synergistic or enhanced cytotoxic effect compared to either agent alone. The effect of TQ on cell cycle progression was examined by flow cytometry after propidium iodide staining, as well as the protein expression of cell cycle genes (such as cyclin D1 and cyclin E) by Western blot. The purpose was to investigate whether TQ would cause cell cycle arrest in breast cancer cells. Flow cytometry (annexin V staining) and Western blot (PARP cleavage) were used to examine the pro-apoptotic effect of TQ in breast cancer cells. Moreover, the protein expression of caspases and Bcl-2 family genes, such as Bcl-xL, Bcl-2 and Bax, were examined by Western blot. This was to study the effect of TQ on the apoptotic pathway.

'Wound-healing' assay was used to study the effect of TQ on breast cancer cell migration, whereby the ability of TQ-treated cells to fill the 'wound' area was evaluated. The effect of TQ on breast cancer cell invasion was studied with commercial invasion assay kit. The purpose of these experiments was to investigate the anti-metastatic effect of TQ in breast cancer cells.

According to the data from our collaborator, TQ was found to form interaction with several residues of PPAR-γ molecule (Woo et al., 2011). Therefore, whether PPAR-γ involved in the anticancer activities of TQ in breast cancer

cells was also investigated. Luciferase assay was used to measure the activity of various PPARs in TQ-treated cells. If there is any induction in PPARs after TQ treatment, a respective specific inhibitor would be used to determine the specificity of this induction. After determining that TQ was able to induce PPAR-γ specifically, our next objective was to study its effect on the PPAR-γ pathway in greater detail. Dose- and time-response effects of TQ on PPAR-γ activity were examined by luciferase assay. Whether PPAR-γ activation involved in TQ-induced apoptosis was examined with PPAR-γ-specific inhibitor in TQ-treated cells. Real time RT-PCR and Western blot were used to examine the mRNA and protein expression, respectively, of PPAR-γ-regulated genes in TQ-treated cells with or without PPAR-γ-specific inhibitor. This was to study whether PPAR-γ activation was involved in the suppression of these genes induced by TQ. In addition to PPAR-γ-specific inhibitor, a genetic approach, i.e. PPAR-γ dominant negative, was also used to study the role of PPAR-γ activation in the mechanism of action of TQ. This genetic approach was to over-express faulty PPAR-γ receptor, which in turn, resulting in reduced PPAR-γ activation. The mRNA and protein expression of PPAR-γ-regulated genes in TQ-treated cells were examined with or without PPAR-γ dominant negative transfection.

Our next phase of study was to determine whether ROS involved in the anticancer activities of TQ. The level of ROS in TQ-treated cells was measured with flow cytometry after Mitosox staining. After determining that TQ was able to induce ROS production in breast cancer cells, NAC, a strong antioxidant, was used to study the role of ROS in TQ's anticancer effects. NAC was used in TQ-treated cells to study whether it would make any significant difference to TQ's growth inhibition and pro-apoptotic effects. Moreover, whether ROS induction involved in the suppression of anti-apoptotic genes induced by TQ was also examined by using NAC in TQ-treated cells.

Next, the effect of TQ on p38 activation was investigated. Western blot and p38 ELISA kit were used to examine the expression level of p-p38/p38 in TQ-treated cells. Following this, p38-specific inhibitor, SB203580, was used in TQ-treated cells to study whether the growth inhibition and pro-apoptotic

effects of TQ required p38 activation. Moreover, the possible role of TQ-induced p38 activation in the suppression of anti-apoptotic genes was also investigated with p38-specific inhibitor. In addition to pharmacological inhibitor, a genetic approach, i.e. p38 siRNA gene silencing, was also used to study the role of p38 activation in TQ-induced apoptosis.

In addition of evaluating the role of PPAR-γ, ROS and p38 MAPK in the anticancer activities of TQ, our next interest was to study the possible interaction between these pathways. Whether the use of PPAR-γ-specific inhibitor would affect the level of TQ-induced ROS and TQ-induced p38 activation was examined. Similarly, whether the use of NAC would affect the level of TQ-induced PPAR-γ expression and TQ-induced p38 activation was also investigated. A p38-specific inhibitor was also used to determine whether p38 activation was involved in TQ-induced PPAR-γ expression and TQ-induced ROS production.

After determining the anticancer activities of TQ and its possible mechanisms of action in breast cancer cells, our next interest was to examine these effects in an animal model. Breast tumor xenograft mouse model was used to study the antitumor effects of TQ and its combination with a chemotherapeutic drug. Breast cancer cells were injected subcutaneously to the right flank region of female nude mice to develop breast tumor xenograft. When the size of tumors was about 100 mm^3, treatments were started for a total of two weeks. Tumor size and body weight were measured twice per week. After euthanisation of the mice, tumor tissues were collected for histological, immunohistochemical and Western blot analysis. Apoptosis marker (DNA fragmentation) and cell proliferation marker (Ki67) of tumor tissues were examined by TUNEL staining and immunohistochemistry, respectively. The protein expression of genes of interest (such as p-p38/p38, PPAR-γ and various anti-apoptotic genes) in the tumor tissues were examined by Western blot. These data would be compared to the *in vitro* findings. Mouse liver tissues were also collected for enzymatic assays to measure the level of several antioxidant enzymes/molecules, which aimed to study the pro-oxidant/anti-oxidant effect of TQ.

2 MATERIALS AND METHODS

2.1 Chemicals and antibodies

Trypsin EDTA, trypan blue, thiazoyl blue tetrazolium bromide (MTT), propidium iodide, RNase, insulin, hydrocortisone, thymoquinone and N-acetylcysteine were purchased from Sigma-Aldrich (St. Louis, MO, USA), while doxorubicin was purchased from Euroasian Chemical Private Ltd. (Mumbai, India). SB203580 was purchased from Promega (WI, USA). 15d-PGJ2 and GW9662 were purchased from Cayman (Michigan, USA). GSK0660 and GW0742 were purchased from Tocris Bioscience (Ellisville, MO, USA). RPMI1640, DMEM, DMEM/F12, Opti-MEM and fetal bovine serum were purchased from Hyclone (Loughborough, UK). Antibiotic-antimycotic mixture was purchased from Gemini Bio-products (West Sacramento, CA, USA). Dimethyl sulfoxide was purchased from MP Biomedicals (Solon, OH, USA). BD matrigel was purchased from BD Biosciences (Franklin Lakes, NJ, USA). Antibodies to Bcl-2, Bcl-xL, PPAR-γ, cyclin D1, cyclin E, p53, p21, p27, caspase 7, Ki67, XIAP, JNK, p-JNK, ERK, p-ERK and PARP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), while caspase 8, caspase 9, survivin, p38, p-p38 and β-actin were purchased from Cell Signaling (Beverly, MA, USA). Chicken anti-rabbit IgG HRP-conjugated, chicken anti-mouse IgG HRP-conjugated, chicken anti-rabbit IgG TR-conjugated antibodies, p38 siRNA and control siRNA-A were purchased from Santa Cruz Biotechnology.

2.2 Cell lines

MCF-7, MDA-MB-231 and BT-474 breast cancer cell lines as well as MCF-10A normal breast epithelial cell line were purchased from ATCC (Manassas, VA, USA). MCF-7 and MDA-MB-231 cells were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic. BT-474 cells were cultured in DMEM medium supplemented with 12.5% FBS and 1% antibiotics-antimycotic. MCF-10A cells were cultured in DMEM/F12 medium supplemented with 5% FBS, 1% antibiotics-

antimycotic, 10 μg/ml insulin, 20 ng/ml EGF and 0.5 μg/ml hydrocortisone. All cell culture were maintained at 37 ℃ and 5% CO_2 in a humidified atmosphere.

2.3 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay

The cytotoxic effect of TQ was assessed by MTT assay. The principal of this assay is that live cells can reduce the yellow dye, MTT, to purple formazan crystals for quantification. Briefly, breast cancer cells were seeded (10^4 cells/well) in a 96-well microtiter plate followed by overnight incubation. After appropriate treatment, 10 μl MTT solution (5 mg/ml) was added into each well for 4 h. The mixture was then removed carefully via pipette, and the remaining formazan crystals formed were dissolved with 100 μl DMSO. The absorbance of each well was read after 30 mins at 570 nm by a microplate reader (Tecan Infinite M200, Mannedorf, Switzerland).

2.4 Cell cycle analysis

Cell cycle progression of breast cancer cells was examined by flow cytometry after propidium iodide (PI) staining. Cell cycle phase can be differentiated by measuring their DNA content, where G1 phase is 2n, S phase is between 2n and 4n, and G2 phase is 4n. PI can diffuse into cell nucleus and binds to DNA proportionately to its amount; thus, by measuring PI signal, the DNA content of each cell can be determined. Briefly, cells were seeded (1.5×10^5 cells/well) in a 6-well microtiter plate followed by overnight incubation. The cells were synchronized by starvation in serum-free medium for 24 h. After appropriate treatment, the cells were detached and fixed with 70% ethanol for at least 2 h before staining with PI solution (mixed with RNase) under dark condition for 20 mins. The cells were then analyzed with a flow cytometer (CyAn^{TM} ADP from Beckman Coulter, Brea, CA, USA).

2.5 Annexin V assay

The level of apoptosis of cancer cells was assessed with Annexin V-PI kit from Miltenyi Biotec (Bergisch Gladbach, Germany). Annexin V can specifically bind to phosphatidylserine. Cells undergoing apoptosis will experience diffusion of phosphatidylserine from inner cell membrane to outer cell membrane, whereby these proteins can be quantified by targeting them with fluorescence-tagged Annexin V. The experimental procedures were carried out according to the manufacturer's protocol. Briefly, cells were seeded (2.6 X 10^5 cells/well) in a 6-well microtiter plate followed by overnight incubation. After appropriate treatment, the cells were trypsinized, washed, and incubated with Annexin V-FITC solution for 15 mins under dark condition. After a washing step, the cells were analyzed with a flow cytometer (CyAn^{TM} ADP from Beckman Coulter, Brea, CA, USA) immediately after adding of PI solution.

2.6 Western blot analysis

The protein expression of genes of interest in breast cancer cells and tumor tissues were measured by Western blot. The principal behind of this technique is to transfer proteins from an acrylamide-gel to a membrane, which they can be specifically targeted by antibodies of interest. These antibodies are tagged with enzyme to cleave substrate for chemiluminescence signal. The chemiluminescence signal, which is proportionately to the quantity of antibody, is measured by exposing substrate-added membrane to X-ray film or Gel-Doc machine. Briefly, cells were seeded (2.6 X 10^5 cells/well) in a 6-well microtiter plate followed by overnight incubation. After appropriate treatment, the cells were trypsinized followed by whole cell lysate extraction (cell lysis buffer: 5 M NaCl, 1 M HEPES, 0.1 M EGTA, 0.5 M EDTA, 1 % Triton-X). For *in vivo* study, the tumor tissues were homogenized for tissue lysate extraction (tissue lysis buffer: 20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.1 % Triton X, 0.1 % SDS, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4). Both cell lysate and tissue lysate were centrifuged at 13,000 rpm and the resulting supernatants were collected.

After protein estimation with Bio-Rad protein assay (Hercules, CA, USA), a calculated volume of lysate was mixed with laemmli sample buffer, whereby the mixture was resolved by 10% or 12% SDS/PAGE gel and then electroblotted onto a nitrocellulose membrane. The membrane was blocked with 5% fat-free milk for 1 h before washed with TBST buffer. The membrane was probed with primary antibody for overnight at 4 ℃, and then washed and probed with HRP-conjugated secondary antibody for 1 h at room temperature. The membrane was examined for its chemiluminescence signal by ECL analysis system (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Densitometric analysis of Western blot bands was performed with Image J software. Band intensity was expressed as percentage relative to untreated control after normalization to β-actin.

2.7 Cell migration assay

The "wound-healing" assay was used to investigate the migration of cancer cells after appropriate treatment. The principal behind is to create a "wound" or long strip within cell culture, which follow by the examination for the ability of cells to grow into the "wound" area. Briefly, cells were seeded in a 6-well microtiter plate and incubated until about 80-90% confluence. A 'wound' was created using pipette tip and rinsed with PBS to remove detached cells. Media with appropriate treatment was added for 48 h incubation. The observation of the cells was recorded at 0, 24 and 48 h with a fluorescence microscope (Olympus BX51, Shinjuku, Japan).

2.8 Invasion assay

The invasiveness of breast cancer cells were examined with BD Matrigel^{TM} Invasion Chamber 24-well Plate 8 μm (BD Biosciences, San Diego, MA, USA). The idea is to seed breast cancer cells at one side of matrigel chamber follow by the introduction of chemo-attractant at the opposite side. The invasiveness of cancer cells was investigated by examining the number of cells that had migrated through matrigel toward the chemo-attractant. Briefly,

the chambers supplied by the manufacturer were allowed to rehydrate in serum-free media for 2 h before being placed in a 24-well microtiter plate filled with 5% FBS media. A total of 6 X 10^4 breast cancer cells were resuspended in serum-free media before added into the chamber. The cells were allowed to settle for 4–6 h inside the chamber before exposure to appropriate concentrations of TQ for 24 h or 48 h. The media was removed carefully and the chamber was transferred into a well filled with 4% paraformaldehyde for cell fixation. After 30 mins incubation, the migratory cells, which attached at the bottom of the chamber, were stained with 0.5% crystal violet solution for 15 mins. The chamber was then washed with deionized water for 5 mins. The observation of the stained migratory cells was recorded with a fluorescence microscope (Olympus BX51, Shinjuku, Japan). The number of the migratory cells after TQ treatment was normalized against untreated group.

2.9 Luciferase assay

The activity of PPARs was investigated by two-step luciferase assay as described previously (Ramachandran et al., 2012). GAL4 sequence is incorporated into the PPAR promoter region. GAL4-luc is incorporated into DNA to produce GAL4 binding domain to regulate luciferase signal. Upon binding of agonist, PPAR proteins will move into the nucleus and bind to peroxisome proliferator response elements (PPRE) to initiate transcription. GAL4 protein will be produced and this protein will then bind to GAL4 binding domain inducing luciferase signal. Briefly, cells were seeded (6 X 10^4 cells/well) in a 12-well microtiter plate followed by overnight incubation. The cells were incubated in DMEM medium for 1 h before plasmid transfection. The cells were transfected with GAL4-PPAR-α LBD, GAL4-PPAR-γ LBD or GAL4-PPAR-β/δ LBD plasmid (were kindly provided by Dr. Javier F. Piedrafita, Torrey Pines Institute for Molecular Studies, San Diego, CA, USA), together with GAL4-Luc and Renilla plasmids (internal control).

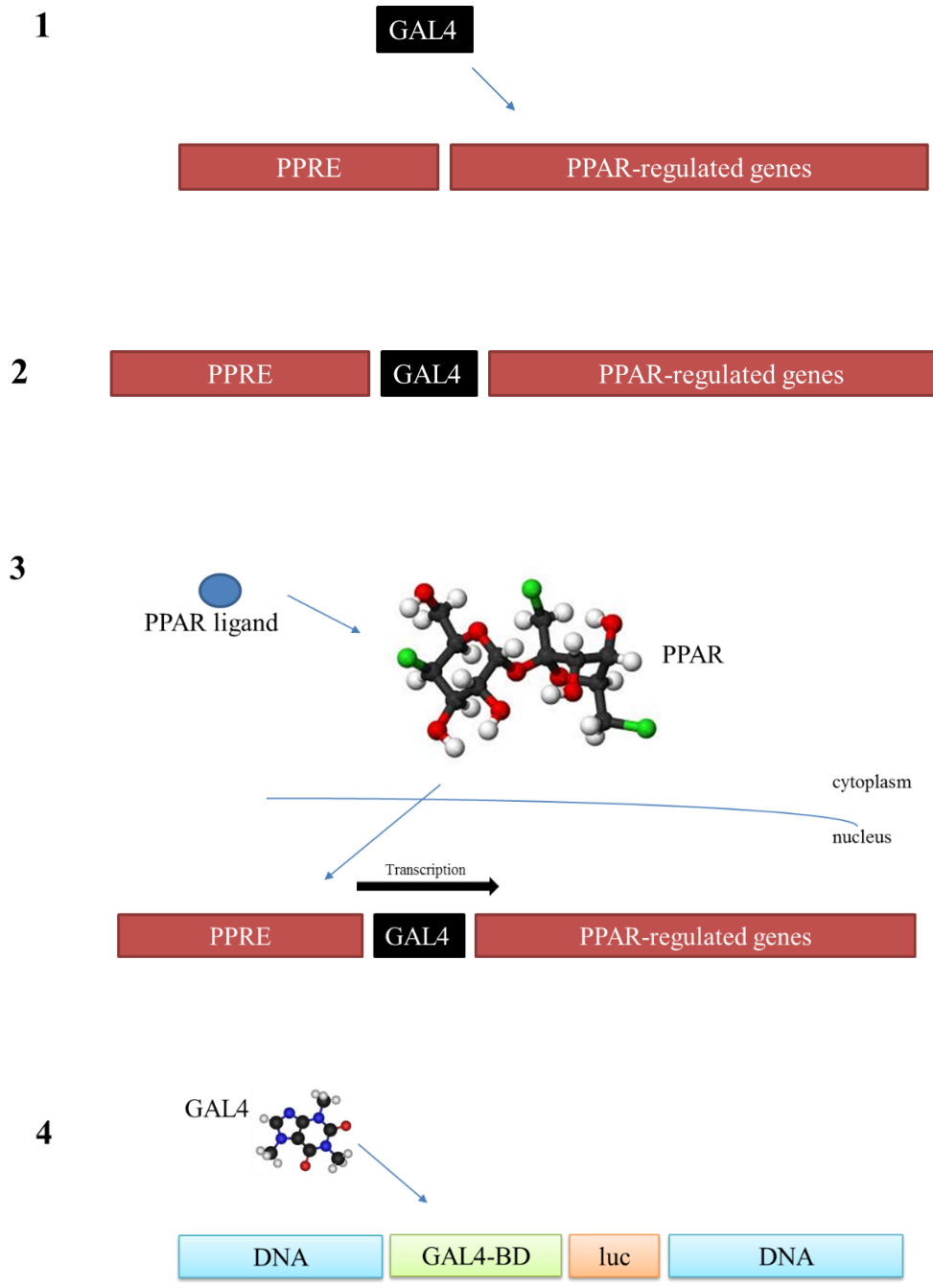


Figure 2.1 Schematic diagram for two-step luciferase assay.

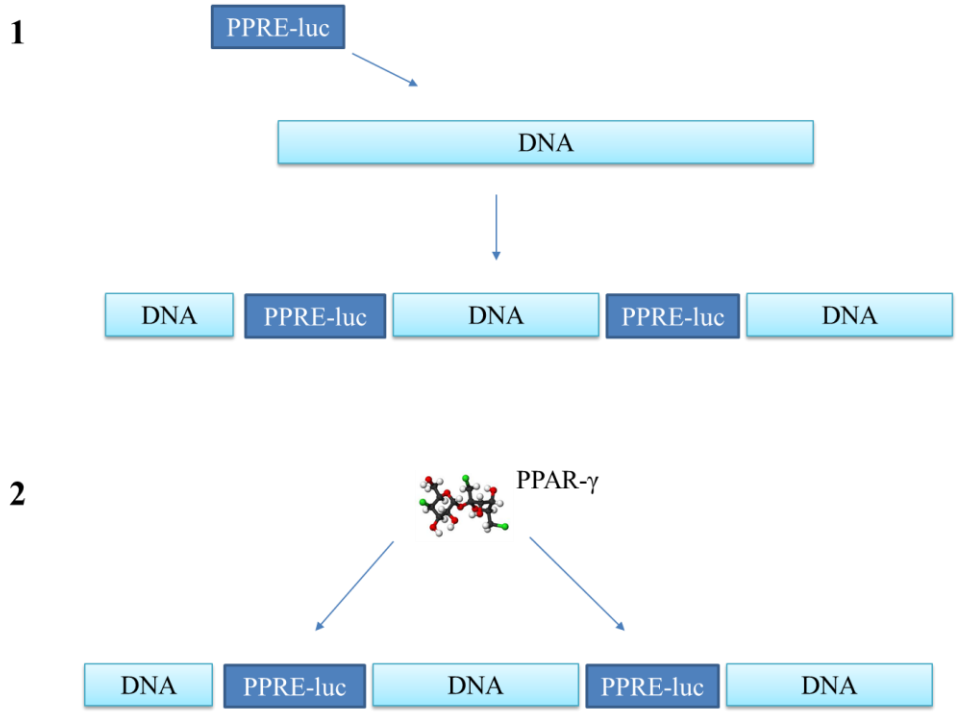


Figure 2.2 Schematic diagram for one-step luciferase assay.

For dominant negative experiment, one-step luciferase assay was used (Kumar et al., 2008). The main idea is to fuse pPPRE-tk-luc (three PPREs from rat acyl-CoA oxidase promoter under the control of the Herpes simplex virus thymidine kinase promoter) to DNA, whereby upon binding of PPAR-γ, induces luciferase signal. Briefly, cells were seeded (6 X 10^4 cells/well) in a 12-well microtiter plate followed by overnight incubation. The cells were incubated in DMEM medium for 1 h before plasmid transfection. The cells were transfected with PPAR-γDN (PPARγC126A/E127A containing amino acid substitutions in the DNA binding domain that abolish binding to PPAR-γ response elements) or pCMX-mPPARγ plasmid (a cDNA clone encoding the mouse PPARγ), together with pPPRE-tk-Luc and Renilla plasmid as an internal control. PPAR-γDN was a generous gift by Dr. Christopher K. Glass (UCSD, San Diego, CA, USA) while pCMX-mPPARγ was kindly provided by Dr. Ronald M. Evans (The Salk Institute for Biological Studies, San Diego, CA, USA).

For both two step and one step luciferase assays, the cells were transfected with 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 appropriate treatment, the cells were harvested using ice-cold reporter lysis buffer. The lysate was then centrifuged at 12,000 rpm for 3 mins. 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.

2.10 Real time RT-PCR (reverse transcription-polymerase chain reaction)

The mRNA expression of genes of interest was examined by real time RT-PCR. The total mRNA will first be converted to cDNA by reverse transcription. Next, fluorescence-tagged primer probe will be used to specifically bind to gene of interest to allow gene amplification through polymerase chain reaction. Thus, the fluorescence level will represent the level of mRNA of gene of interest. Briefly, cells were seeded (2 X 10^5 cells/well) in a 6-well microtiter plate followed by overnight incubation. After appropriate treatment, 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 reaction of mRNA to cDNA. 1 μg of total RNA was mixed with reaction mixture (RT buffer, $MgCl_2$, dNTPs, random hexamers, RNase inhibitors, RTase and H_2O) to make a total volume of 10 ml. The reverse transcription reaction was carried out over a PCR thermal cycle (25 ℃ for 10 mins, 37 ℃ for 60 mins and 95 ℃ for 5 mins). The resulting cDNA solution was then mixed with TaqMan® Universal Master Mix 2.0 (Applied Biosystems, Foster City, CA, USA) together with target primer probes (purchased as kits from Applied Biosystems). The polymerase chain reaction was carried out with ABI Prism

7500 (Applied Biosystems, Foster City, CA, USA). All reactions were carried out in duplicate and the mRNA expression was normalized against 18S rRNA as an internal control.

2.11 Mitosox assay

The level of ROS was examined with Mitosox assay (Invitrogen, Carlsbad, CA, USA). Breast cancer cells were seeded (2.6 X 10^5 cells/well) in a 6-well microtiter plate followed by overnight incubation. After appropriate treatment, the cells were trypsinized, washed and incubated with Mitosox-added serum free medium at 37℃ for 15 mins under dark condition. The cells were then analyzed with a flow cytometer (BD LSRII, Franklin Lakes, NJ, USA).

2.12 PathScan® Phospho-p38 MAPK (Thr180/Tyr182) Sandwich ELISA Kit

The p-p38 protein level of cancer cells was measured with PathScan® Phospho-p38 MAPK (Thr180/Tyr182) Sandwich ELISA Kit (Cell Signaling, Beverly, MA, USA). Incubation of cell lysate in the supplied plate will allow the p-p38 protein to be captured by coated antibody. The captured p-p38 protein is then targeted by p-p38 specific antibody follow by HRP-secondary antibody. Finally, the HRP enzyme on the secondary antibody will cleave substrate for color signal. The experimental procedures were carried out according to the manufacturer's protocol. Briefly, cells were seeded (2.6 X 10^5 cells/well) in a 6-well microtiter plate followed by overnight incubation. After appropriate treatment, lysis buffer (Cell Signaling, Beverly, MA, USA) was added for whole cell extraction. The cell lysate was subjected to centrifugation at 13,000 rpm. The resulting supernatant was added into the wells supplied by the manufacturer. After 4 h incubation at 37℃, the wells were washed with wash buffer for 4 times. p38 MAPK rabbit detection antibody was then added for 1 h at 37℃. The washing step was repeated, followed by 30 mins incubation with HRP-linked secondary antibody at 37℃. The washing step was again repeated, followed by 10 mins incubation with TMB substrate at

37℃. STOP solution was then added into each well for 5 mins. The absorbance was read at 450 nm with a microplate reader (Tecan Infinite M200, Mannedorf, Switzerland).

2.13 Gene silencing with siRNA

The expression of gene of interest can be suppressed using siRNA gene silencing. The mRNAs of gene of interest are bind by specific siRNA with complementary nucleotide sequence creating double-stranded RNA which will then be degraded by endo- and exo-nucleases. Briefly, cells were seeded (1.7 X 10^5 cells/well) in a 6-well microtiter plate followed by overnight incubation. The cells were tranfected with 30 nM of p38 siRNA or control siRNA-A using Oligofectamine tranfection reagent (Invitrogen, Carlsbad, CA, USA) for 6 h according to the manufacturer's protocol. The cells were let to recover in serum-added medium for at least 24 h before exposure to appropriate treatment.

2.14 Breast tumor xenograft mouse model

Female nude mice (BALB/c OlaHsd-foxn1) were purchased from Biological Resource Centre Biopolis (Singapore). The animal protocol used in this experiment was approved by The NUS Institutional Animal Care and Use Committee (protocol no. 065/11). Upon arrival, all nude mice were kept in individual disposable cages with ventilation, and given food and water ad lib. All mice were acclimatisated for at least 7 days before used. Breast cancer cells were sent for infection check before used in this experiment. After trypsinized, the cells were washed with serum-free medium twice before resuspended in matrigel-added serum-free medium. Each mouse was injected subcutaneously with 10^7 MDA-MB-231 human breast cancer cells at the right flank region. The tumor was allowed to grow until about 100 mm^3 (Volume = ½ X $width^2$ X length) before introducing any treatment. The mice were divided into five different treatment groups (n=5) as the following table.

Table 2.1: Treatment protocol of tumor-induced mice

Groups	Treatments	Times per week
Vehicle	Saline water (i.p.)	6
4 mg/kg TQ	4 mg/kg TQ (i.p.)	6
8 mg/kg TQ	8 mg/kg TQ (i.p.)	6
2.5 mg/kg Dox	2.5 mg/kg Dox (i.p.)	1
Combined	4 mg/kg TQ (i.p.) + 2.5 mg/kg Dox (i.p.)	6 + 1

The tumor volume (measured by Vernier caliper) and body weight were measured twice per week. All mice were euthanized with CO_2 asphyxiation after two weeks of treatment. Tumor tissues were collected for histological, immunohistochemical and Western blot analysis, while liver tissues were collected for enzymatic assays.

2.15 Hematoxylin and Eosin (H&E) staining

H&E staining was used to study the tissue structure of mouse tumors. This method can stain nucleus to purple color and cytoplasm to pink color. Briefly, the tumor tissues were placed in 10% neutral buffered formalin solution (Sigma-Aldrich, St. Louis, MO, USA) for at least 1 day before being processed and paraffinized. The sample tissues were sectioned at 4 μm thick. Next, the tissue section was deparaffinized with Histo-Clear (National Diagnostics, Atlanta, GA, USA) for 5 mins. The tissue section was rehydrated with decreasing concentrations of ethanol, i.e. 100% ethanol, 95% ethanol, 80% ethanol and 70% ethanol. The tissue section was then rinsed with running water for 5 mins before stained with Hematoxylin (Merck, Germany) for 8 mins. Next, the tissue section was rinsed with acid alcohol for 3 seconds followed by running water for 2 mins. The tissue section was then rinsed with weak ammonia for 10 seconds followed by running water for 10 mins. In the following step, the tissue section was stained with Eosin (Merck, Germany) for 5 mins. The tissue section was then dehydrated with increasing

concentrations of ethanol, i.e. 70% ethanol, 80% ethanol, 95% ethanol and 100% ethanol. Next, the tissue section was rinsed with Histo-Clear for 3 mins before covered with a cover slip using mounting medium. The tissue section was examined and photographed with a fluorescence microscope (Olympus BX51, Shinjuku, Japan).

2.16 TUNEL staining

The level of apoptosis of tumor tissues was examined with TUNEL staining (Promega, WI, USA). This method can detect DNA fragmentation which is a characteristic hallmark of apoptosis. Fragmented DNA exposes 3' OH ends which can be targeted by terminal deoxynucleotidyl transferase (TdT), follow by incorporation of fluorescence-tagged dUTP. The experimental procedures were carried out according to the manufacturer's protocol. Briefly, the tissue section was deparaffinized with Histo-Clear (National Diagnostics, Atlanta, GA, USA) before rehydration with decreasing concentrations of ethanol. After washing with 0.85% NaCl and PBS, the tissue section was fixed with 4% formaldehyde for 15 mins. After another PBS wash, the tissue section was covered with Proteinase K solution for 8-10 mins. Following another PBS wash, the tissue section was again fixed with 4% formaldehyde for 5 mins. After PBS wash, the tissue section was covered with equilibrium buffer for 5-10 mins before addition of TdT reaction mixture for 1 h incubation under dark condition. Next, the tissue section was incubated with SSC solution for 15 mins, followed by a final PBS wash. The tissue section was counterstained with DAPI (Sigma-Aldrich, St. Louis, MO, USA) for 5 mins. Immediately after drying, the tissue section was examined and photographed with a fluorescence microscope (Olympus BX51, Shinjuku, Japan). The graph showed the average number of TUNEL-positive cells from three photographs in each treatment group.

2.17 Ki67 immunohistochemistry

Ki67 protein is a cellular marker for proliferation. By using Ki67 antibody, this protein can be specifically targeted and be quantified through fluorescence signal on secondary antibody. Firstly, the tissue section was deparaffinized with Histo-Clear (National Diagnostics, Atlanta, GA, USA) before undergoing antigen retrieval step with citrate buffer. The tissue section was next blocked with 2% fetal bovine serum for 20-30 mins. The tissue section was then incubated with rabbit anti-Ki67 antibody for 1 h at room temperature. After PBS wash, the tissue section was incubated with chicken anti-rabbit IgG TR-conjugated antibody for 1 h under dark condition. The tissue section was then counterstained with DAPI (Sigma-Aldrich, St. Louis, MO, USA) for 5 mins. Immediately after drying, the tissue section was examined and photographed with a fluorescence microscope (Olympus BX51, Shinjuku, Japan). The graph showed the average number of Ki67-positive cells from three photographs in each treatment group.

2.18 Catalase assay

Catalase is an antioxidant enzyme that catalyzes the conversion of hydrogen peroxide to oxygen and water. The catalase level in mouse liver tissues was measured by using catalase assay kit from Cayman Chemical (Ann Arbor, Michigan, USA). The experimental procedures were carried out according to the manufacturer's protocol. Briefly, the liver tissues were homogenized in cold buffer (50 mM potassium phosphate, 1 mM EDTA, pH 7). The supernatant was collected after 10 mins centrifugation at 10,000 g. The sample was mixed to diluted assay buffer and methanol in a 96-well microtiter plate. The reaction was initiated by adding diluted hydrogen peroxide for 20 mins with constant shaking. Diluted potassium hydroxide and catalase purpald were then added followed by 10 mins incubation with constant shaking. Next, catalase potassium periodate was added for 5 mins incubation with constant shaking. The absorbance was then read at 540 nm with a microplate reader (Tecan Infinite M200, Mannedorf, Switzerland).

2.19 Superoxide dismutase (SOD) assay

SOD is an antioxidant enzyme which catalyzes the conversion of superoxide to oxygen and hydrogen peroxide. The SOD level in mouse liver tissues was measured using the SOD assay kit from Cayman Chemical (Ann Arbor, Michigan, USA). The experimental procedures were carried out according to the manufacturer's protocol. Briefly, the liver tissues were homogenized in HEPES buffer (20 mM HEPES buffer, 1 mM EGTA, 210 mM mannitol, 70 mM sucrose, pH 7.2). The supernatant was collected after 5 mins centrifugation at 1,500 g. The sample was mixed to diluted radical detector in a 96-well microtiter plate. The reaction was initiated by adding diluted xanthine oxidase for 20 mins with constant shaking. The absorbance was then read at 450 nm with a microplate reader (Tecan infinite M200, Mannedorf, Switzerland).

2.20 Glutathione assay

Glutathione is a tripeptide which serves as a reducing agent. The thiol group can donate electron to unstable molecules (e.g. reactive oxygen species) thus resulting in the conversion of glutathione to glutathione disulfide. The glutathione level in mouse liver tissues was measured using the glutathione assay kit from Cayman Chemical (Ann Arbor, Michigan, USA). The experimental procedures were carried out according to the manufacturer's protocol. Briefly, the liver tissues were homogenized in cold buffer (50 mM phosphate, 1 mM EDTA, pH 6-7). The supernatant was collected after 10 mins centrifugation at 10,000 g. The sample was first deproteinated by mixing with triethanolamine. The sample mixture was then mixed to assay cocktail in a 96-well microtiter plate and incubated immediately for 25 mins under dark condition with constant shaking. The absorbance was then read at 405 nm with a microplate reader (Tecan Infinite M200, Mannedorf, Switzerland).

2.21 Statistical analysis

Statistical analysis was performed by one way analysis of variance (ANOVA) and Student's *t test*. Tukey's *t test* was used after one way ANOVA to determine the significance between the groups. A p-value of less than 0.05 was considered to be statistically significant.

3 RESULTS

3.1 Studies on the cytotoxic effects of TQ in breast cancer cells

3.1.1 Growth inhibition effect of TQ

TQ was first examined for its growth inhibition effect in several breast cancer cell lines (MCF-7, MDA-MB-231 and BT-474) and a normal breast cell line (MCF-10A) by using MTT assay. As shown in Figure 3.1.1, TQ induced potent growth inhibition effect in all tested cell lines in a dose- and time-dependent manner. The IC_{50} values of TQ in MCF-7 cells after 12 h, 24 h and 48 h exposures were 48, 40 and 32 μM, respectively. In MDA-MB-231 cells, the IC_{50} values of TQ after 12 h, 24 h and 48 h exposures were 24, 14 and 11 μM, respectively. On the other hand, the IC_{50} values of TQ after 12 h, 24 h and 48 h exposures in BT-474 cells were 38, 18 and 21 μM, respectively. The IC_{50} values of TQ in MCF-10A cells after 12 h, 24 h and 48 h exposure were 46, 30 and 32 μM, respectively. From the results, the sensitivity of MCF-10A cells to the cytotoxicity of TQ was comparable to MCF-7 cells, however, this normal breast cell line was less sensitive when compared to MDA-MB-231 and BT-474 cell lines. Overall, the IC_{50} values of TQ were less than 50 μM suggesting the strong potency of this drug in inhibiting the growth of breast cancer cells.

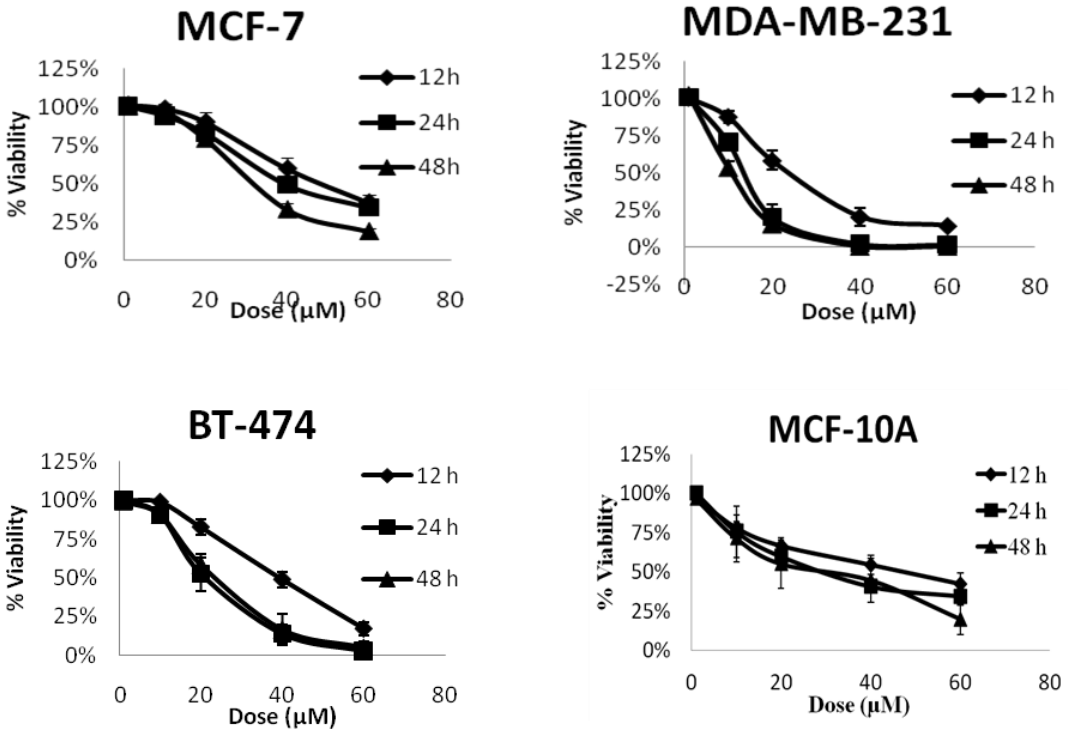


Figure 3.1.1 The dose- and time-response curves of TQ treatment in several breast cancer cell lines and a normal breast cell line.

Cells were treated with increasing concentrations of TQ for 12 h, 24 h and 48 h before analyzed with MTT assay. Values are means ± S.E.M. of at least three independent experiments.

Table 3.1: IC_{50} values of TQ in different breast cell lines after 12 h, 24 h and 48 h exposures

Cell lines	12 h (µM)	24 h (µM)	48 h (µM)
MCF-7	48	40	32
MDA-MB-231	24	14	11
BT-474	38	18	21
MCF-10A	46	30	32

3.1.2 Effect of the combination of TQ and chemotherapeutic drugs

The effect of the combination of TQ and chemotherapeutic drugs on breast cancer cell growth was investigated with MTT assay. MCF-7 cells were treated with TQ alone or in combination with doxorubicin, 5-fluorouracil or paclitaxel. Our results showed that the combination of TQ with doxorubicin or 5-fluorouracil could produce enhanced growth inhibition effect than either agent alone (Figure 3.1.2). A low dose of TQ (20 μM) was found to increase the cytotoxicity of doxorubicin by 2.6 fold and 5-fluorouracil by 2.7 fold. However, the combination of TQ and paclitaxel did not produce significant difference in growth inhibition than either agent alone (Figure 3.1.2). Together, these findings demonstrate the possible use of TQ as a complement agent in combination therapy.

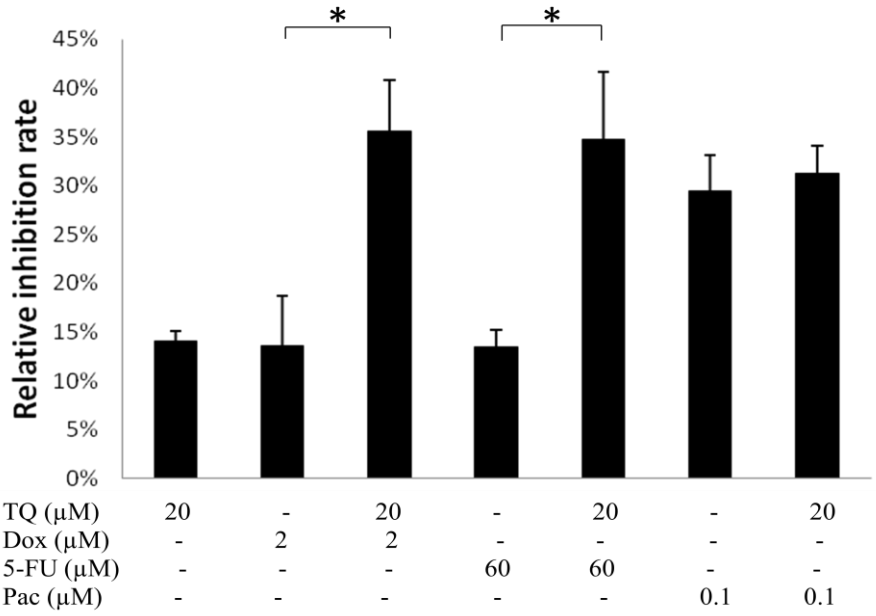
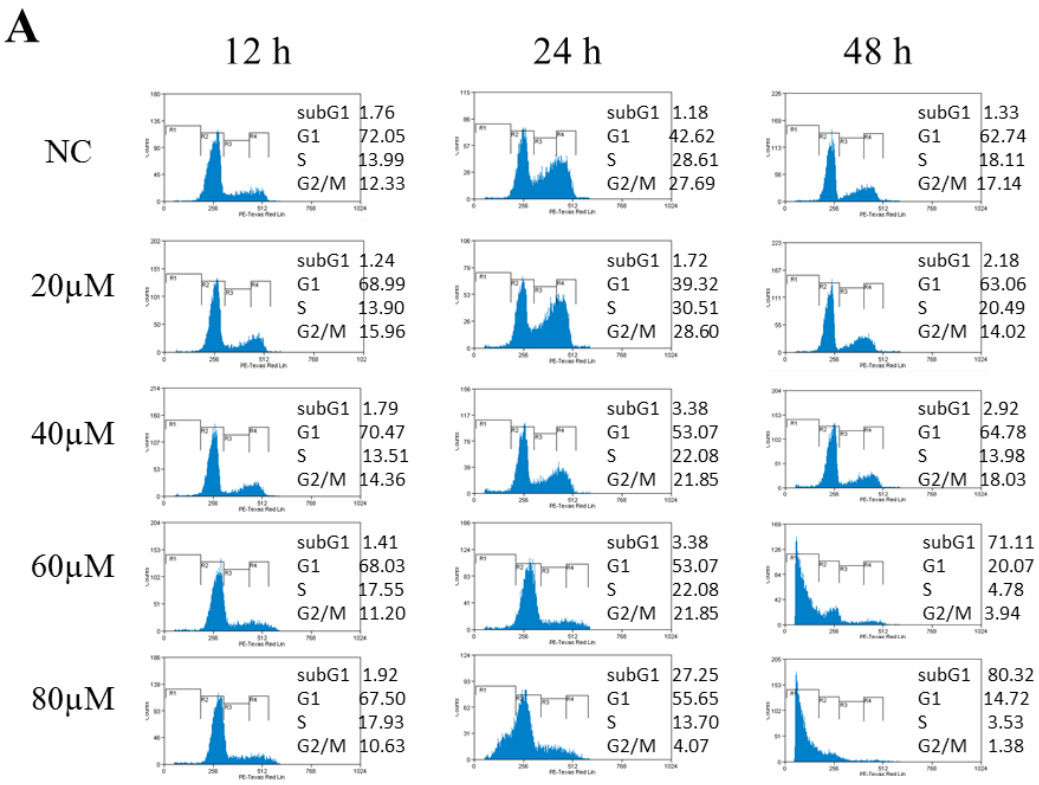


Figure 3.1.2 Growth inhibition rate of the combination of TQ and chemotherapeutic drugs.

MCF-7 cells were treated with 20 μM TQ with or without the indicated dose of chemotherapeutic drugs for 24 h before analyzed with MTT assay. Values are means ± S.E.M. of at least three independent experiments. * $p<0.05$ vs. control.

3.1.3 Effect of TQ on cell cycle progression

Flow cytometry analysis was used to study the chronology of the cellular and molecular events of TQ-treated MCF-7 cells. After TQ treatment, the cells were stained with propidium iodide before analyzed with flow cytometry. As shown in Figure 3.1.3A, no cell cycle arrest was observed after TQ treatment, however, subG1 accumulation was observed at 80 µM TQ after 24 h exposure, and at 60 µM and 80 µM TQ after 48 h exposure. SubG1 phase represents genetic material less than 2n which indicates nuclear fragmentation, a characteristic of apoptosis. In addition, the protein expression of cell cycle genes was also investigated with Western blot. Our results showed that TQ treatment did not cause significant change in the protein expression of cyclin D1 and cyclin E in MCF-7 cells (Figure 3.1.3B).



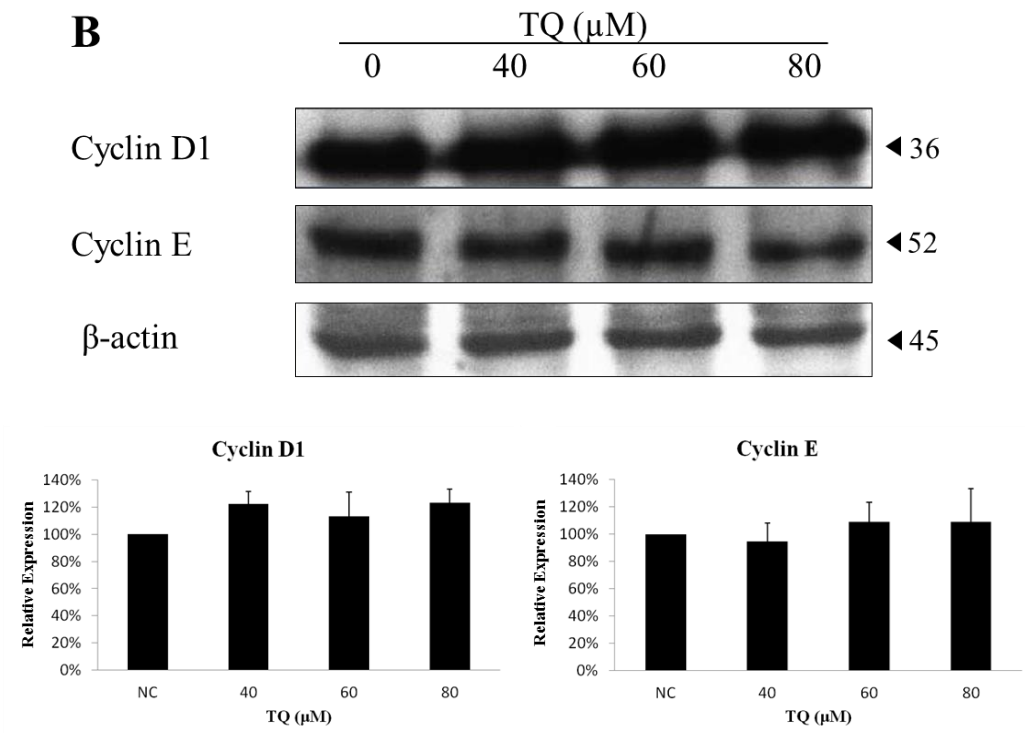


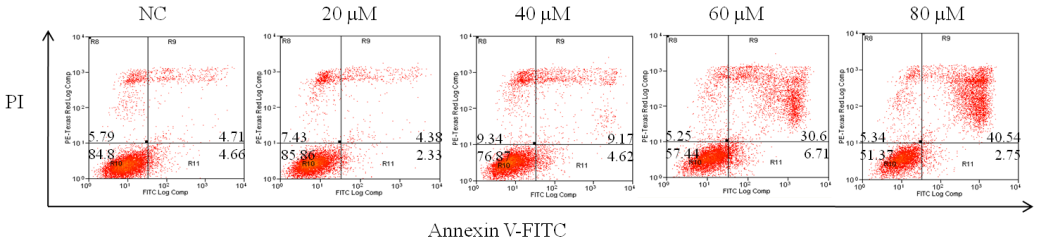
Figure 3.1.3 Effect of TQ on cell cycle progression and cell cycle genes.

(A) Effect of TQ on cell cycle progression. MCF-7 cells were treated with increasing concentrations of TQ for 12 h, 24 h and 48 h before analysis with flow cytometry. The data are representative of three independent experiments. (B) Effect of TQ on the protein expression of cell cycle genes. MCF-7 cells were treated with increasing concentrations of TQ for 12 h. Whole cell lysate was then used for Western blot analysis. The data are representative of at least three independent experiments.

3.1.4 Pro-apoptotic effect of TQ

Upon apoptosis, phosphatidylserine proteins, which are normally in the inner cell membrane, will be exposed to the cell surface. These phospholipid components have a high affinity for protein molecule like annexin V. Thus, by labeling annexin V with fluorescein, the cell population with exposed phosphatidylserine can be detected for apoptosis measurement. Our data showed that TQ increased the percentage of annexin V-positive cells in a dose-dependent manner (Figure 3.1.4A). Interestingly, TQ treatment appeared to increase the percentage of annexin V-positive cells at the upper right quadrant (known as late apoptosis) than the lower right quadrant (known as early apoptosis). In addition to annexin V assay, PARP protein cleavage was also used to examine apoptosis. Poly (ADP-ribose) polymerase (PARP) is a family of protein that is involved in many cellular processes such as DNA repair and cell death. Its inactivation through caspase-dependent cleavage indicates apoptosis induction. As shown in Figure 3.1.4B, TQ treatment was found to induce the cleavage of PARP protein in a dose-dependent manner. Together, these results suggested that TQ was able to induce apoptosis in breast cancer cells.

A



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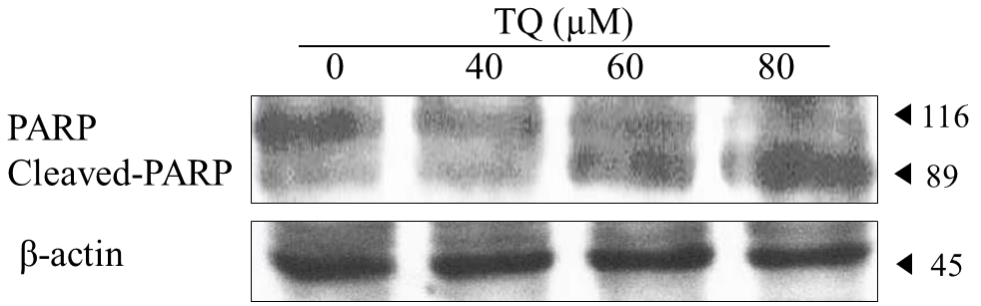


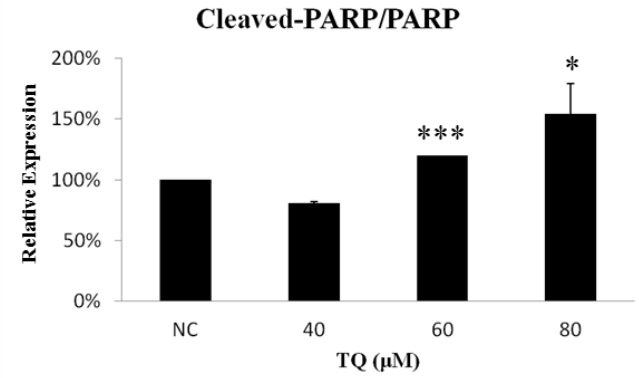


Figure 3.1.4 Effect of TQ on apoptosis induction.

(A) Effect of TQ on annexin V positive cell population. MCF-7 cells were treated with increasing concentrations of TQ for 12 h before analyzed with annexin V assay. The data are representative of three independent experiments. (B) Effect of TQ on the protein expression of cleaved-PARP/PARP. MCF-7 cells were treated with increasing concentrations of TQ for 12 h. Whole cell lysate was then used for Western blot analysis. The data are representative of at least three independent experiments. * $p<0.05$, *** $p<0.001$ vs. negative control.

3.1.5 Effect of TQ on apoptotic pathway

After having determined that TQ is pro-apoptotic, our next interest was to study the effect of TQ on various caspases and Bcl-2 family proteins. TQ was found to reduce the protein expression of procaspase 8, 9 and 7 in a dose-dependent manner (Figure 3.1.5), suggested that cleavage events were occurred. Moreover, the protein expression of Bcl-2 was decreased after TQ treatment, with a slight increase in Bax. This would resulting in an increase of Bax/Bcl-2 ratio (Figure 3.1.5). These results explained the pro-apoptotic effect of TQ in breast cancer cells.

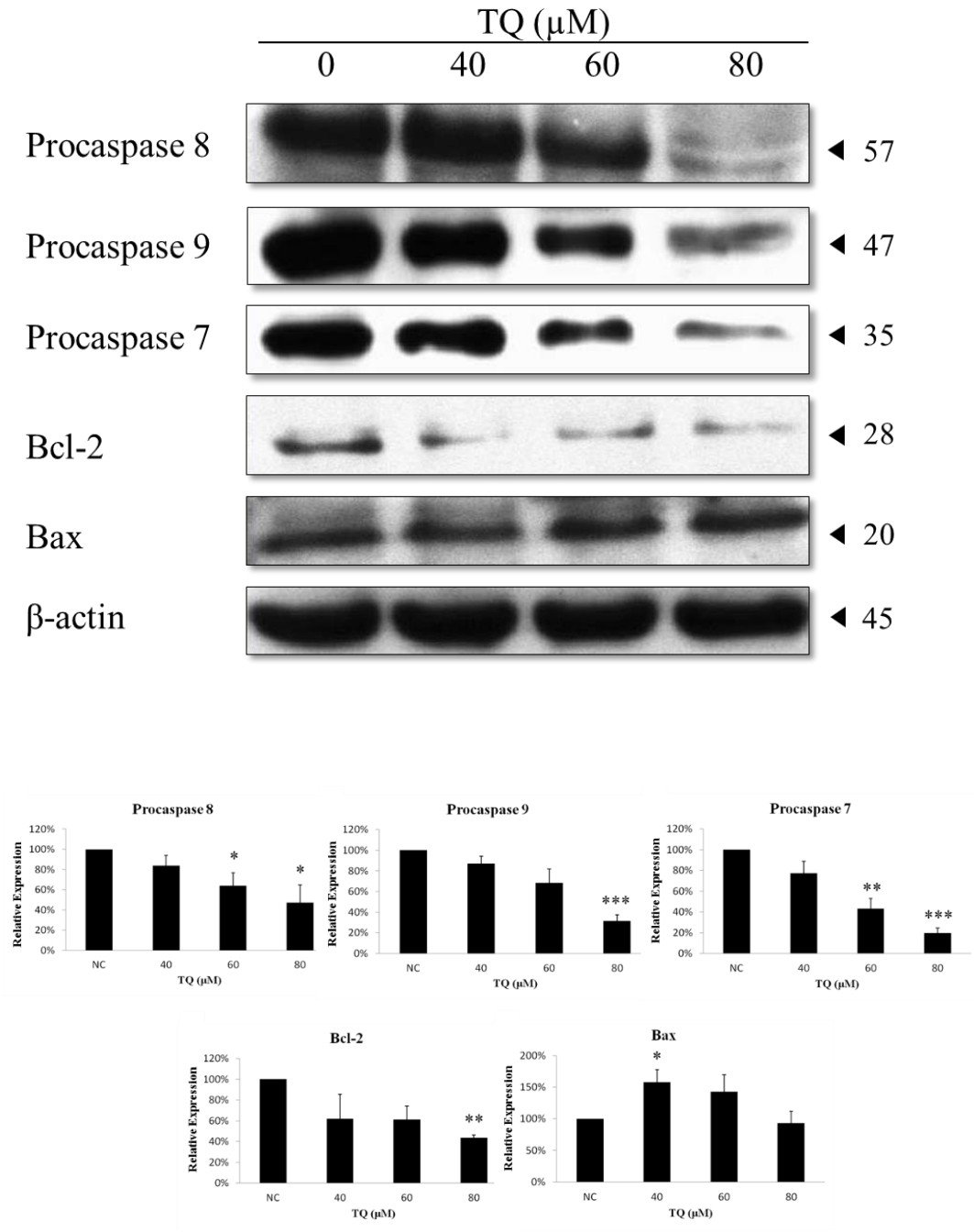


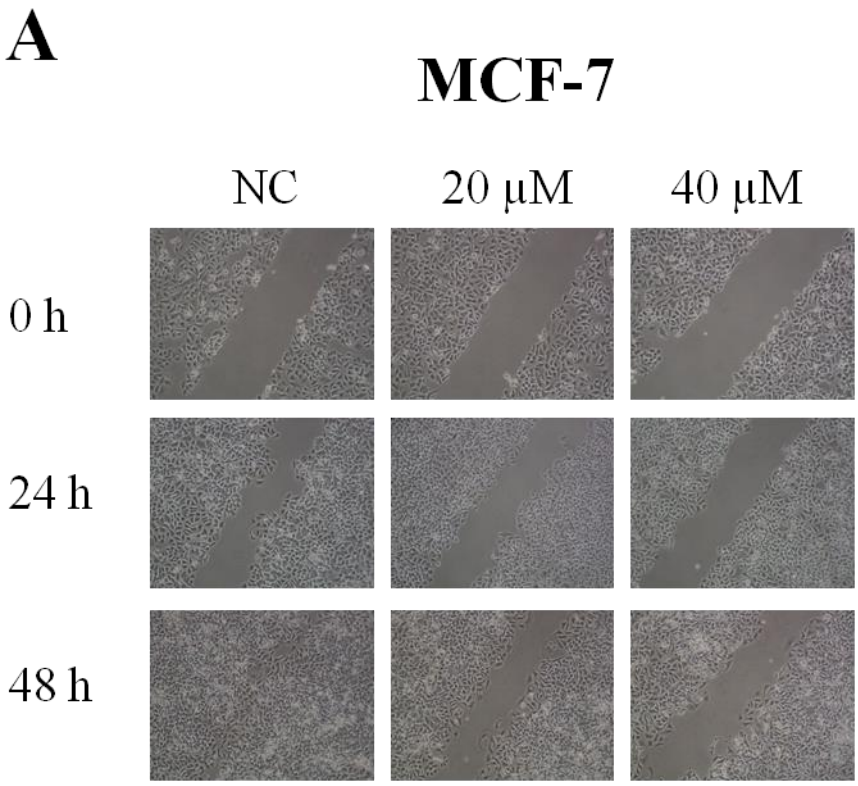
Figure 3.1.5 Effect of TQ on the protein expression of caspases and Bcl-2 family proteins.

MCF-7 cells were treated with increasing concentrations of TQ for 12 h. Whole cell lysate was then used for Western blot analysis. The data are representative of at least three independent experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. negative control.

3.2 Studies on the anti-metastatic effect of TQ in breast cancer cells

3.2.1 Effect of TQ on cell migration

In order to study the effect of TQ on breast cancer cell migration, 'wound healing' assay was used. A 'wound' was created within cell culture and the ability of breast cancer cells to fill up the 'wound' after TQ treatment was examined. At 48 h microscopic observation, the 'wound' was barely detected in PBS-treated cells in both MCF-7 and MDA-MB-231 cell lines. In contrast, TQ was found to inhibit the migration of cancer cells from both cell lines to the created 'wound', as shown by the visible 'wound' in the cell culture after 24 h and 48 h TQ exposure (Figure 3.2.1).



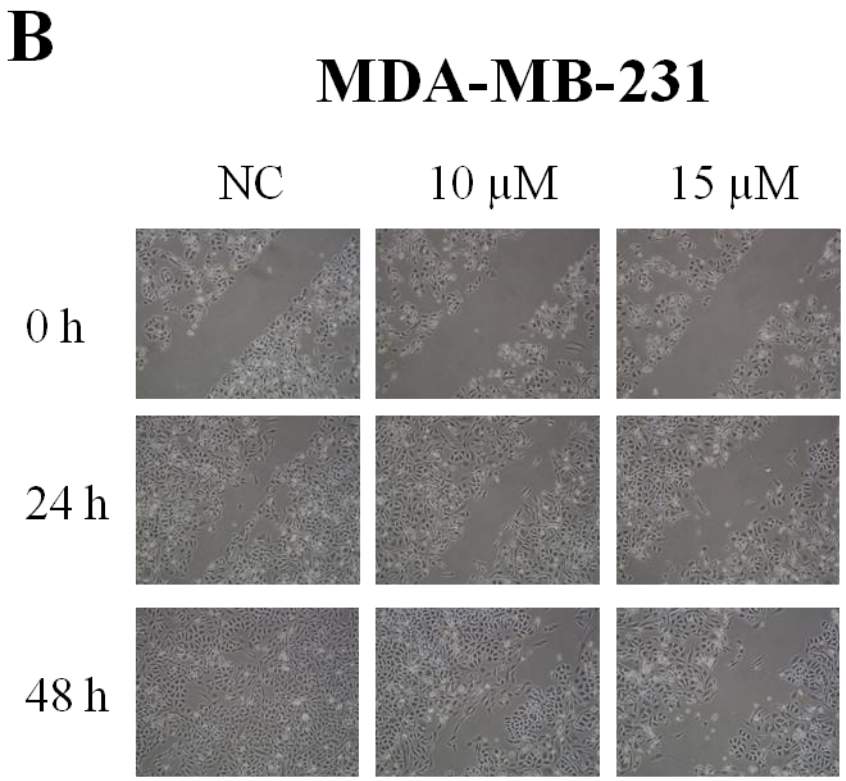
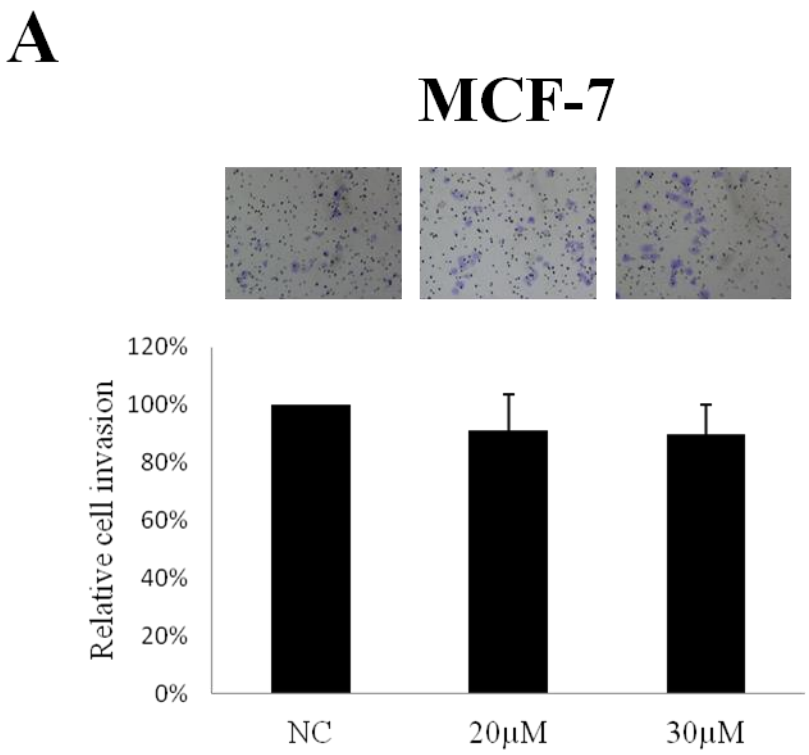


Figure 3.2.1 Effect of TQ on breast cancer cell migration.

(A) MCF-7 and (B) MDA-MB-231 cells were cultured until 80-90% confluent before a 'wound' was created. The cells were treated with indicated concentrations of TQ for 48 h. Microscopy observations were recorded at 0 h, 24 h and 48 h after TQ treatment. The data are representative of three independent experiments.

3.2.2 Effect of TQ on cell invasion

The invasiveness of breast cancer cells after TQ treatment was examined with a commercial invasion assay kit (BD Matrigel™ Invasion Chamber 24-well Plate 8 micron). As shown in Figure 3.2.2, TQ reduced the number of stained cells in a dose-dependent manner in MDA-MB-231 cells but not MCF-7 cells. This could be due to the strong invasive property of MDA-MB-231 cells but low to medium in MCF-7 cells.



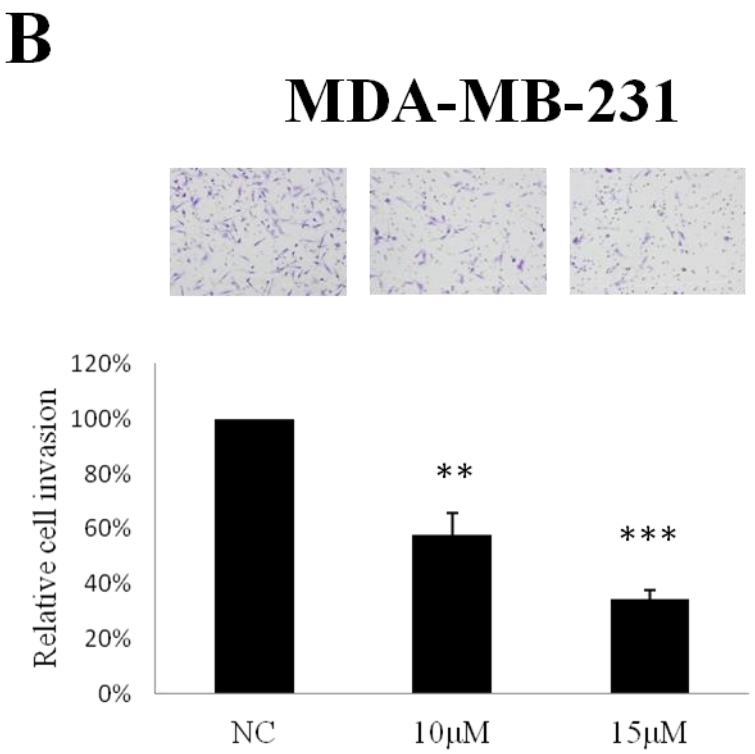


Figure 3.2.2 Effect of TQ on breast cancer cell invasion.

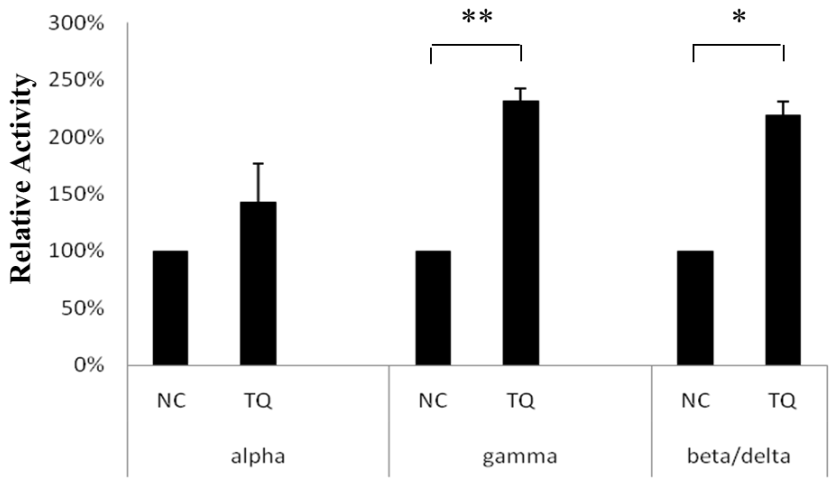
BD Matrigel™ Invasion Chamber 24-well plate 8 micron from BD Biosciences was used according to the manufacturer's protocol. Indicated concentrations of TQ were added into the chamber for 48 h in (A) MCF-7 cells or 24 h in (B) MDA-MB-231 cells. The number of the migratory cells of TQ-treated groups was normalized against the untreated group. Values are means ± S.E.M. of two or three independent experiments. ** $p<0.01$, *** $p<0.001$ vs. negative control.

3.3 Studies on the role of PPAR-γ in the anticancer activities of TQ

3.3.1 Effect of TQ on the activity of PPARs

Since TQ was found to form interaction with several residues of PPAR-γ molecule (Woo et al., 2011), thus whether the effects of TQ involved PPARs were investigated. The effect of TQ on the activity of PPARs was first tested by using two-step luciferase assay as described in Materials and Methods. As shown in Figure 3.3.1A, TQ significantly increased the activity of PPAR-γ and PPAR-β/δ in MCF-7 cells. Although the activity of PPAR-α was increased after TQ treatment, however this increase was not significant. Next, whether these inductions were specific were examined by using respective pharmacological agonist and antagonist. The activity of PPAR-γ was found to both be induced by TQ and PPAR-γ agonist, 15d-PGJ2, as shown in Figure 3.3.1B. These inductions could then be reversed by pre-treatment with PPAR-γ antagonist, GW9662 (Figure 3.3.1B). Similarly, the activity of PPAR-β/δ could both be induced by TQ and PPAR-β/δ agonist, GW0742 (Figure 3.3.1C). Pre-treatment with PPAR-β/δ antagonist, GSK0660, could reverse GW0742-induced PPAR-β/δ activity but not TQ-induced PPAR-β/δ activity.

A



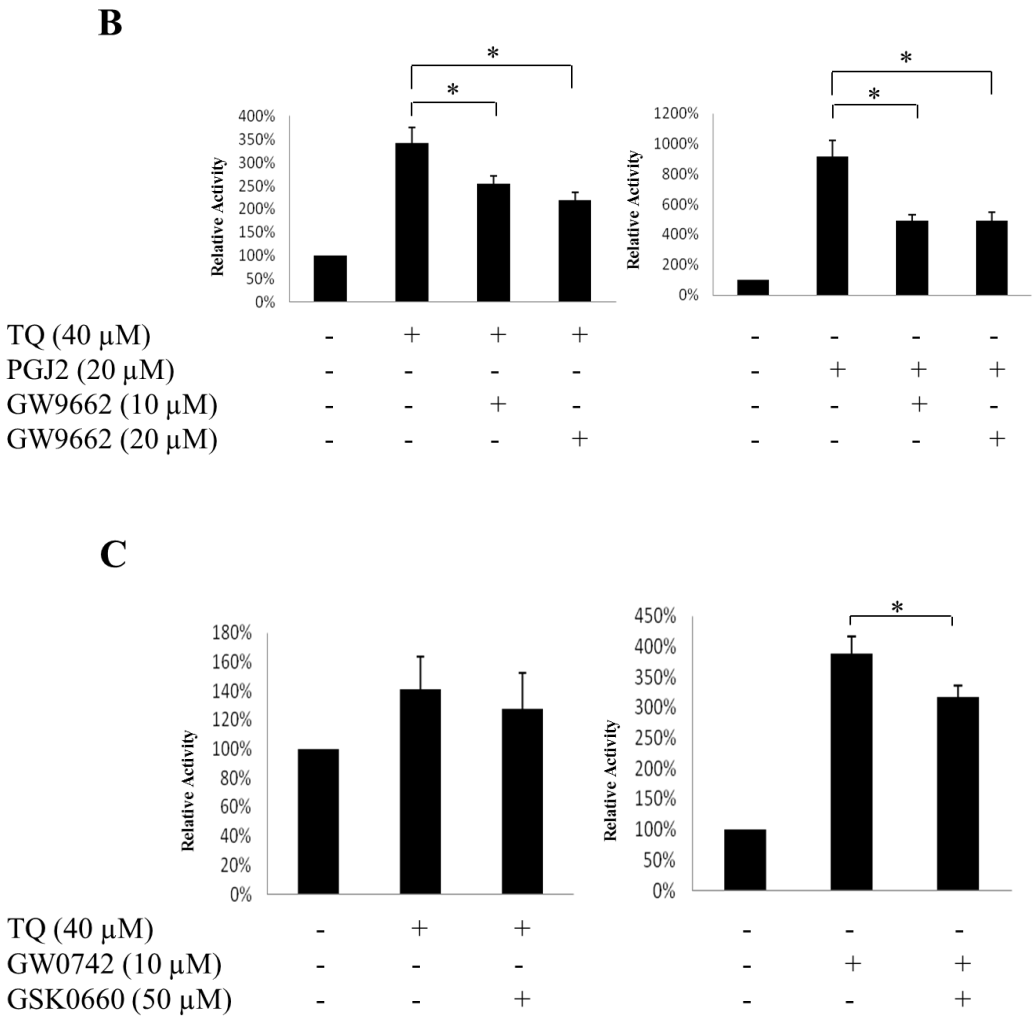


Figure 3.3.1 Effect of TQ on various PPARs.

Two step luciferase assay was used whereby MCF-7 cells were transfected with either of GAL4-PPAR-α LBD, GAL4-PPAR-γ LBD or GAL4-PPAR-β/δ LBD plasmids, together with GAL4-Luc and Renilla plasmids for 12-14 h before appropriate treatment. (A) Effect of TQ on the activity of various PPARs. Transfected cells were treated with 40 μM TQ for 18 h. Whole cell lysate was then used for luciferase assay. The data are expressed as percentages in relative to the control. Values are means ± S.E.M. of two or three independent experiments. * $p<0.05$, ** $p<0.01$ vs. control. (B) Study on the specificity of TQ on PPAR-γ activity. Transfected cells were pre-treated with 10 μM or 20 μM GW9662 for 2 h before treatment with 40 μM TQ or 20 μM 15d-PGJ2 for 18 h. Whole cell lysate was then used for luciferase assay. The data are expressed as percentages in relative to the control. Values are means ± S.E.M. of two or three independent experiments. * $p<0.05$. (C) Study on the specificity of TQ on PPAR-β/δ activity. Transfected cells were pre-treated with 50 μM GSK0660 for 4 h before treatment with 40 μM TQ or 10 μM GW0742 for 18 h. Whole cell lysate was then used for luciferase assay. The data are expressed as percentages in relative to the control. Values are means ± S.E.M. of two or three independent experiments. * $p<0.05$.

3.3.2 Effect of TQ on PPAR-γ activity

Since the effect of TQ on PPAR-γ activity was specific, our next interest was to study the effect of TQ on PPAR-γ pathway in a greater detail. One-step luciferase assay was used to measure PPAR-γ activity as described in Materials and Methods section. As shown in Figure 3.3.2, TQ induced PPAR-γ activity in a dose- and time-dependent manner in MCF-7 cells. TQ at 40 μM could significantly increase the level of PPAR-γ activity as early as 6 h exposure.

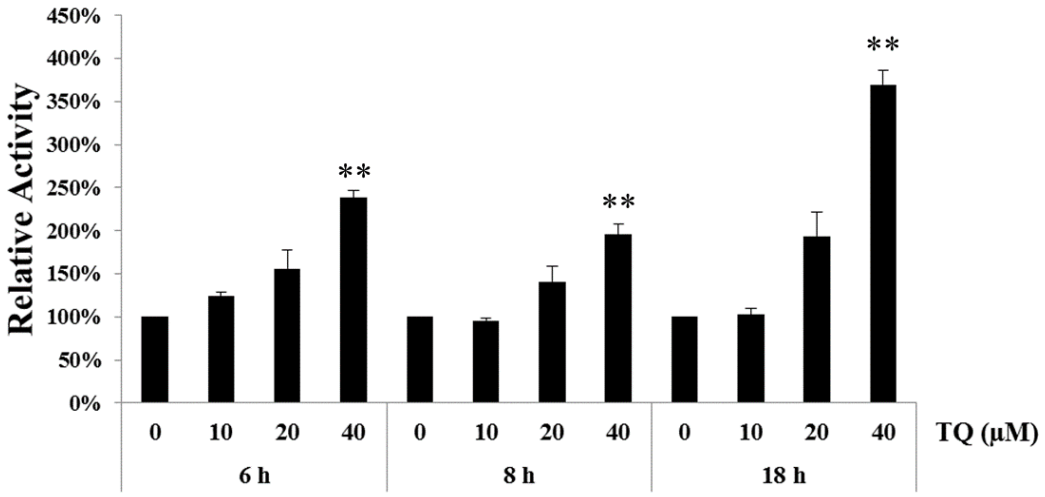


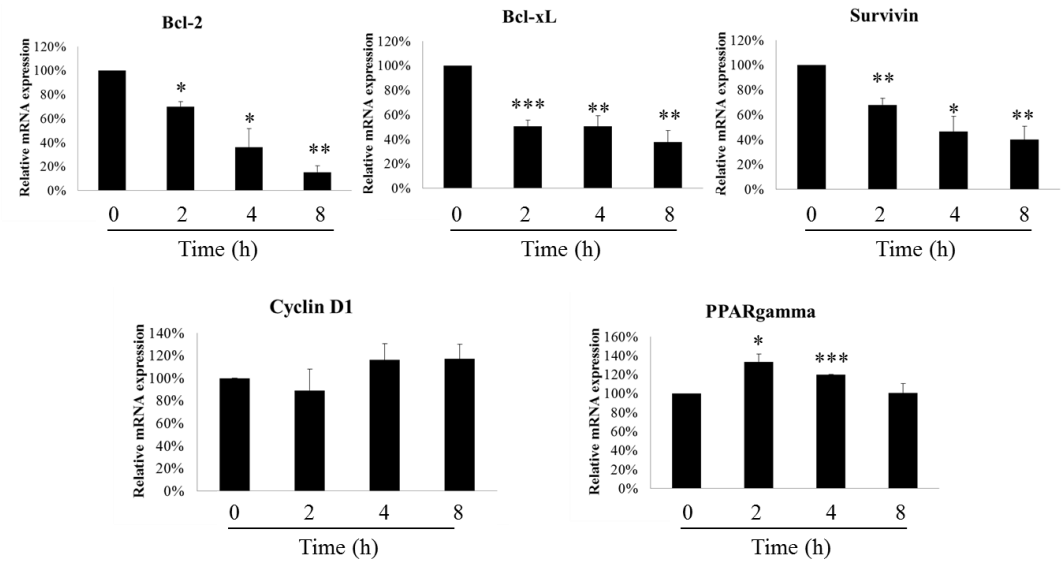
Figure 3.3.2 The dose- and time-response effects of TQ on PPAR-γ activity.

One step luciferase assay was used whereby MCF-7 cells were transfected with pPPRE-tk-Luc and Renilla plasmids for 12-14 h before treatment with increasing concentrations of TQ for 6, 8 and 18 h. Whole cell lysate was then used for luciferase assay. The data are expressed as percentages in relative to the control. Values are means ± S.E.M. of two or three independent experiments. ** $p<0.01$ vs. negative control.

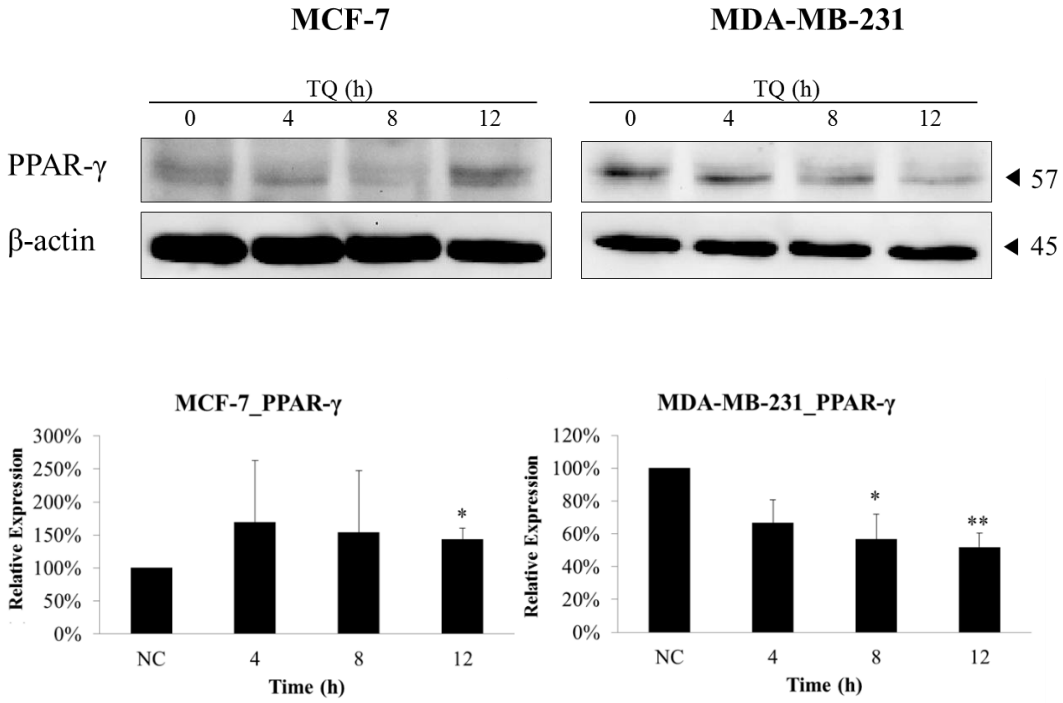
3.3.3 Effect of TQ on the expression of PPAR-γ and PPAR-γ-regulated genes

Genes such as Bcl-2, Bcl-xL, cyclin D1 and survivin are important in cell cycle progression and apoptotic pathway. Since these genes were found to be modulated by PPAR-γ pathway (Robbins and Nie, 2012), the expression of these genes after TQ treatment was also examined. From the results, the mRNA expression of PPAR-γ in MCF-7 cells was significantly increased at 2 h TQ exposure but gradually decreased till insignificant at 8 h exposure (Figure 3.3.3A). The mRNA expression of PPAR-γ-regulated genes, such as Bcl-2, Bcl-xL and survivin, in MCF-7 cells was significantly decreased in a time-dependent manner after TQ treatment; however no significant change was observed in cyclin D1 (Figure 3.3.3A). Similarly, the protein expression of PPAR-γ in MCF-7 cells was significantly increased after TQ treatment (Figure 3.3.3B). Interestingly, a decreasing trend was observed in MDA-MB-231 cells after TQ treatment (Figure 3.3.3B). The protein expression of PPAR-γ-regulated genes including Bcl-2, Bcl-xL and survivin, but not cyclin D1, was decreased after TQ treatment in MCF-7 cells, in line with the mRNA results (Figure 3.3.3C).

A


Bcl-2
Bcl-xL
Survivin
Cyclin D1
PPARgamma
Relative mRNA expression
Time (h)
0
2
4
8

B


MCF-7
MDA-MB-231
TQ (h)
0
4
8
12
PPAR-γ
β-actin
57
45
MCF-7_PPAR-γ
MDA-MB-231_PPAR-γ
Relative Expression
NC
Time (h)

C

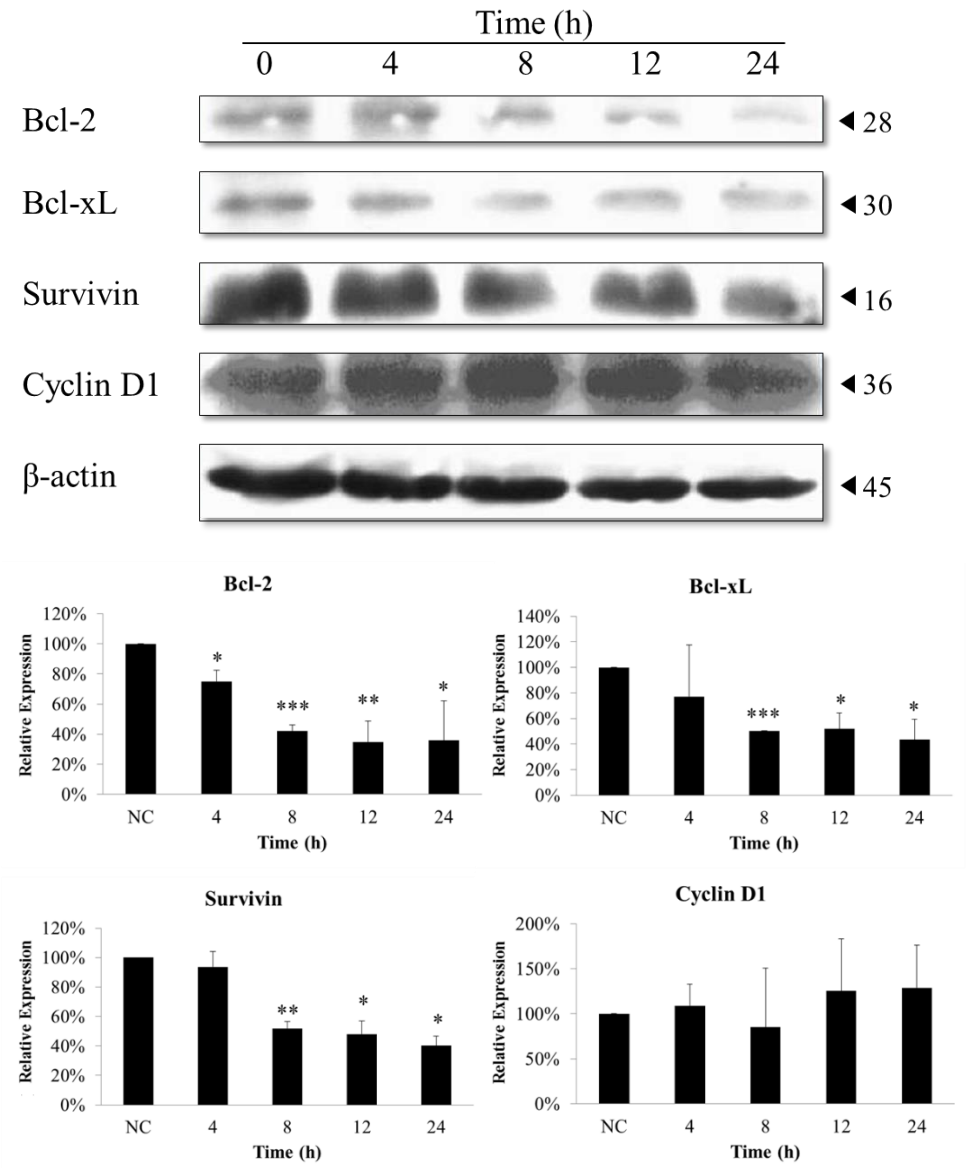
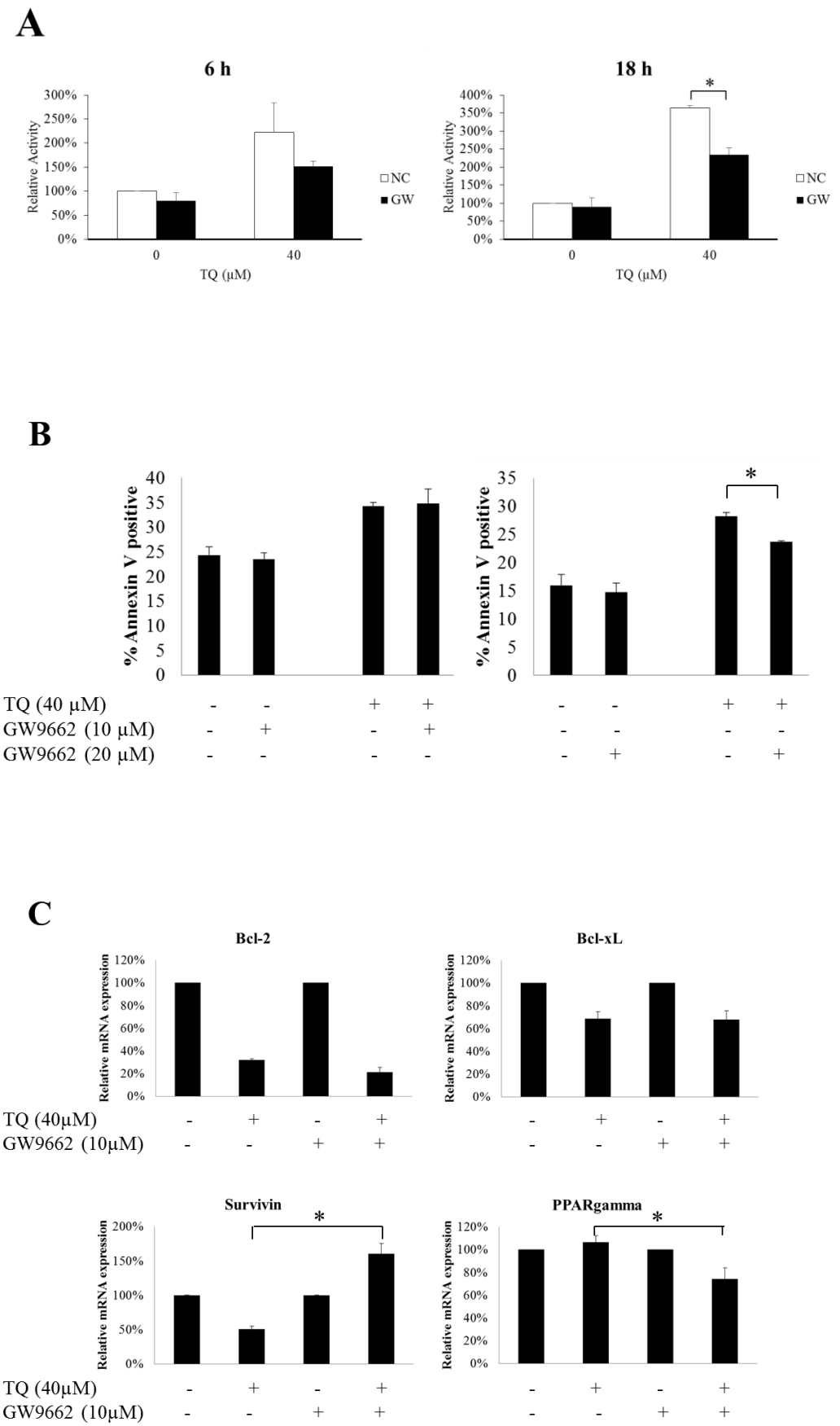


Figure 3.3.3 Effect of TQ on the expression of PPAR-γ and PPAR-γ-regulated genes.

(A) Effect of TQ on the mRNA expression of PPAR-γ and PPAR-γ-regulated genes in MCF-7 cells. Cells were treated with 40 μM TQ for 0, 2, 4 and 8 h. The cells were then harvested for real time RT-PCR. Values are means ± S.E.M. of two or three independent experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. negative control. (B) Effect of TQ on the protein expression of PPAR-γ in MCF-7 and MDA-MB-231 cells. MCF-7 and MDA-MB-231 cells were treated with 40 μM TQ for 0, 4, 8 and 12 h. Whole cell lysate was then used for Western blot analysis. The data are representative of at least three independent experiments. * $p<0.05$, ** $p<0.01$ vs. negative control. (C) Effect of TQ on the protein expression of PPAR-γ-regulated genes in MCF-7 cells. Cells were treated with 40 μM TQ for 0, 4, 8, 12 and 24 h. Whole cell lysate was then used for Western blot analysis. The data are representative of at least three independent experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. negative control.

3.3.4 Effect of GW9662 on TQ-induced apoptosis and TQ-induced suppression of PPAR-γ-regulated genes

A PPAR-γ inhibitor, GW9662, was used to examine whether it could reverse TQ-induced PPAR-γ activation. GW9662 can covalently bind to Cys285 located on helix 3 of the PPAR-γ ligand binding domain resulting in the irreversible loss of ligand binding (Leesnitzer et al., 2002). By using one-step luciferase assay, pre-treatment with GW9662 was found to significantly reverse TQ-induced PPAR-γ activity in MCF-7 cells (Figure 3.3.4A). This reversal, however, was not able to restore PPAR-γ activity to the basal level. In addition, GW9662 could also reverse TQ-induced apoptosis, at least partially, as shown in Figure 3.3.4B. Next, whether the change in PPAR-γ activity would affect the mRNA and protein expressions of PPAR-γ-regulated genes in TQ-treated cells were also investigated. Our data showed that the decrease of survivin mRNA expression, but not Bcl-2 and Bcl-xL, induced by TQ could be reversed by GW9662 (Figure 3.3.4C). Similarly, GW9662 was found to reverse the decrease of survivin protein expression induced by TQ, but not Bcl-2 and Bcl-xL (Figure 3.3.4D), which was in line with their mRNA results. These results suggested that the suppression of survivin could be due to TQ-induced PPAR-γ activation.


A
6 h
18 h
Relative Activity
300%
250%
200%
150%
100%
50%
0%
400%
350%
0
40
TQ (μM)
NC
GW
*
B
% Annexin V positive
40
35
30
25
20
15
10
5
0
TQ (40 μM)
GW9662 (10 μM)
GW9662 (20 μM)
C
Bcl-2
Bcl-xL
Survivin
PPARgamma
Relative mRNA expression
120%
100%
80%
60%
40%
20%
0%
200%
150%
50%
TQ (40μM)
GW9662 (10μM)

D

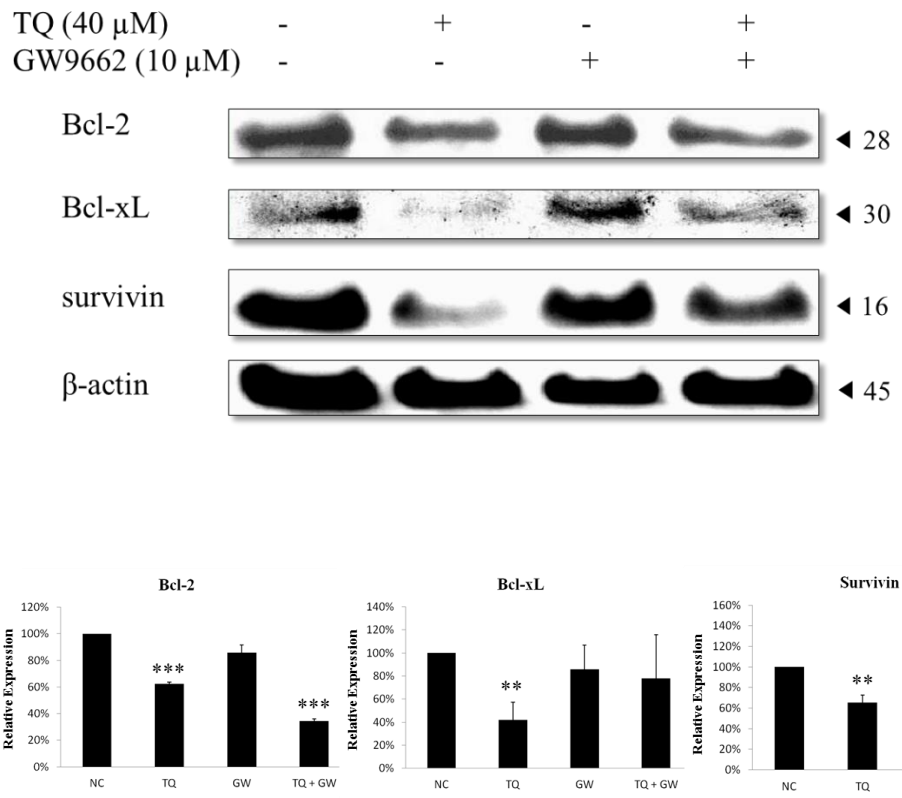


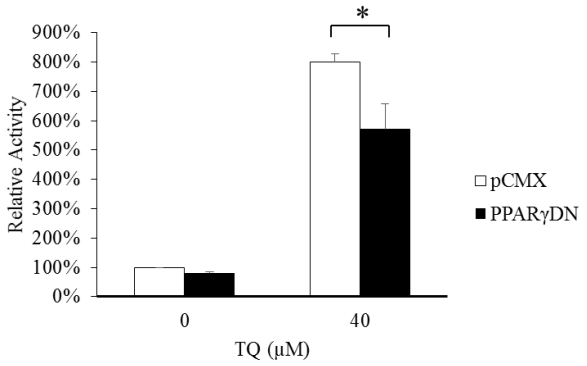
Figure 3.3.4 Effect of GW9662 on the apoptotic effect of TQ and the expression of PPAR-γ-regulated genes after TQ treatment.

(A) Effect of GW9662 on TQ-induced PPAR-γ activity in MCF-7 cells. One-step luciferase assay was used whereby cells were transfected with pPPRE-tk-Luc and Renilla plasmids for 12-14 h. The cells were pre-treated with 10 μM GW9662 for 2 h before treatment with 40 μM TQ for 6 and 18 h. Whole cell lysate was then used for luciferase assay. The data are expressed as percentages in relative to the control. Values are means ± S.E.M. of two or three independent experiments. * $p<0.05$. (B) Effect of GW9662 on TQ-induced apoptosis in MCF-7 cells. Cells were pre-treated with 10 or 20 μM GW9662 for 2 h before treatment with 50 μM TQ for 12 h. The cells were then analyzed with annexin V assay. Values are means ± S.E.M. of three independent experiments. * $p<0.05$. (C) Effect of GW9662 on the mRNA expression of PPAR-γ and PPAR-γ-regulated genes after TQ treatment. MCF-7 cells were pre-treated with 10 μM GW9662 for 2 h before treatment with 40 μM TQ for 8 h. The cells were then harvested for real time RT-PCR. The data are expressed as the percentage relative to their respective control. Values are means ± S.E.M. of two or three independent experiments. * $p<0.05$. (D) Effect of GW9662 on the protein expression of PPAR-γ-regulated genes after TQ treatment. MCF-7 cells were pre-treated with 10 μM GW9662 for 2 h before treatment with 40 μM TQ for 24 h. Whole cell lysate was then used for Western blot analysis. The data are representative of at least three independent experiments. ** $p<0.01$, *** $p<0.001$ vs. negative control.

3.3.5 Effect of PPAR-γ dominant negative on TQ-induced suppression of PPAR-γ-regulated genes

In addition to pharmacological approach (PPAR-γ inhibitor), a genetic approach, i.e. PPAR-γ dominant negative, was also used to suppress PPAR-γ activity which aimed to investigate the role of TQ-induced PPAR-γ activation on TQ's effects. The increase of PPAR-γ protein expression (Figure 3.3.5C) confirmed the successful transfection of PPAR-γ dominant negative (PPAR-$\gamma^{C126A/E127A}$) plasmids, whereby this increase represented the over-expression of faulty PPAR-γ receptor (Kumar et al., 2009; Kumar et al., 2013). This type of faulty receptor would bind to PPAR-γ ligands but would not initiate downstream signaling. As shown in Figure 3.3.5A by one-step luciferase assay, PPAR-γ dominant negative significantly reversed TQ-induced PPAR-γ activity in MCF-7 cells, though this reversal was not completely. A small reduction in PPAR-γ activity was observed in vehicle-control cells after PPAR-γ dominant negative transfection, suggesting that the antagonism effect on wild type PPAR-γ was not completely. By using real time RT-PCR, PPAR-γ dominant negative was found to reverse the decrease of survivin mRNA expression induced by TQ, but not Bcl-2 and Bcl-xL (Figure 3.3.5B). Similarly, the decrease of survivin protein expression induced by TQ could also be reversed by PPAR-γ dominant negative, but not Bcl-2 and Bcl-xL (Figure 3.3.5C). These trends were similar to those in the results of PPAR-γ inhibitor, suggesting that the suppression of survivin was indeed due to TQ-induced PPAR-γ activation.

A



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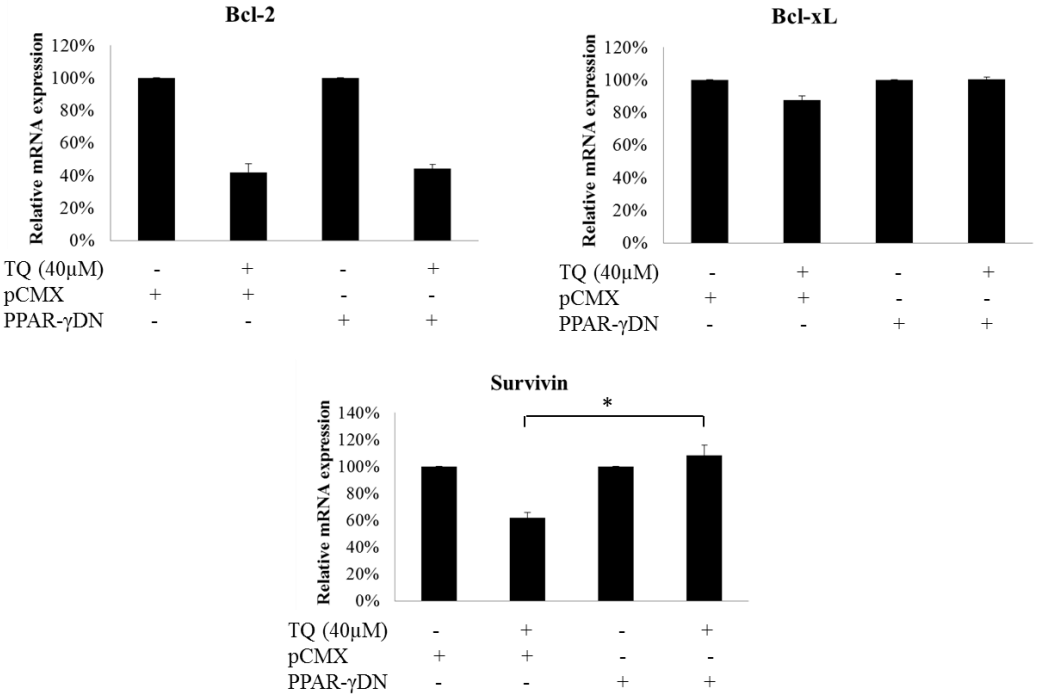
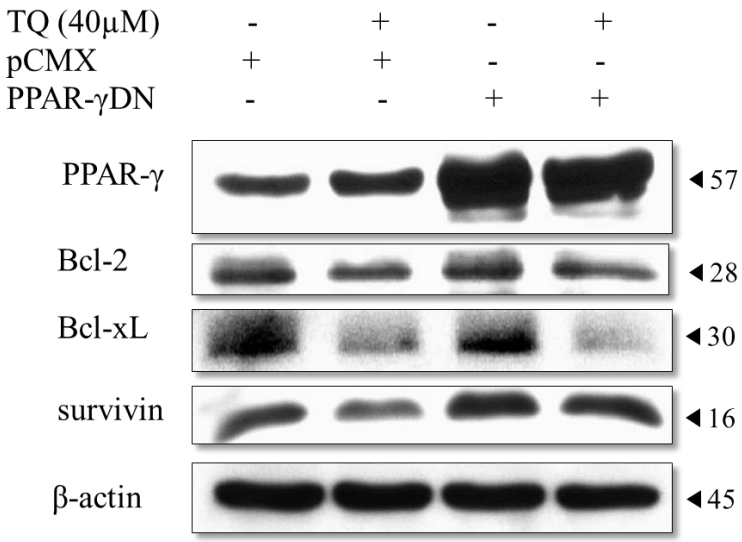

Bcl-2
Relative mRNA expression
120%
100%
80%
60%
40%
20%
0%
TQ (40μM) - + - +
pCMX + + - -
PPAR-γDN - - + +
Bcl-xL
Relative mRNA expression
120%
100%
80%
60%
40%
20%
0%
TQ (40μM) - + - +
pCMX + + - -
PPAR-γDN - - + +
Survivin
*
Relative mRNA expression
140%
120%
100%
80%
60%
40%
20%
0%
TQ (40μM) - + - +
pCMX + + - -
PPAR-γDN - - + +

C


TQ (40μM) - + - +
pCMX + + - -
PPAR-γDN - - + +
PPAR-γ
Bcl-2
Bcl-xL
survivin
β-actin
57
28
30
16
45

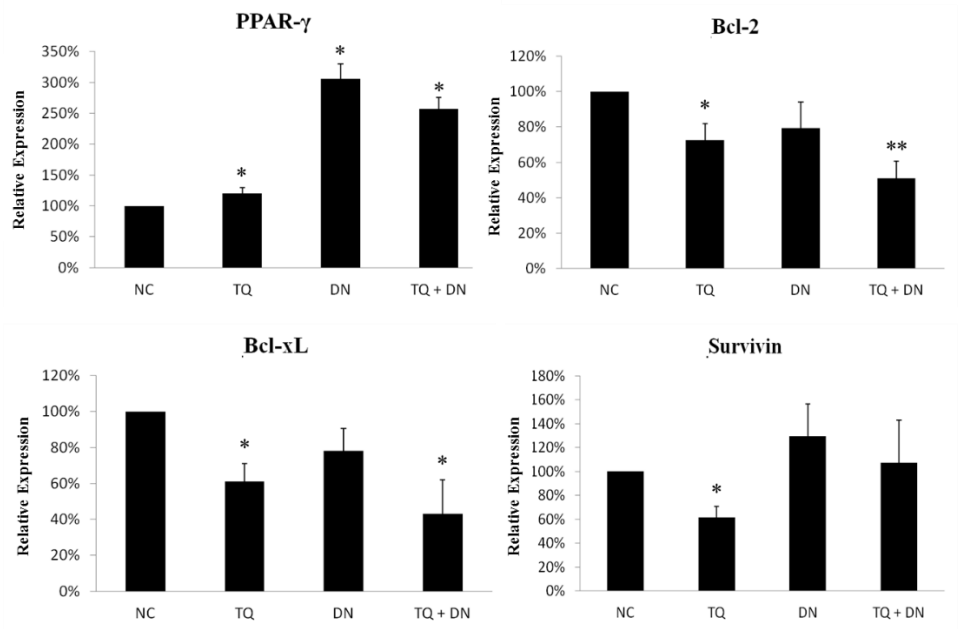


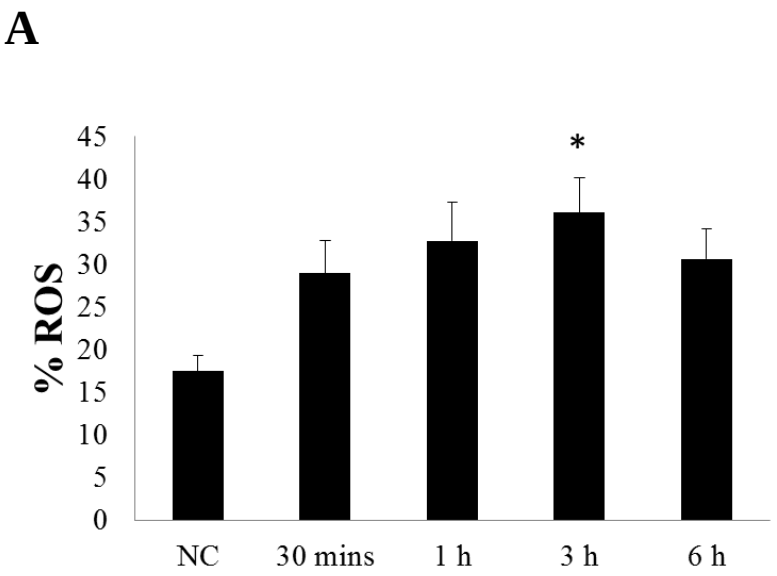
Figure 3.3.5 Effect of PPAR-γ dominant negative on the expression of PPAR-γ-regulated genes after TQ treatment.

(A) Effect of PPAR-γ dominant negative on TQ-induced PPAR-γ activity. One step luciferase assay was used whereby MCF-7 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 TQ for 18 h. Whole cell lysate was then used for luciferase assay. The data are expressed as percentages in relative to the control. Values are means ± S.E.M. of two or three independent experiments. * $p<0.05$. (B) Effect of PPAR-γ dominant negative on the mRNA expression of PPAR-γ-regulated genes after TQ treatment. MCF-7 cells were transfected with PPAR-γDN or pCMX plasmids for 12-14 h before treatment with 40 μM of TQ for 8 h. The cells were then harvested for real time RT-PCR. The data are expressed as the percentages relative to their respective control. Values are means ± S.E.M. of two or three independent experiments. * $p<0.05$. (C) Effect of PPAR-γ dominant negative on the protein expression of PPAR-γ and PPAR-γ-regulated genes after TQ treatment. MCF-7 cells were transfected with PPAR-γDN or pCMX plasmids for 12-14 h before treatment with 40 μM TQ for 24 h. Whole cell lysate was then collected for Western blot analysis. The data are representative of at least three independent experiments. * $p<0.05$, ** $p<0.01$ vs. negative control.

3.4 Studies to investigate the role of ROS in the anticancer activities of TQ

3.4.1 Effect of TQ on ROS production

The induction effect of TQ on ROS production as a mechanism to cause cancer cell death has been reported in primary effusion lymphoma cells, DLD-1 human colon cancer cells, C4-2B prostate cancer cells and Jurkat cells (Woo et al., 2012). Whether ROS induction was also involved in TQ-induced apoptosis in breast cancer cells was investigated. By using flow cytometry after Mitosox staining, our data showed that TQ significantly induced ROS level in a time-dependent manner (Figure 3.4.1A). ROS level was maximal at 3 h TQ exposure and slightly decreased at 6 h. Pre-treatment with N-acetylcysteine (NAC), a strong anti-oxidant, could abrogate TQ-induced ROS production in both MCF-7 (Figure 3.4.1B) and MDA-MB-231 cell lines (Figure 3.4.1C).



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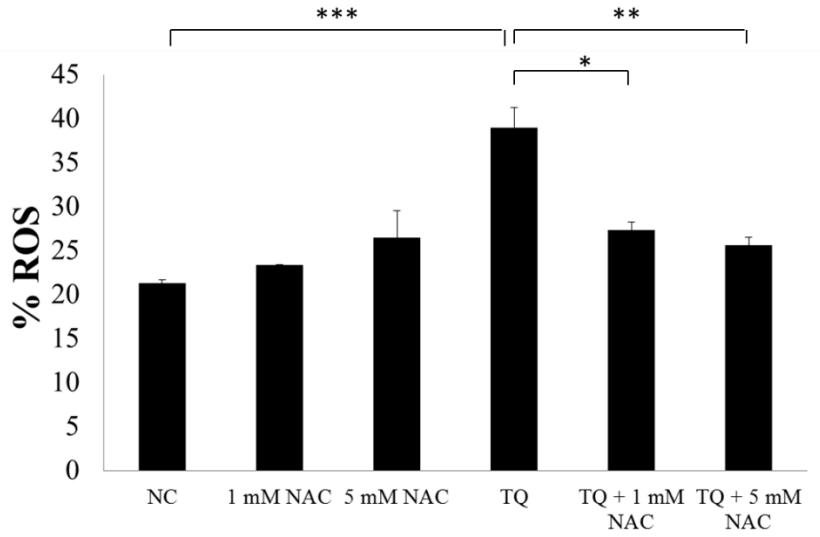

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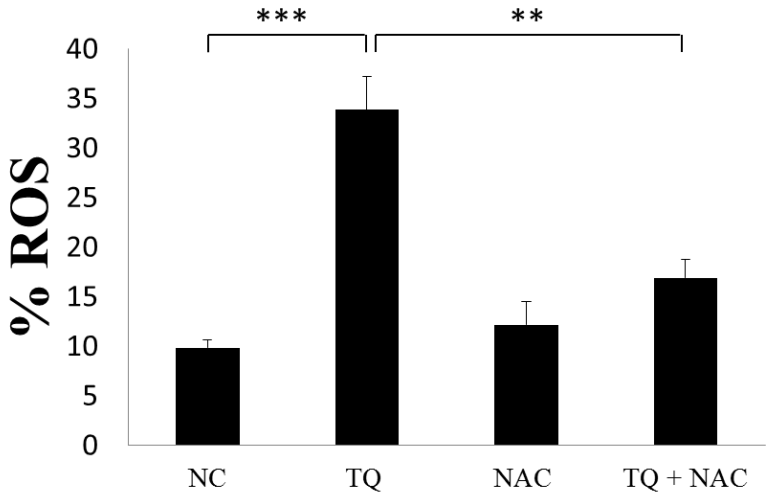


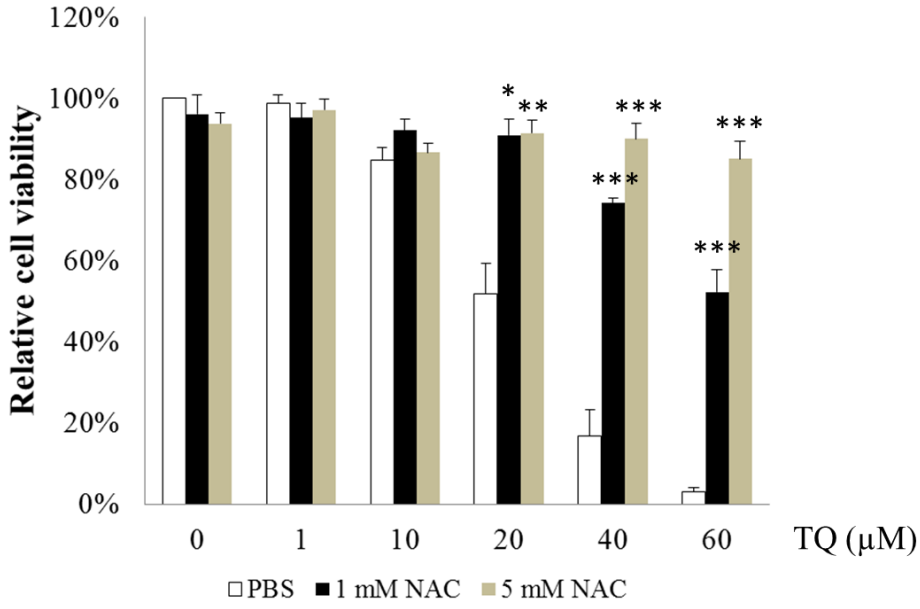
Figure 3.4.1 Effect of TQ on ROS production in breast cancer cells.

(A) Time-response effect of TQ on ROS level. MCF-7 cells were treated with 40 µM TQ for various incubation periods ranging up to 6 h. The cells were analyzed by flow cytometry after Mitosox staining. Values are means ± S.E.M. of at least three independent experiments. * $p<0.05$ vs. negative control. (B) Effect of NAC on TQ-induced ROS production in MCF-7 cells. Cells were pre-treated with 1 or 5 mM NAC for 2 h before exposure to 40 µM TQ for 3 h. The cells were analyzed by flow cytometry after Mitosox staining. Values are means ± S.E.M. of at least three independent experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. (C) Effect of NAC on TQ-induced ROS production in MDA-MB-231 cells. Cells were pre-treated with 5 mM NAC for 2 h before exposure to 40 µM TQ for 3 h. The cells were analyzed by flow cytometry after Mitosox staining. Values are means ± S.E.M. of at least three independent experiments. ** $p<0.01$, *** $p<0.001$.

3.4.2 The role of ROS in the cytotoxic effect of TQ

In order to study the role of TQ-induced ROS in the cytotoxicity of TQ, NAC was used to block the induction of ROS. By using MTT assay, our data showed that TQ's growth inhibition effect could be abrogated by NAC in a dose-dependent manner. Pre-treatment with 5 mM NAC could reverse TQ-induced growth inhibition almost completely in both MCF-7 (Figure 3.4.2A) and MDA-MB-231 cells (Figure 3.4.2B). These results demonstrate that TQ's cytotoxicity was mediated via the increase in ROS.

A



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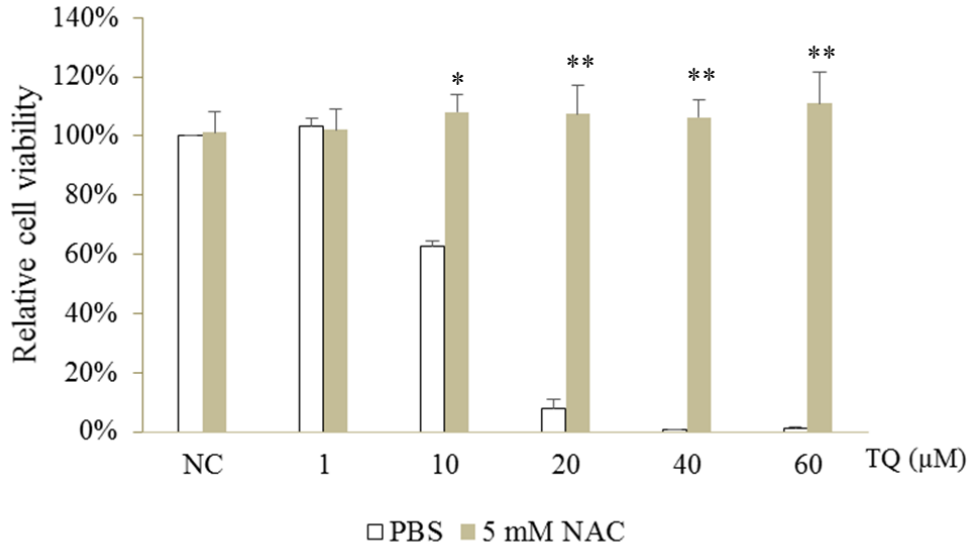
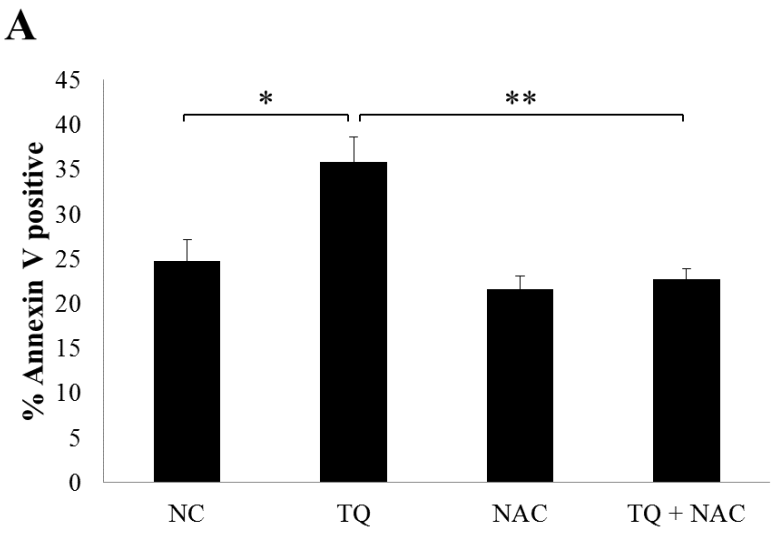


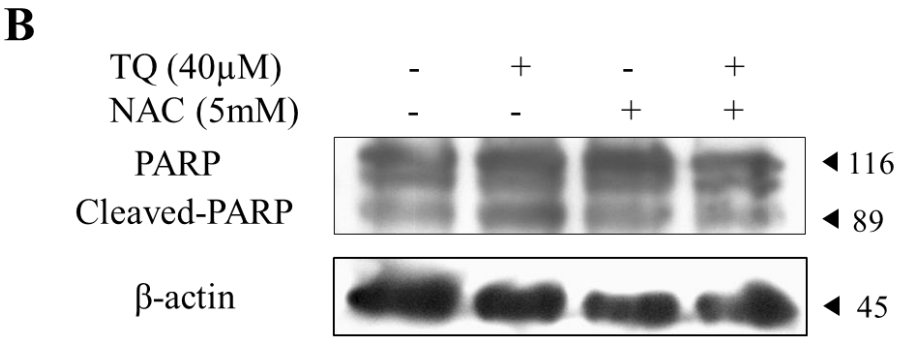
Figure 3.4.2 Effect of NAC on TQ-induced cytotoxicity in breast cancer cells.

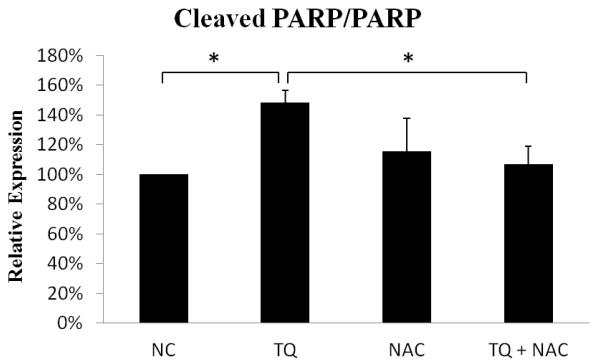
(A) MCF-7 and (B) MDA-MB-231 cells were pre-treated with 1 or 5 mM NAC for 2 h before exposure to increasing concentrations of TQ for 24 h. The cells were then analyzed with MTT assay. Values are means ± S.E.M. of at least three independent experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. PBS control.

3.4.3 The role of ROS in TQ-induced apoptosis

Whether TQ-induced ROS played a role in the pro-apoptotic effect of TQ was also investigated. Our results showed that the increased percentage of annexin V positive cells induced by TQ could be completely reversed by NAC pre-treatment (Figure 3.4.3A). Moreover, the cleavage of PARP protein induced by TQ in MCF-7 (Figure 3.4.3B) and MDA-MB-231 cells (Figure 3.4.3C) could also be reversed by NAC pre-treatment. These results suggest that ROS indeed play a crucial role in TQ-induced apoptosis.







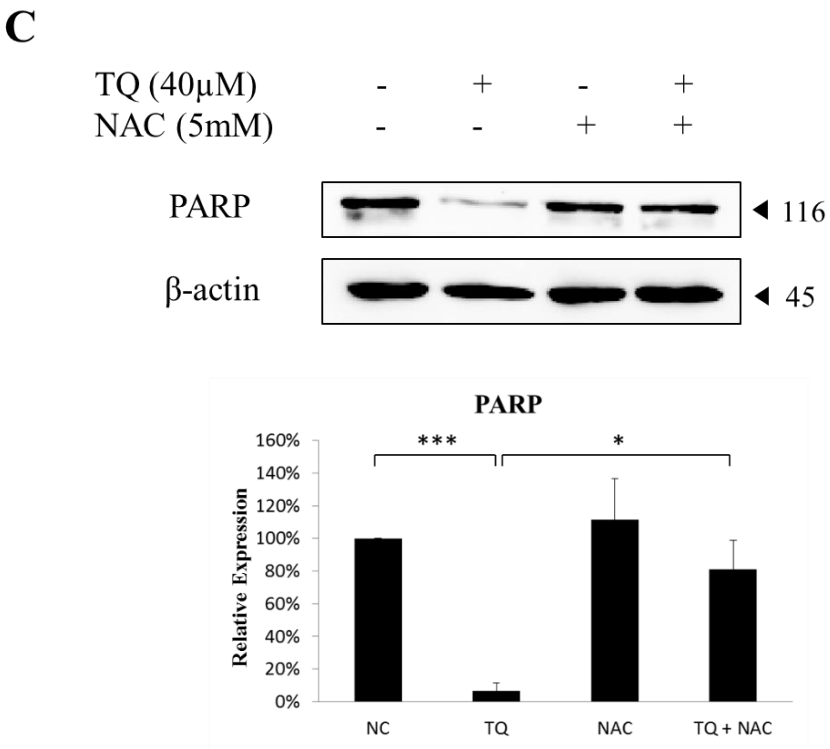


Figure 3.4.3 The role of ROS in TQ-induced apoptosis.

(A) Effect of NAC on TQ-induced increased percentage of annexin V positive cells. MCF-7 cells were pre-treated with 5 mM NAC for 2 h before exposure to 40 μM TQ for 12 h. The cells were then analyzed with annexin V assay. Values are means ± S.E.M. of at least three independent experiments. * $p<0.05$, ** $p<0.01$. (B) Effect of NAC on TQ-induced PARP-cleavage in MCF-7 cells. Cells were pre-treated with 5 mM NAC for 2 h before exposure to 40 μM TQ for 12 h. Whole cell lysate was collected for Western blot analysis. Data are a representative of at least three independent experiments. * $p<0.05$. (C) Effect of NAC on TQ-induced PARP-cleavage in MDA-MB-231 cells. Cells were pre-treated with 5 mM NAC for 2 h before exposure to 40 μM TQ for 12 h. Whole cell lysate was collected for Western blot analysis. Data are a representative of at least three independent experiments.

3.4.4 The role of ROS in mediating the effect of TQ on various anti-apoptotic genes

Previous experiments have shown that TQ treatment was able to suppress the protein expression of various anti-apoptotic genes including survivin, XIAP, Bcl-xL and Bcl-2. Whether ROS involved in these suppressions was also examined. NAC was used to block the induction of ROS. As shown in Figure 3.4.4, our data showed that NAC pre-treatment could reverse the suppression of survivin, XIAP, Bcl-xL and Bcl-2 protein expression induced by TQ. These results demonstrate that TQ-induced apoptosis was mediated via induction of ROS, which in turn, suppressing the expression of these anti-apoptotic gene products leading to apoptosis initiation.

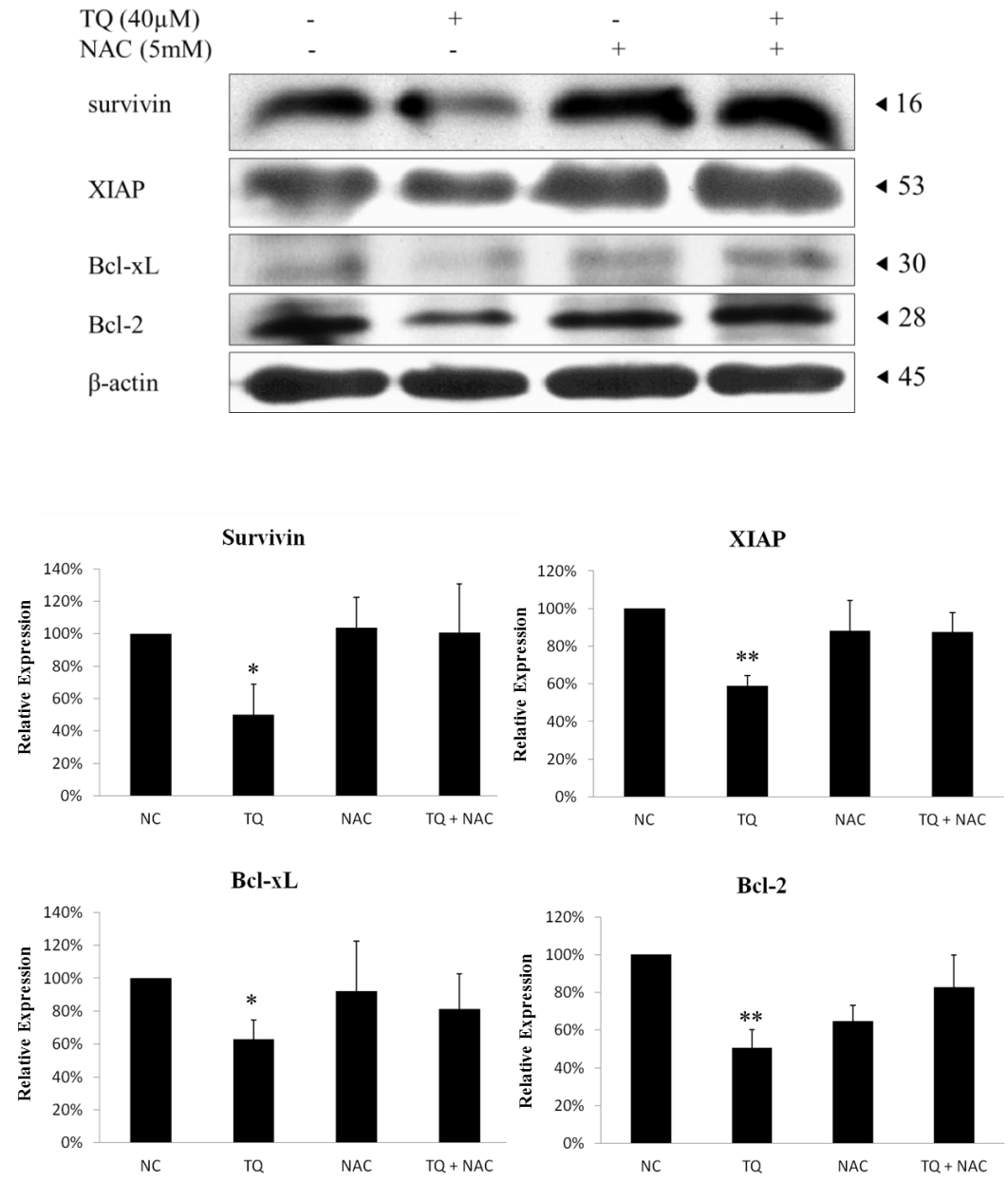


Figure 3.4.4 Effect of TQ-induced ROS on the protein expression of various anti-apoptotic genes.

MCF-7 cells were pre-treated with 5 mM NAC for 2 h before exposure to 40 μM TQ for 12 h. Whole cell lysate was collected for Western blot analysis. Data are a representative of at least three independent experiments. * $p<0.05$, ** $p<0.01$ vs. negative control.

3.4.5 The relationship between ROS and PPAR-γ in the mechanism of action of TQ

Since ROS and PPAR-γ were found to be both involved in the anticancer activities of TQ, whether these two pathways linked to each other was also investigated. Our results showed that NAC pre-treatment could reverse the increase of PPAR-γ expression induced by TQ in MCF-7 cells, and also reverse the decrease of PPAR-γ expression induced by TQ in MDA-MB-231 cells (Figure 3.4.5A). These results suggested that the changes in PPAR-γ expression were due to the increase in ROS production induced by TQ. On the other hand, pre-treatment with GW9662 did not cause any significant change in TQ-induced ROS level. Together, these results suggest that PPAR-γ could be the downstream effector of ROS in the mechanism of action of TQ.

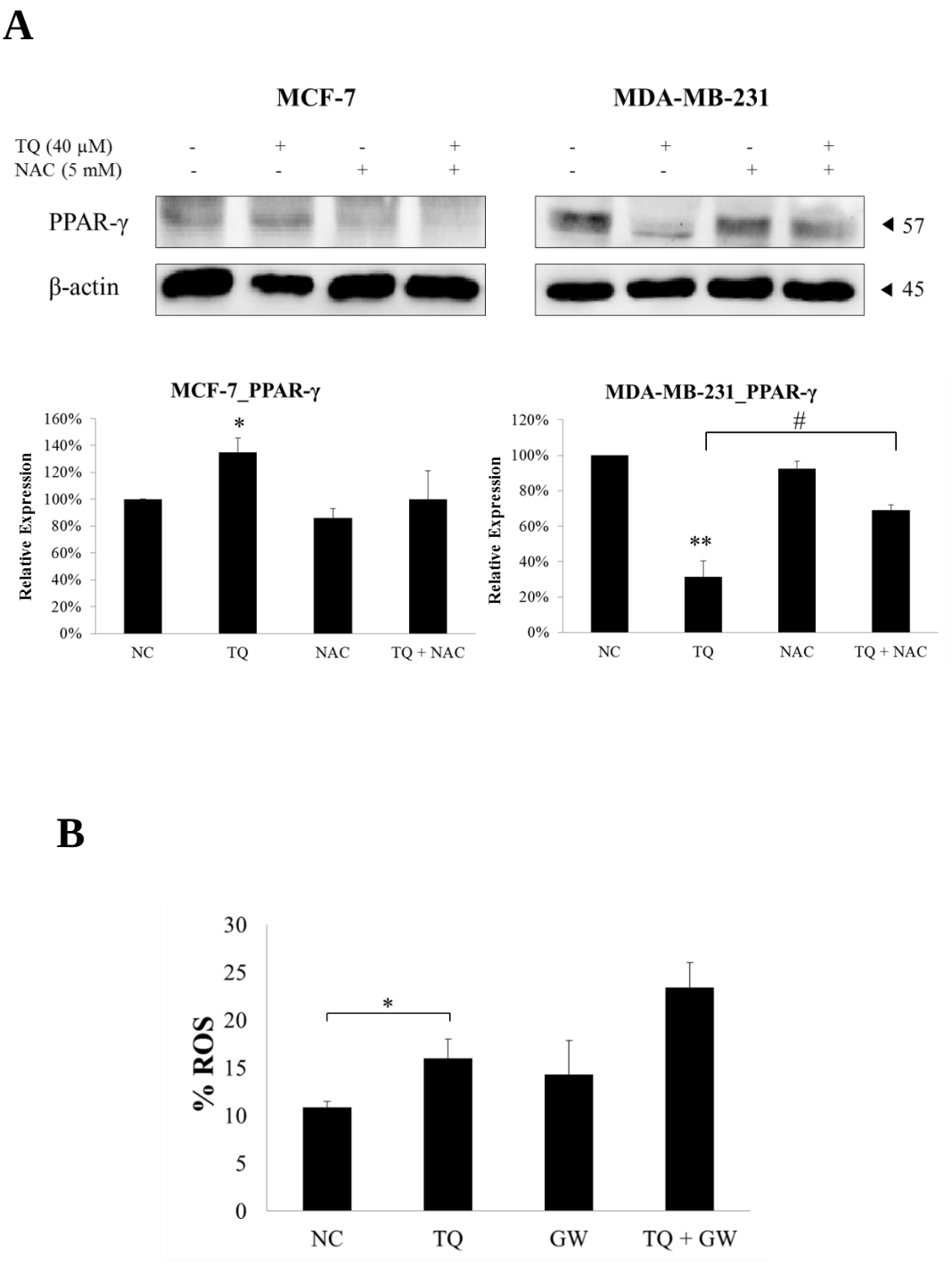


Figure 3.4.5 The relationship between ROS and PPAR-γ in the mechanism of action of TQ.

(A) Effect of NAC on PPAR-γ expression after TQ treatment. MCF-7 and MDA-MB-231 cells were pre-treated with 5 mM NAC for 2 h before exposure to 40 μM TQ for 12 h. Whole cell lysate was collected for Western blot analysis. Data are a representative of at least three independent experiments. * $p<0.05$, ** $p<0.01$ vs. negative control. # $p<0.05$. (B) Effect of GW9662 on TQ-induced ROS level. MCF-7 cells were pre-treated with 10 μM GW9662 for 2 h before exposure to 40 μM TQ for 3 h. The cells were then analyzed with flow cytometry after Mitosox staining. Values are means ± S.E.M. of at least two or three independent experiments. * $p<0.05$.

3.5 Studies on the role of p38 MAPK in the anticancer activities of TQ

3.5.1 Effect of TQ on various MAPKs

The effect of TQ on MAPKs pathway was investigated by examined the protein expression of p-ERK/ERK, p-JNK/JNK and p-p38/p38 after TQ treatment. Our data showed that TQ treatment increased the phosphorylation of ERK, JNK and p38 in MCF-7 cells with no change in their respective total protein. The increase of JNK phosphorylation started as early as 4 h TQ exposure and this increase was maintained till 12 h TQ exposure. The increase of p38 phosphorylation occurred in a time-dependent manner, and was maximal at 12 h TQ exposure. The increase of ERK phosphorylation peaked at 4 h and gradually decreased till 12 h.

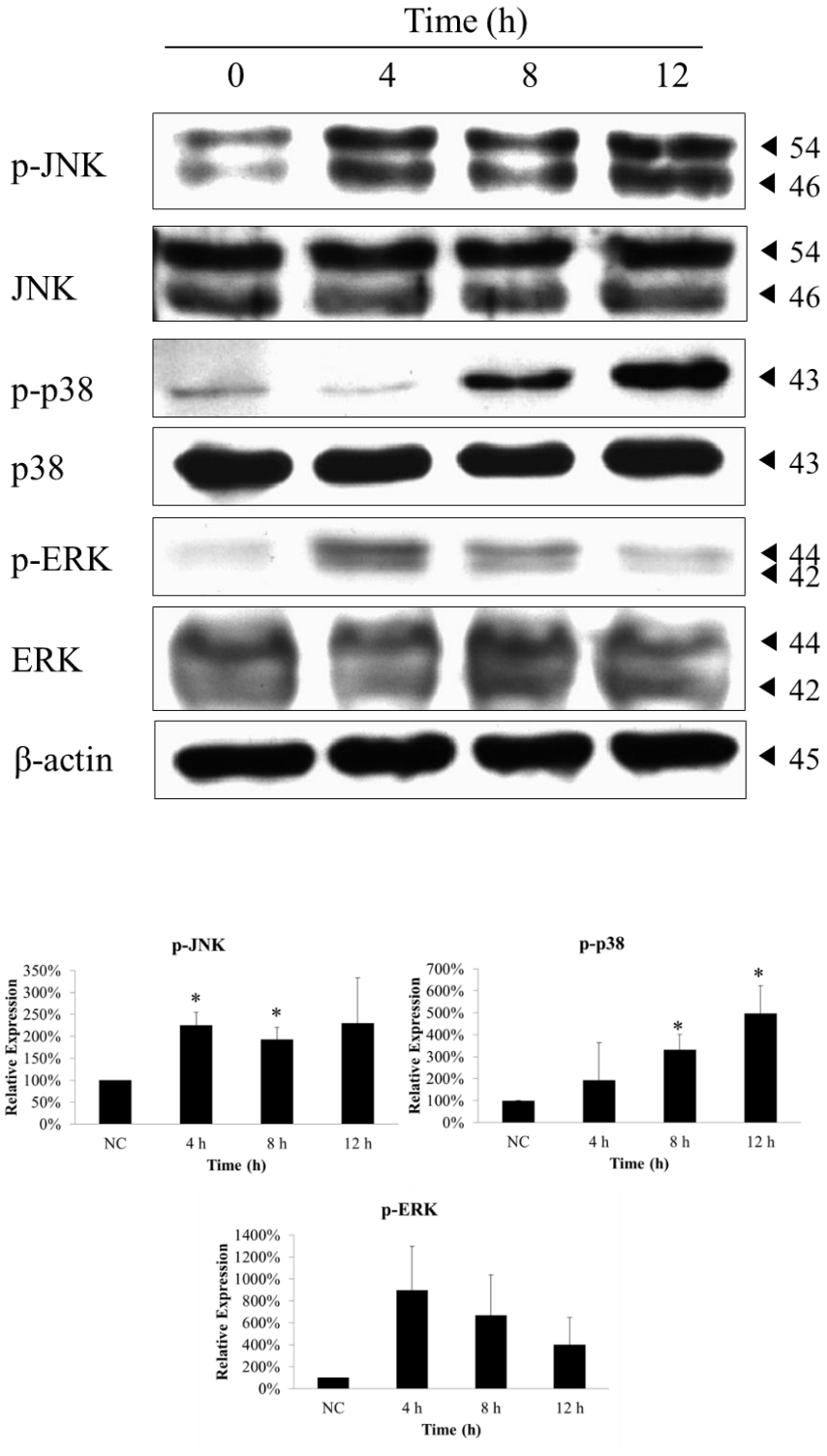


Figure 3.5.1 Effect of TQ on the phosphorylation status of various MAPKs.

MCF-7 cells were treated with 40 μM TQ for different incubation periods ranging up to 12 h. Whole cell lysate was collected for Western blot analysis. The data are representative of at least three independent experiments. * $p<0.05$ vs. negative control.

3.5.2 Effect of TQ on p38 activation

Since p38 is a potential tumor suppressor gene and is a possible downstream effector of ROS, its role in the anticancer activities of TQ was next investigated. A p38-specific pharmacological inhibitor, SB203580, was used to investigate whether it could reverse TQ-induced p38 activation. Our results showed that the increased phosphorylation of p38 induced by TQ in MCF-7 and MDA-MB-231 cells could be abrogated by SB203580 pre-treatment (Figure 3.5.2A). Similar finding was also obtained with p38 ELISA kit whereby SB203580 could abrogate TQ-induced increased level of p-p38 (Figure 3.5.2B).

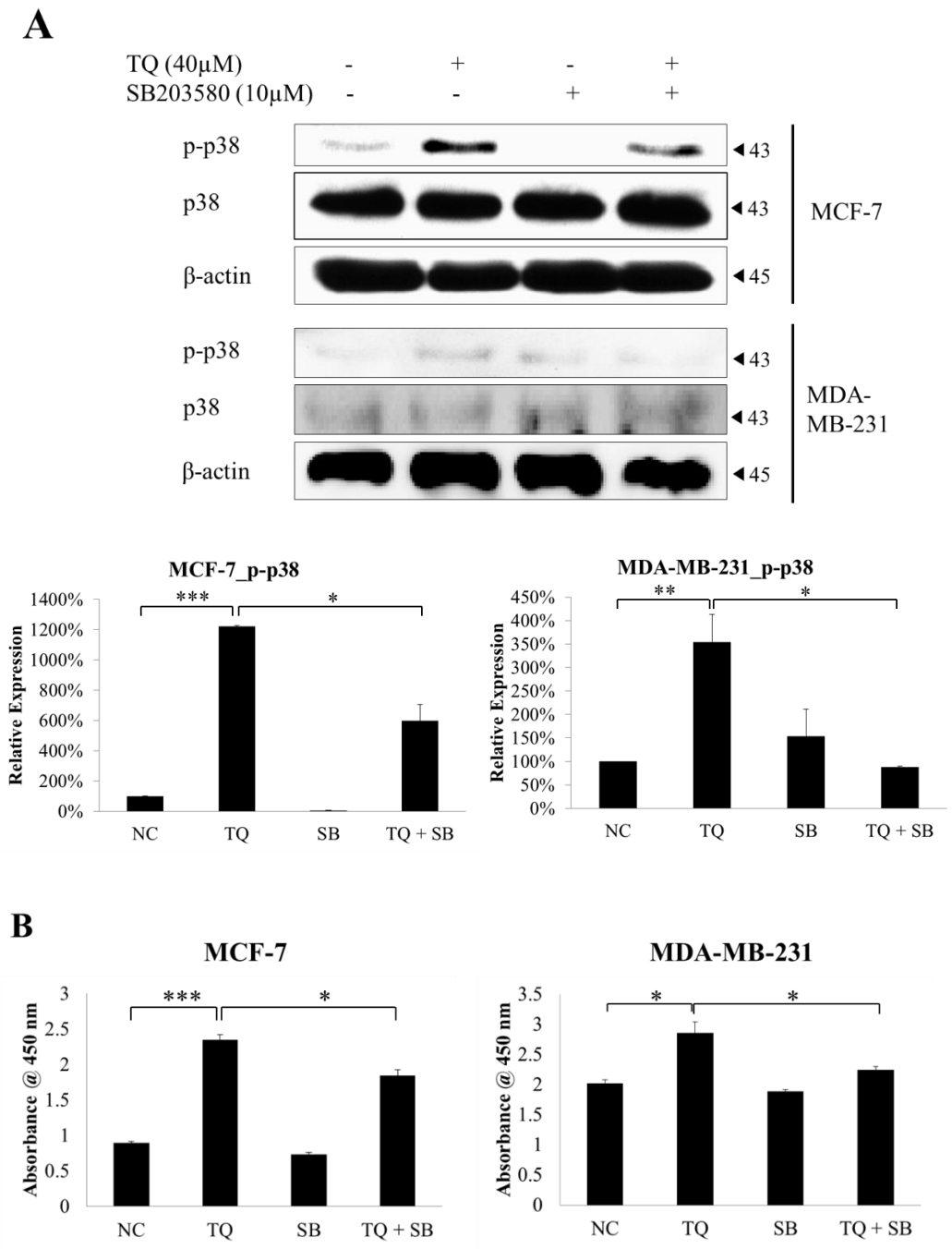


Figure 3.5.2 Effect of SB203580 on TQ-induced p38 activation.

(A) Effect of SB203580 on TQ-induced p38 phosphorylation. MCF-7 and MDA-MB-231 cells were pre-treated with 10 μM SB203580 for 1 h before exposure to 40 μM TQ for 12 h. Whole cell lysate was collected for Western blot analysis. Data are a representative of at least three independent experiments. * $p<0.05$, ** $p<0.01$. (B) The effect of SB203580 on TQ-induced p-p38 level. MCF-7 and MDA-MB-231 cells were pre-treated with 10 μM SB203580 for 1 h before exposure to 40 μM TQ for 12 h. Whole cell lysate was analyzed with PathScan® phospho-p38 MAPK (Thr180/Tyr182) sandwich ELISA kit as described under Materials and Methods section. Values are means ± S.E.M. of at least three independent experiments. * $p<0.05$, *** $p<0.001$.

3.5.3 The role of p38 activation on the cytotoxicity of TQ

We next investigated the role of p38 activation in the cytotoxic effect of TQ. SB203580 was used to block TQ-induced p38 activation. Our data showed that TQ-induced growth inhibition could be abrogated by SB203580 pre-treatment, at least partially, in both MCF-7 and MDA-MB-231 cell lines (Figure 3.5.3). These results suggest that p38 activation is partially involved in the cytotoxic effect of TQ.

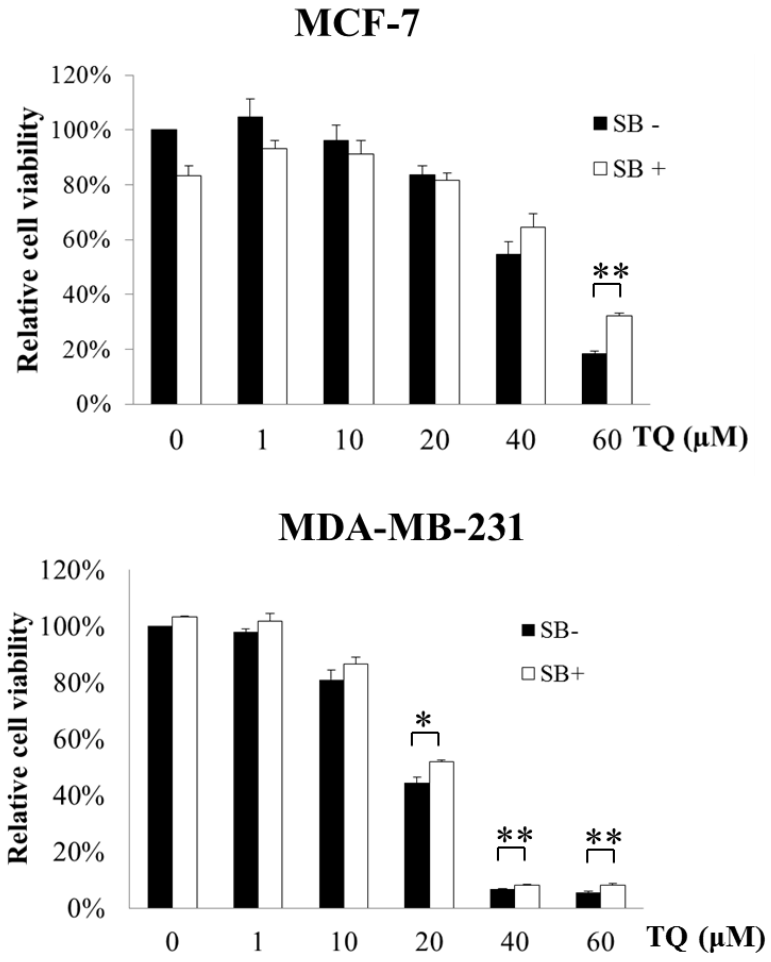


Figure 3.5.3 Effect of SB203580 on TQ's growth inhibition effect.

MCF-7 and MDA-MB-231 cells were pre-treated with 10 μM SB203580 for 1 h before exposure to increasing doses of TQ for 24 h. The cells were then analyzed by MTT assay. Values are means ± S.E.M. of at least three independent experiments. * $p<0.05$, ** $p<0.01$.

3.5.4 The role of p38 activation on TQ-induced apoptosis

We also investigated whether p38 activation is involved in TQ-induced apoptosis. As shown in Figure 3.5.4A, TQ-induced increased percentage of annexin V-positive cells could be reversed by SB203580 pre-treatment in both MCF-7 and MDA-MB-231 cells. Moreover, the PARP cleavage induced by TQ in both MCF-7 and MDA-MB-231 cells could also be reversed by SB203580 pre-treatment (Figure 3.5.4B). These results demonstrate that p38 activation indeed plays an important role in the pro-apoptotic effect of TQ.

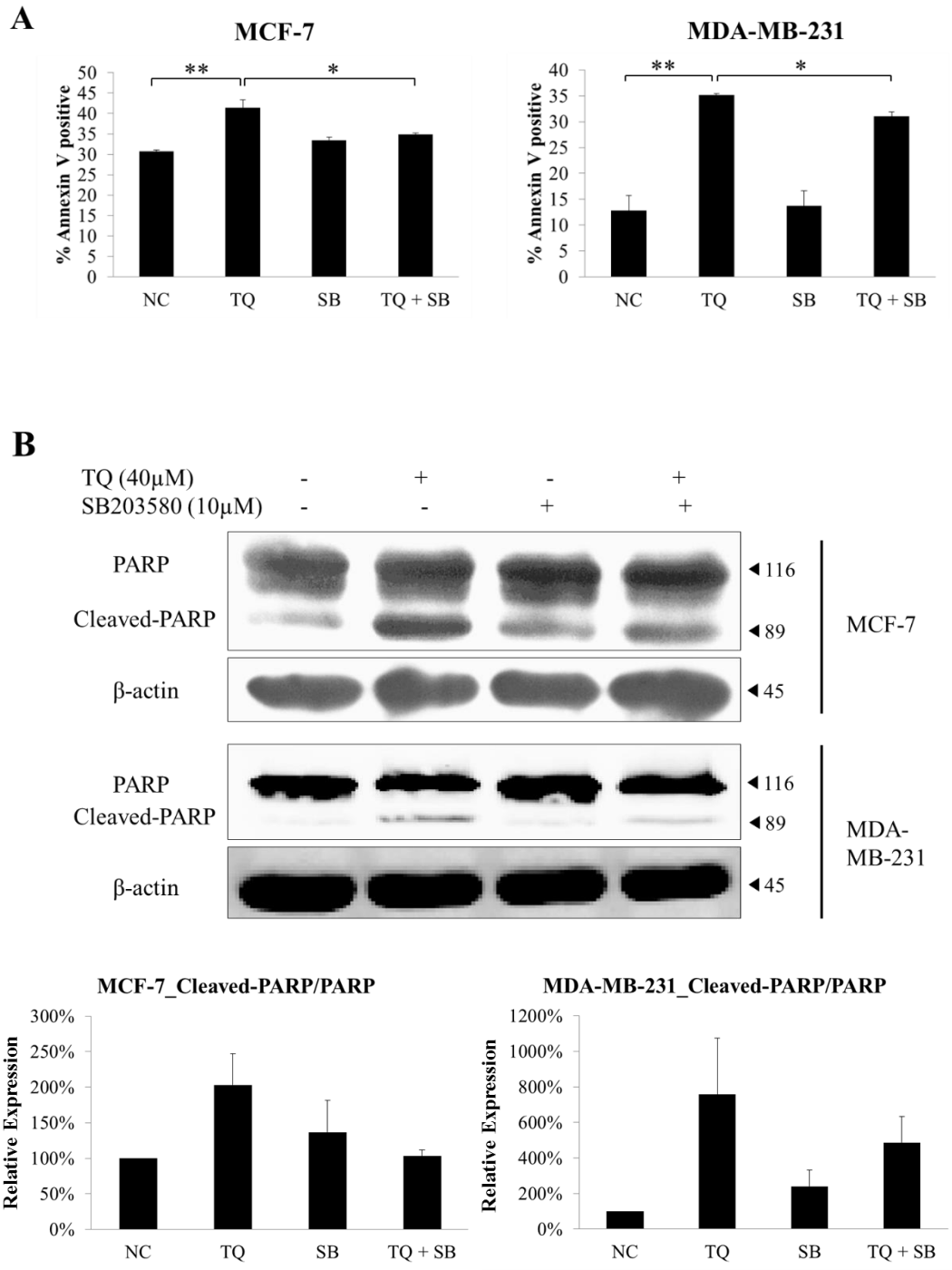
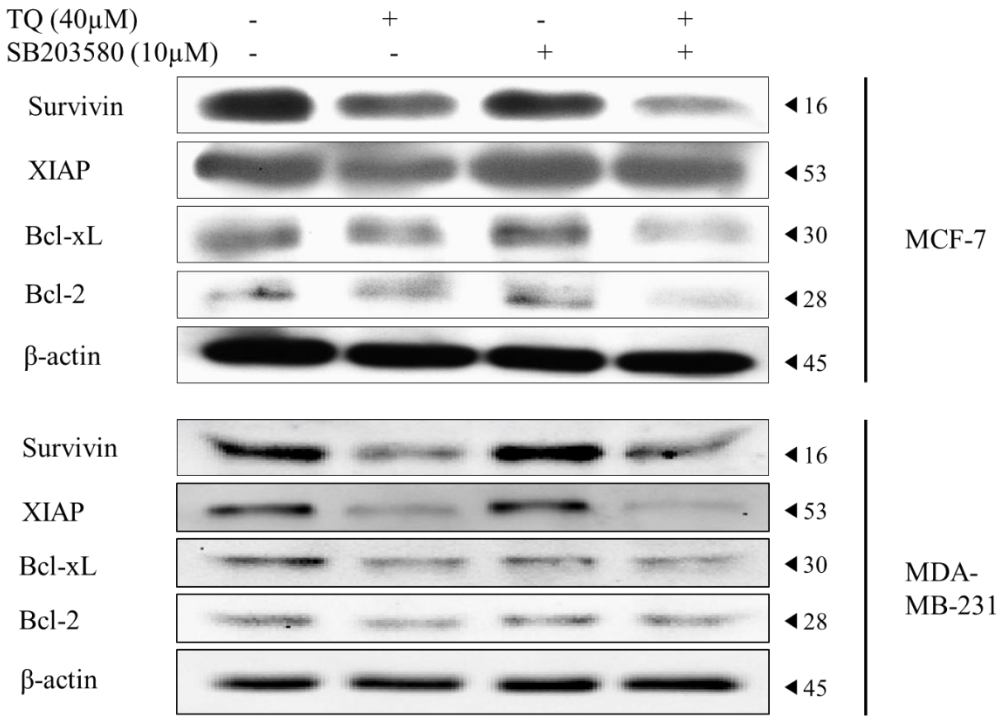


Figure 3.5.4 The role of p38 activation on TQ-induced apoptosis.

(A) Effect of SB203580 on TQ-induced increased percentage of annexin V-positive cells. MCF-7 and MDA-MB-231 cells were pre-treated with 10 μM SB203580 for 1 h before exposure to 40 μM (for MDA-MB-231) or 50 μM (for MCF-7) TQ for 12 h. The cells were then analyzed with annexin V assay. Values are means ± S.E.M. of at least three independent experiments. * $p<0.05$, ** $p<0.01$. (B) Effect of SB203580 on TQ-induced PARP-cleavage. MCF-7 or MDA-MB-231 cells were pre-treated with 10 μM SB203580 for 1 h before exposure to 40 μM TQ for 12 h. Whole cell lysate was collected for Western blot analysis. Data are a representative of at least three independent experiments.

3.5.5 Effect of TQ-induced p38 activation on various anti-apoptotic genes

The effect of TQ-induced p38 activation on various anti-apoptotic genes, such as survivin, XIAP, Bcl-xL and Bcl-2, was also examined. We found that these proteins could be suppressed by TQ treatment, whereas pre-treatment with SB203580 could reverse some of these reductions (Figure 3.5.5). The decrease of XIAP induced by TQ in MCF-7 cells was found to be reversed by SB203580. On the other hand, the decrease of survivin and Bcl-2 induced by TQ in MDA-MB-231 cells could be reversed by SB203580. This difference could be due to cell line differences such as ER status and p53 status which might interfere with the mechanism of action of TQ on the p38 pathway.



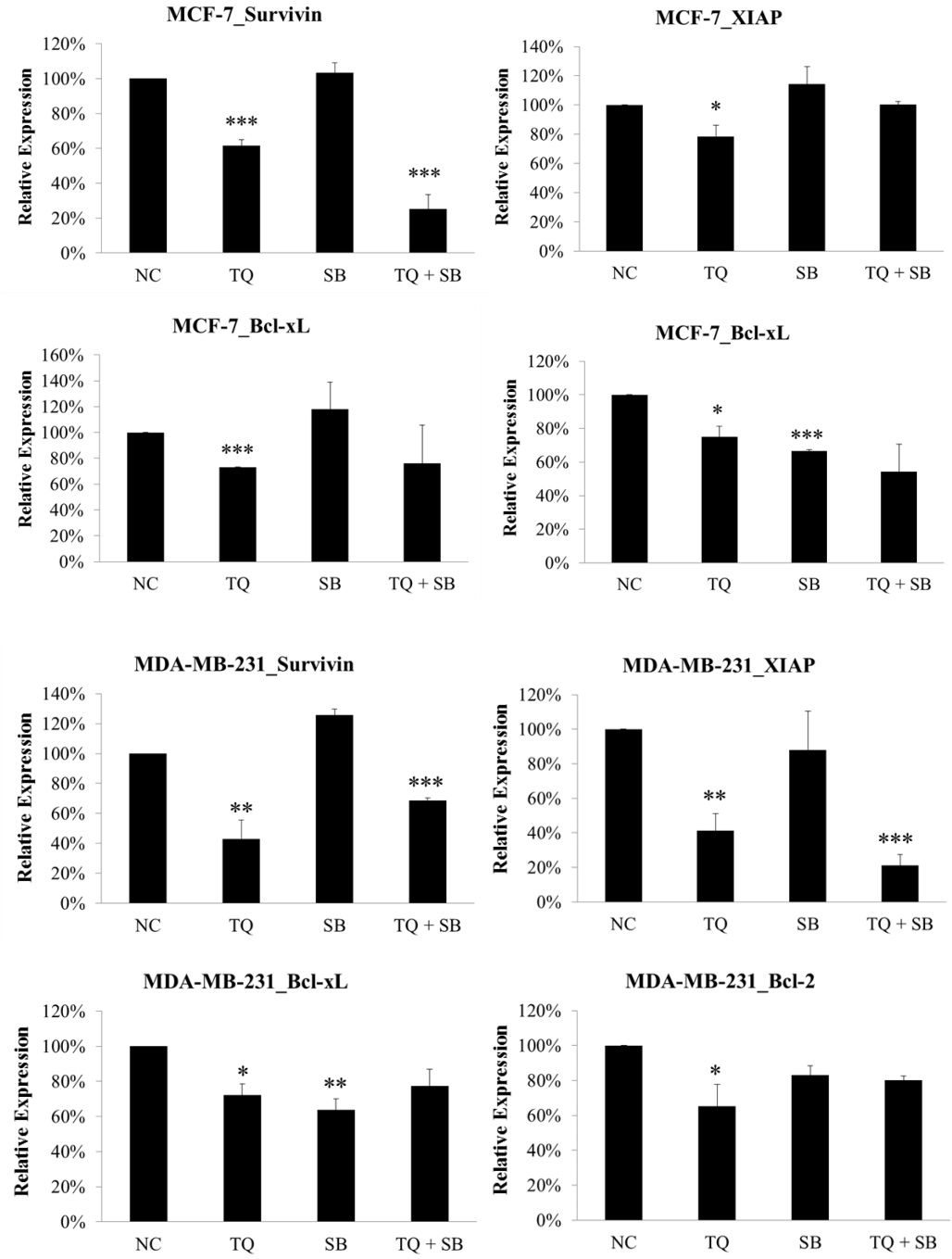
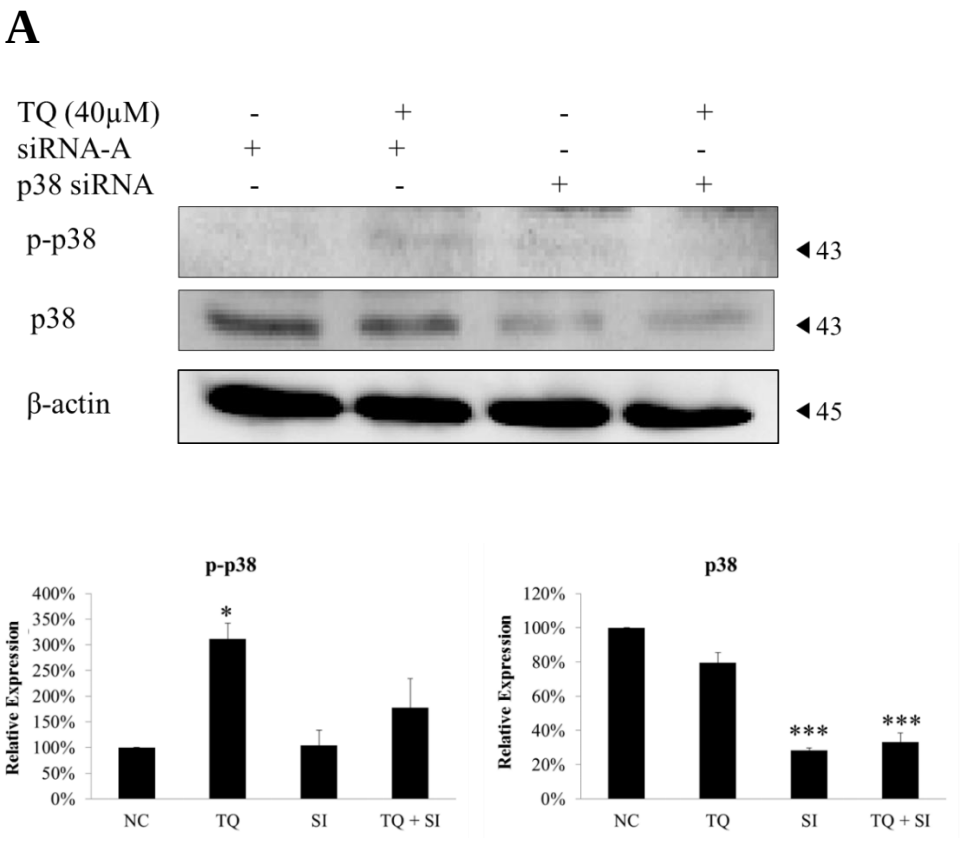


Figure 3.5.5 Effect of TQ-induced p38 activation on the protein expression of various anti-apoptotic genes.

MCF-7 and MDA-MB-231 cells were pre-treated with 10 μM SB203580 for 1 h before exposure to 40 μM TQ for 12 h. Whole cell lysate was collected for Western blot analysis. Data are a representative of at least three independent experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. negative control.

3.5.6 Effect of p38 siRNA gene silencing on TQ-induced apoptosis

In addition to p38-specific inhibitor, we also used p38 siRNA gene silencing to study the role of p38 activation on the pro-apoptotic effect of TQ. The effect of p38 siRNA gene silencing was first validated using Western blot. As shown in Figure 3.5.6A, p38 siRNA gene silencing could successfully suppress both the expression of p-p38 and p38. Next, our data showed that p38 siRNA gene silencing could abrogate TQ-induced increased percentage of annexin V positive cells (Figure 3.5.6B). Moreover, p38 siRNA gene silencing could also reverse TQ-induced PARP cleavage (Figure 3.5.6C). These results confirmed the role of p38 activation in TQ-induced apoptosis.



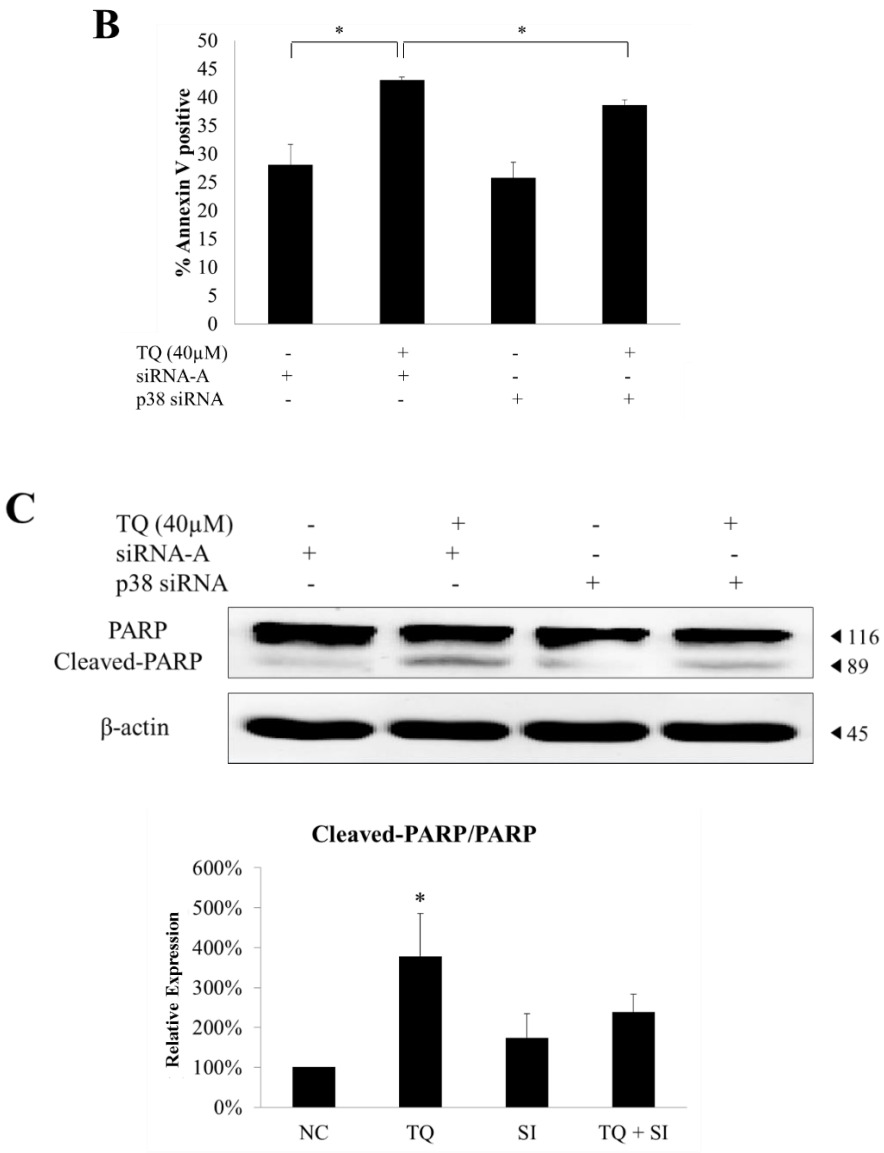


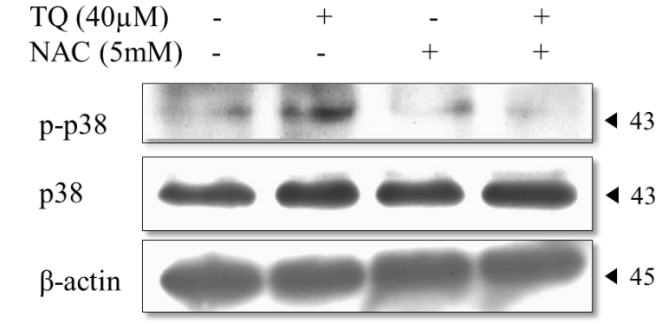
Figure 3.5.6 Effect of p38 siRNA gene silencing on TQ-induced apoptosis.

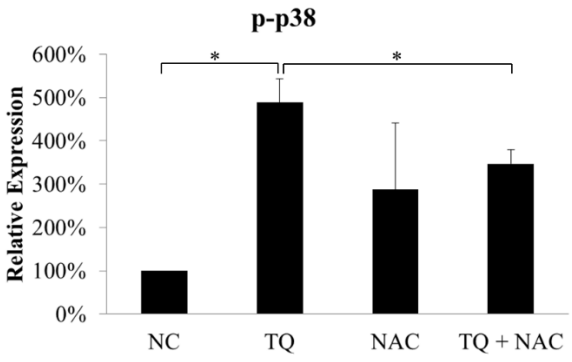
MCF-7 cells were transfected with 30 nM p38 siRNA or siRNA-A for 6 h followed by at least 24 h recovery with serum-added medium. The cells were then treated with 40 μM TQ for 12 h. (A) Effect of p38 siRNA gene silencing on the protein expression of p-p38 and p38. Whole cell lysate was collected for Western blot analysis. Data are a representative of at least three independent experiments. * $p<0.05$, *** $p<0.001$ vs. negative control. (B) Effect of p38 siRNA gene silencing on TQ-induced increased percentage of annexin V positive cells. The cells were analyzed with annexin V assay. Values are means ± S.E.M. of at least three independent experiments. * $p<0.05$. (C) Effect of p38 siRNA gene silencing on TQ-induced PARP cleavage. Whole cell lysate was collected for Western blot analysis. Data are a representative of at least three independent experiments. * $p<0.05$ vs. negative control.

3.5.7 The relationship between ROS and p38 in the mechanism of action of TQ

There are studies suggested that the interaction of ROS and p38 was responsible for certain anticancer activities as shown in several types of carcinoma (Bragado et al 2007; Kang and Lee 2008). Thus, whether the mechanism of action of TQ involved the interaction between ROS and p38 was investigated here. By changing the level of ROS using NAC, TQ-induced increased phosphorylation of p38 was prevented (Figure 3.4.6A). The data from p38 ELISA kit also showed that TQ was able to significantly increase the p-p38 level which could be abrogated by NAC pre-treatment (Figure 3.4.6B). These results demonstrate that TQ-induced ROS indeed play an important role in p38 activation. Next, whether p38 activation interfered the level of ROS was also examined. As shown in Figure 3.4.6C, SB203580 pre-treatment did not cause significant change on TQ-induced ROS level. Together, these results showed that p38 activation is mediated through TQ-induced ROS.

A





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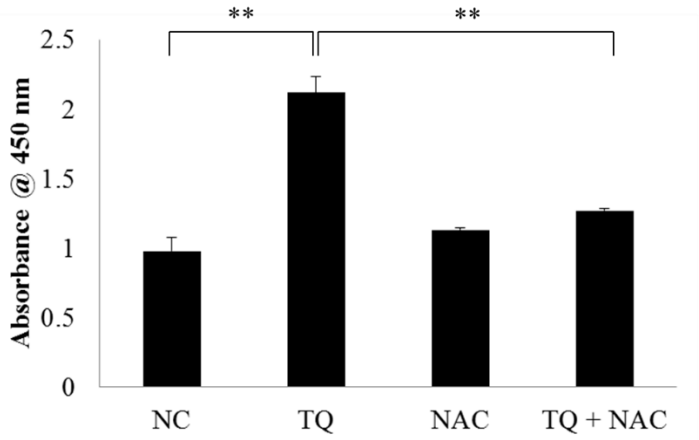

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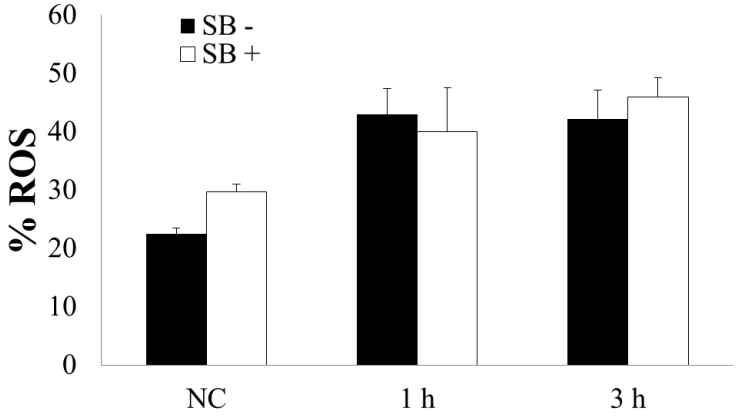

Figure 3.5.7 The relationship between ROS and p38 in the mechanism of action of TQ.

(A) Effect of NAC on TQ-induced p38 phosphorylation. MCF-7 cells were pre-treated with 5 mM NAC for 2 h before exposure to 40 μM TQ for 12 h. Whole cell lysate was collected for Western blot analysis. Data are a representative of at least three independent experiments. * $p<0.05$, ** $p<0.01$ (B) Effect of NAC on TQ-induced p-p38 level. MCF-7 cells were pre-treated with 5 mM NAC for 2 h before exposure to 40 μM TQ for 12 h. Whole cell lysate was analyzed with PathScan® phospho-p38 MAPK (Thr180/Tyr182) sandwich ELISA kit. Values are means ± S.E.M. of at least three independent experiments. ** $p<0.01$. (C) Effect of SB203580 on TQ-induced ROS production. MCF-7 cells were pre-treated with 10 μM SB203580 for 1 h before exposure to 40 μM TQ for 1 or 3 h. The cells were then analyzed with flow cytometry after Mitosox staining. Values are means ± S.E.M. of at least three independent experiments.

3.5.8 The relationship between p38 and PPAR-γ in the mechanism of action of TQ

Since p38 and PPAR-γ are both involved in the anticancer activities of TQ, whether these two pathways linked to each other was also investigated. Our data showed that SB203580 pre-treatment did not cause significant change in the protein expression of PPAR-γ in MDA-MB-231 cells, but slightly increased the PPAR-γ expression in MCF-7 cells (Figure 3.5.7A). Interestingly, GW9662 pre-treatment appeared to increase p38 phosphorylation in MCF-7 cells, while no change was observed in MDA-MB-231 cells (Figure 3.5.7B). These results showed that p38 and PPAR-γ might antagonize each other in the action of TQ in MCF-7 cells.

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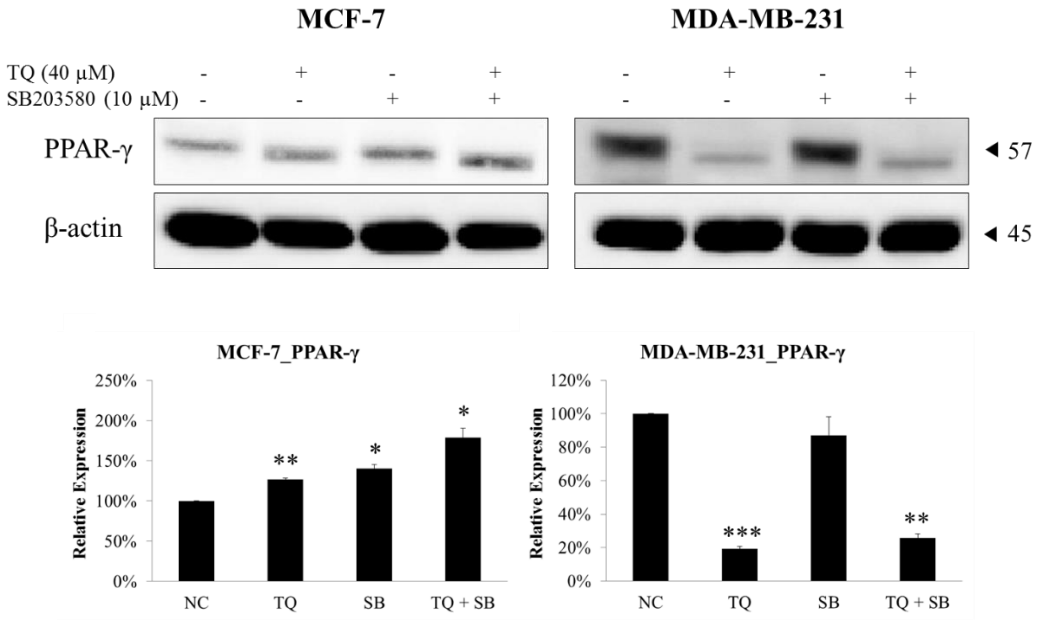

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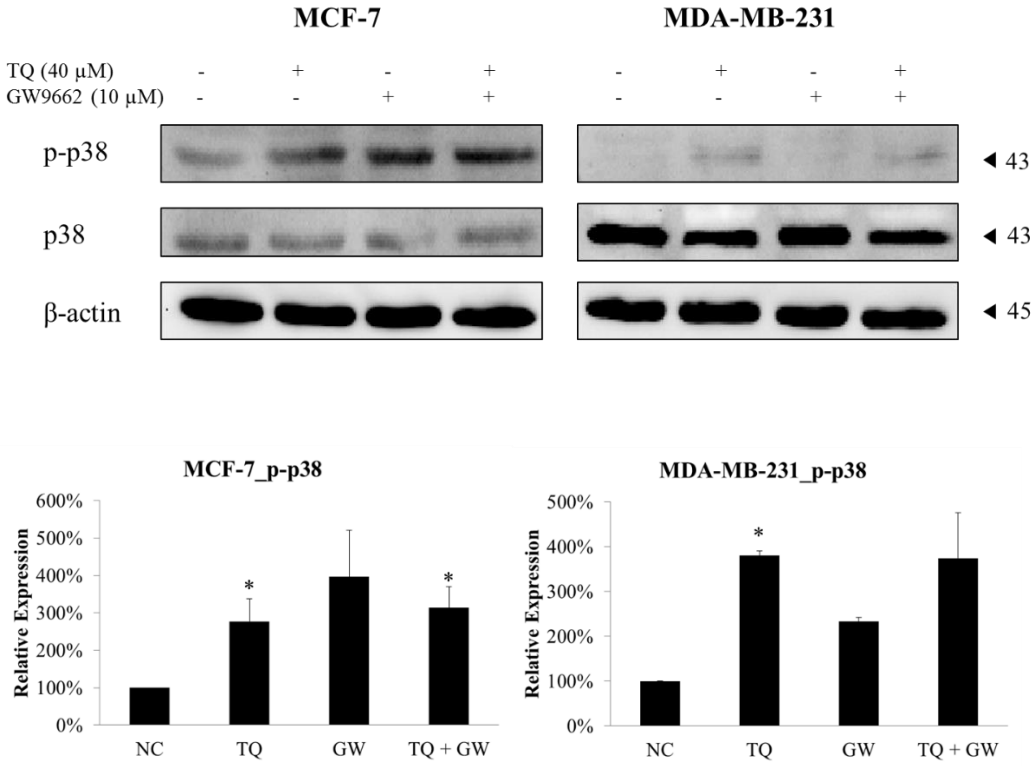


Figure 3.5.8 The relationship between p38 and PPAR-γ in the mechanism of action of TQ.

(A) Effect of SB203580 on the protein expression of PPAR-γ after TQ treatment. MCF-7 and MDA-MB-231 cells were pre-treated with 10 μM SB203580 for 1 h before exposure to 40 μM TQ for 12 h. Whole cell lysate was collected for Western blot analysis. Data are a representative of at least three independent experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. negative control. (B) Effect of GW9662 on the protein expression of p-p38/p38 after TQ treatment. MCF-7 and MDA-MB-231 cells were pre-treated with 10 μM GW9662 for 2 h before exposure to 40 μM TQ for 12 h. Whole cell lysate was collected for Western blot analysis. Data are a representative of at least three independent experiments. * $p<0.05$ vs. negative control.

3.6 Studies on the antitumor effect of TQ in the breast tumor xenograft mouse model

3.6.1 Effect of TQ on the growth of breast tumor xenograft

In addition of *in vitro* experiments, *in vivo* study was also performed to provide clinical relevance for the anticancer activities of TQ. A high number (10^7 cells/mouse) of MDA-MB-231 cells was injected subcutaneously to develop tumor xenograft in the right flank region of female nude mice. The treatment was started when the tumor volume was about 100 mm^3. Our data showed that TQ alone (4 mg/kg TQ, and 8 mg/kg TQ) was able to significantly suppress the tumor growth compared to the vehicle group (Figure 3.6.1A). The effect of TQ alone was also comparable to the positive control (2.5 mg/kg Dox). However, these treatments could not completely eradicate the tumor or maintain the tumor size at the starting level (Figure 3.6.1A). On the other hand, the combined treatment (4 mg/kg TQ + 2.5 mg/kg Dox) not only suppressed tumor growth more significantly than the vehicle group, but also more significantly than either agent alone (Figure 3.6.1A). Although the combined treatment could not completely eradicate the tumor, this treatment maintained the tumor size at the starting level throughout the treatment period (Figure 3.6.1A). Together, these results showed that TQ alone was able to inhibit tumor growth and the combination with chemotherapeutic agent produced greater antitumor effect. Photographs of extracted tumors from each treatment group were shown in Figure 3.6.1B.

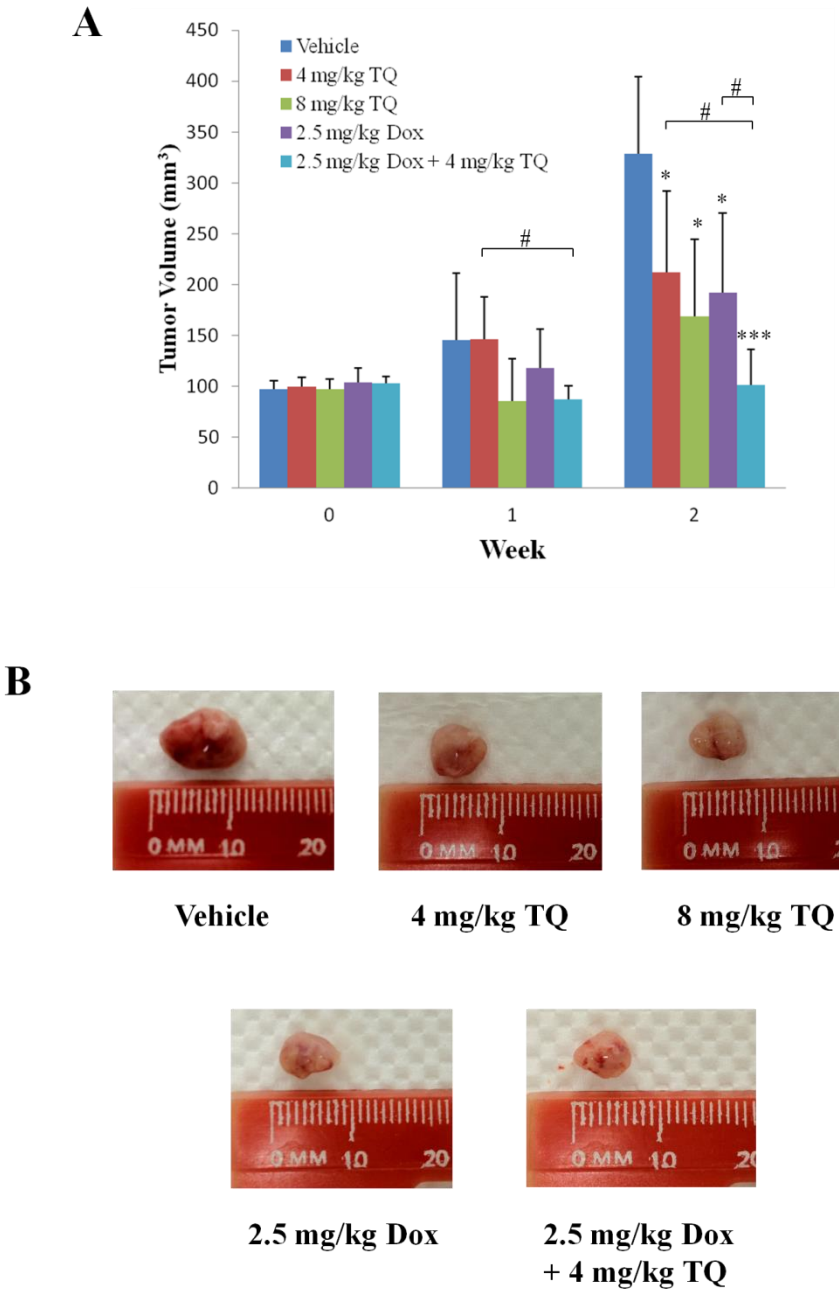


Figure 3.6.1 Changes in tumor volume in different treatment groups.

(A) When tumor volume reached 100 mm^3, the mice were divided into five treatment groups, each n=5. Group I: saline water/vehicle (i.p., 6 days per week), group II: 4 mg/kg TQ (i.p., 6 days per week), group III: 8 mg/kg TQ (i.p., 6 days per week), group IV: 2.5 mg/kg Dox (i.p., once per week), and group V: 2.5 mg/kg Dox (i.p., once per week) + 4 mg/kg TQ (i.p., 6 days per week). Tumor volume was measured with Vernier calipers and calculated with the formula, Volume = ($width^2$ X length)/2. Values are means ± S.D. of each group. * $p<0.05$, *** $p<0.001$ vs. vehicle control group. # $p<0.05$. (B) Photographs of extracted tumors from each treatment group.

3.6.2 Effect of TQ on mouse body weight

Whether TQ treatment caused any possible adverse effect to the mice was also examined. No visible adverse effect was observed during or after drug injection. The slight decrease in mouse weight (< 9% compared to the starting value) in TQ-treated groups suggested that TQ caused little or no toxicity to mouse health (Figure 3.6.2). However, further toxicity study is needed to address this issue in greater detail.

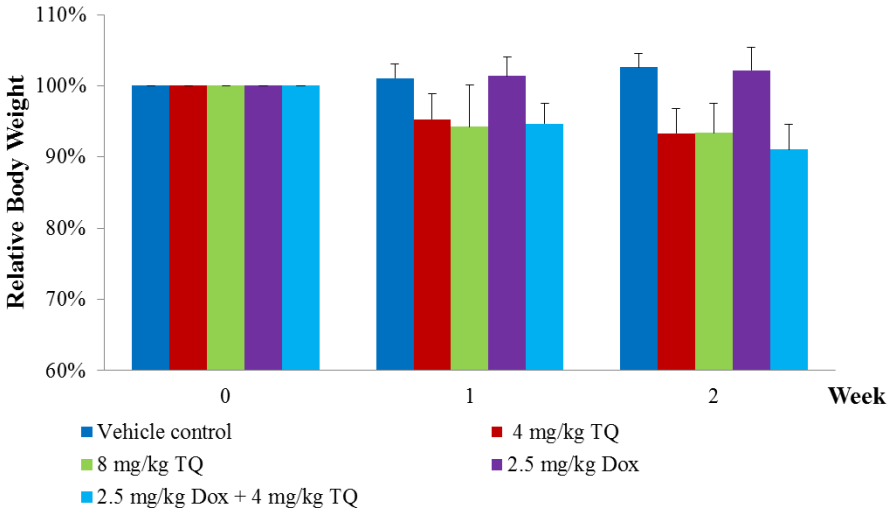
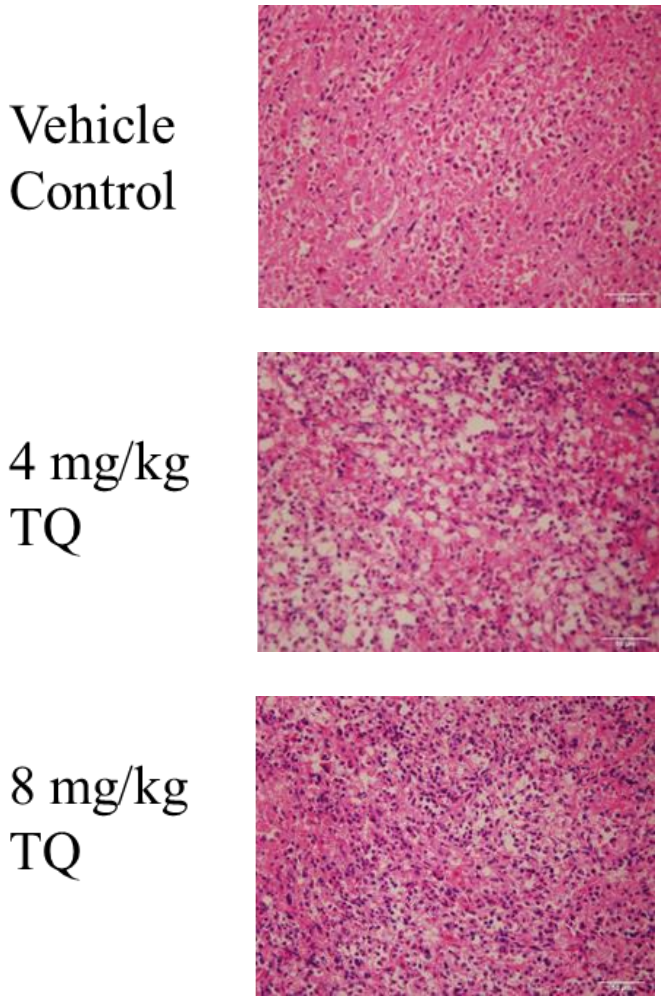


Figure 3.6.2 Mouse body weight relative to the starting measurement.

Values are means ± S.D. of each group.

3.6.3 Effect of TQ on tumor structure (H&E staining)

In order to study the structure of tumors from all treatment groups, the tumor tissues were stained with H&E followed by microscopy observation. As shown in Figure 3.6.3, the tumor observation of vehicle group displayed high grade tumor with irregular cell arrangement and intact tissue structure. In contrast, the tumors from TQ-treated groups were considerably tightly packed cells, decrease in stroma and increase in necrotic cells suggesting tumor destruction (Figure 3.6.3).



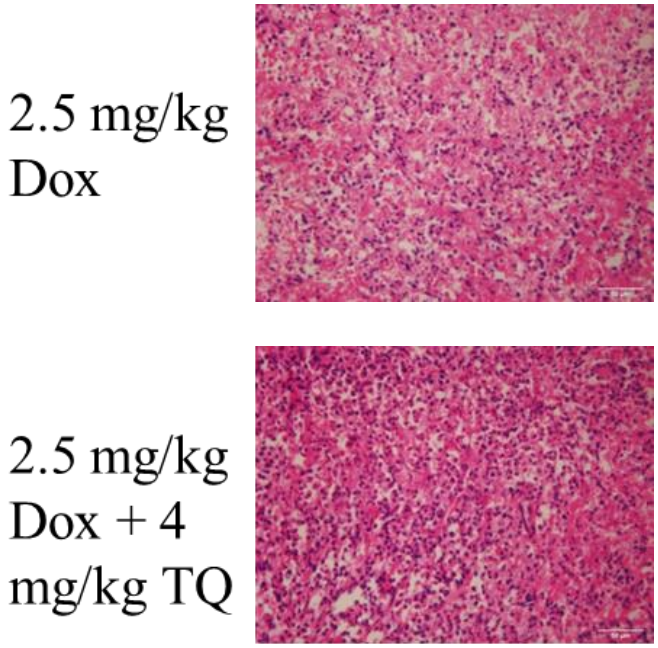
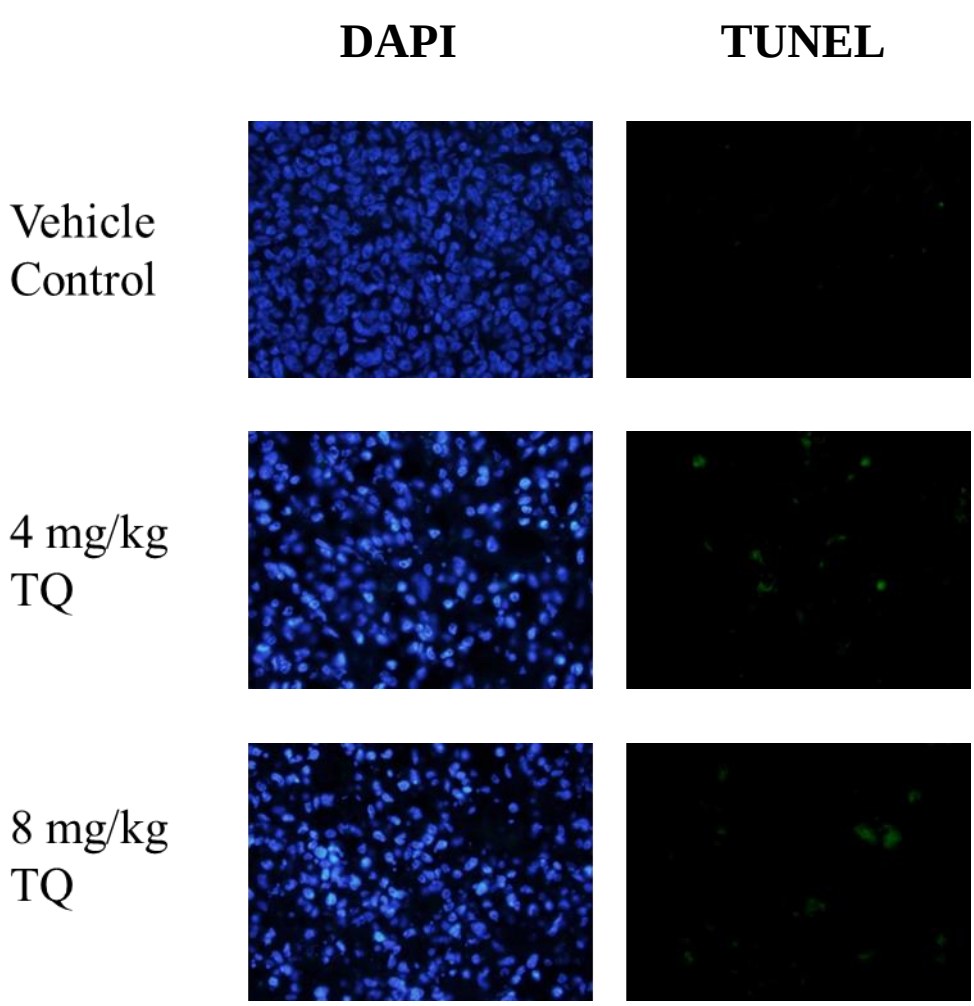


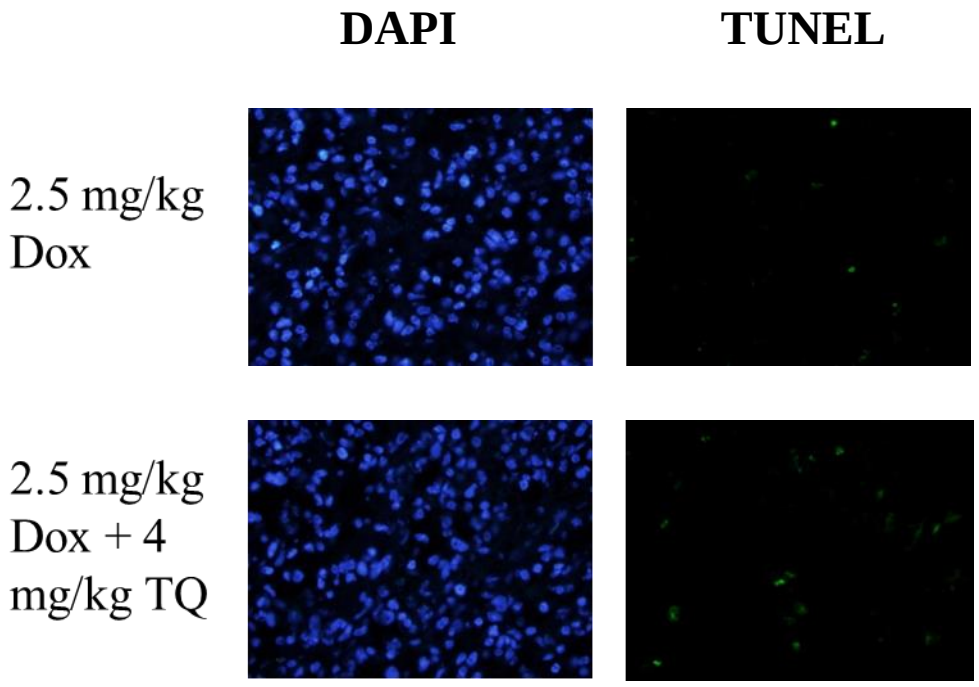
Figure 3.6.3 H&E staining of tumor tissues from each treatment group.

3.6.4 Effect of TQ on the level of apoptosis in tumor tissues (TUNEL staining)

TUNEL staining was used to examine the level of apoptosis in tumor tissues from each treatment group, while DAPI was used to stain nucleus. From the results, TQ-treated groups showed a greater number of fluorescence than the vehicle group (Figure 3.6.4A). By counting the average number of TUNEL positive cells in drug treatment groups as compared to the vehicle group, we found that the single-drug treatment groups (4 mg/kg TQ, 8 mg/kg TQ, and 2.5 mg/kg Dox) had about 2-fold higher number of TUNEL positive cells than the vehicle group, while the combined group showed the highest number of TUNEL positive cells (Figure 3.6.4B). These results showed that the tumor suppression effect of TQ was due to apoptosis.

A





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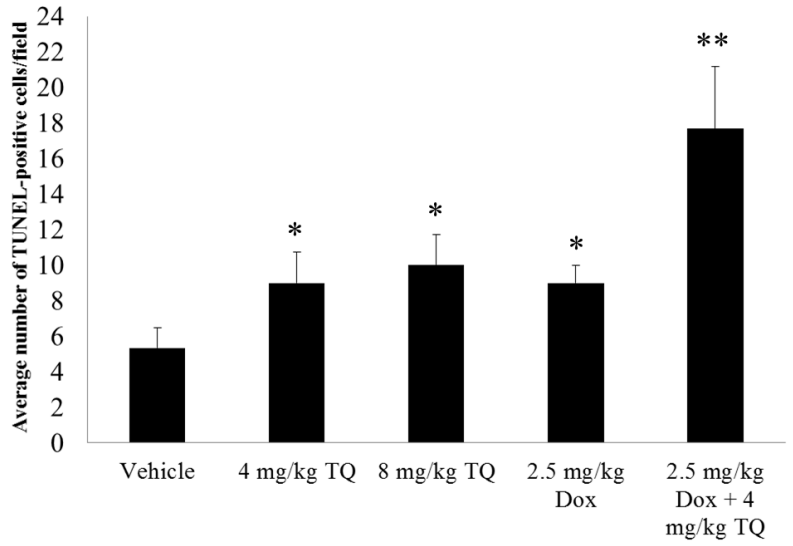


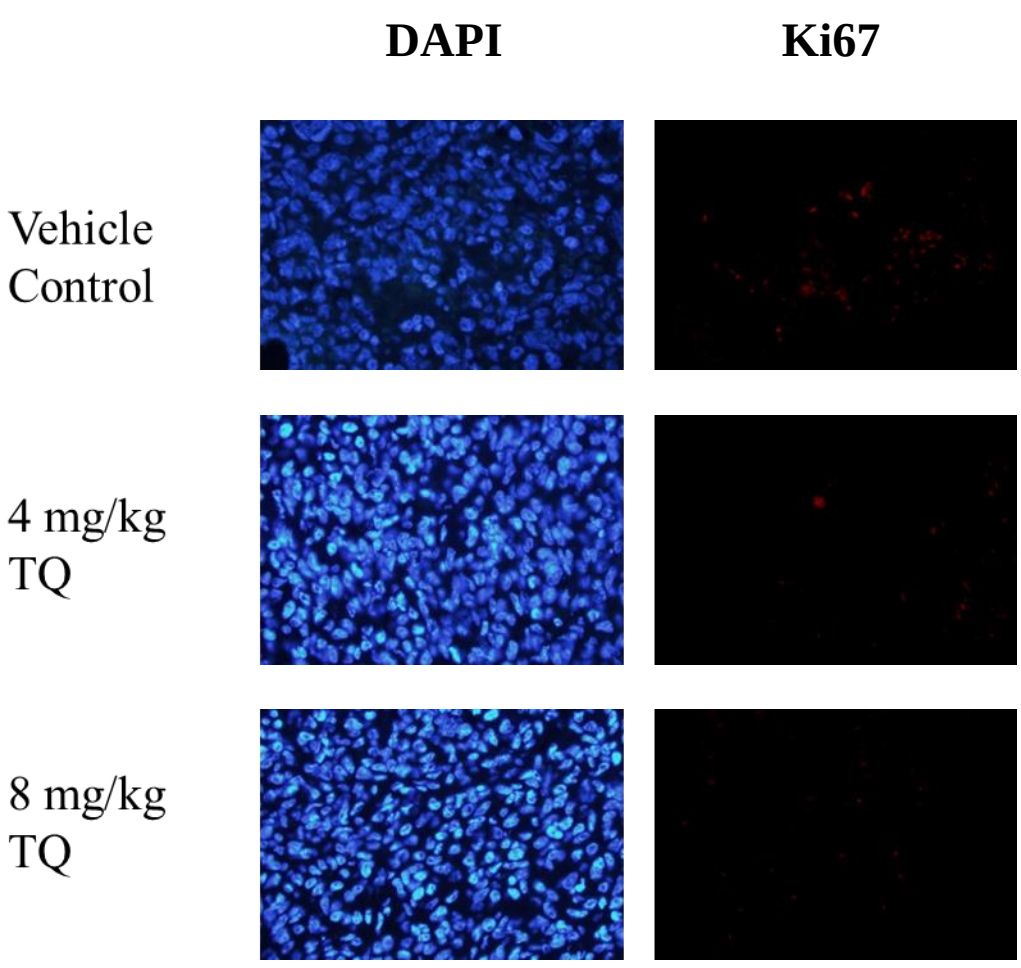
Figure 3.6.4 TUNEL staining of tumor tissues from different treatment groups.

(A) Fluorescence microscopy observation of TUNEL and DAPI staining. (B) The average number of TUNEL-positive cells per field. Values are means ± S.D. of three photographs. * $p<0.05$, ** $p<0.01$ vs. vehicle control group.

3.6.5 Effect of TQ on the proliferation rate of tumor tissues (Ki67 immunohistochemical staining)

Ki67 is a protein marker for cellular proliferation, thus we were interested to study its expression in the tumor tissues after TQ treatment. Our data showed that the tumor tissues of drug treatment groups expressed significantly decreased Ki67 protein compared to the vehicle group (Figure 3.6.5A). By calculating the number of Ki67 positive cells as compared to the vehicle group, we found that the single-drug treatment groups contained at least 60% lesser Ki67 positive cells than the vehicle group, while the combined group showed the lowest number of Ki67 positive cells (Figure 3.6.5B).

A



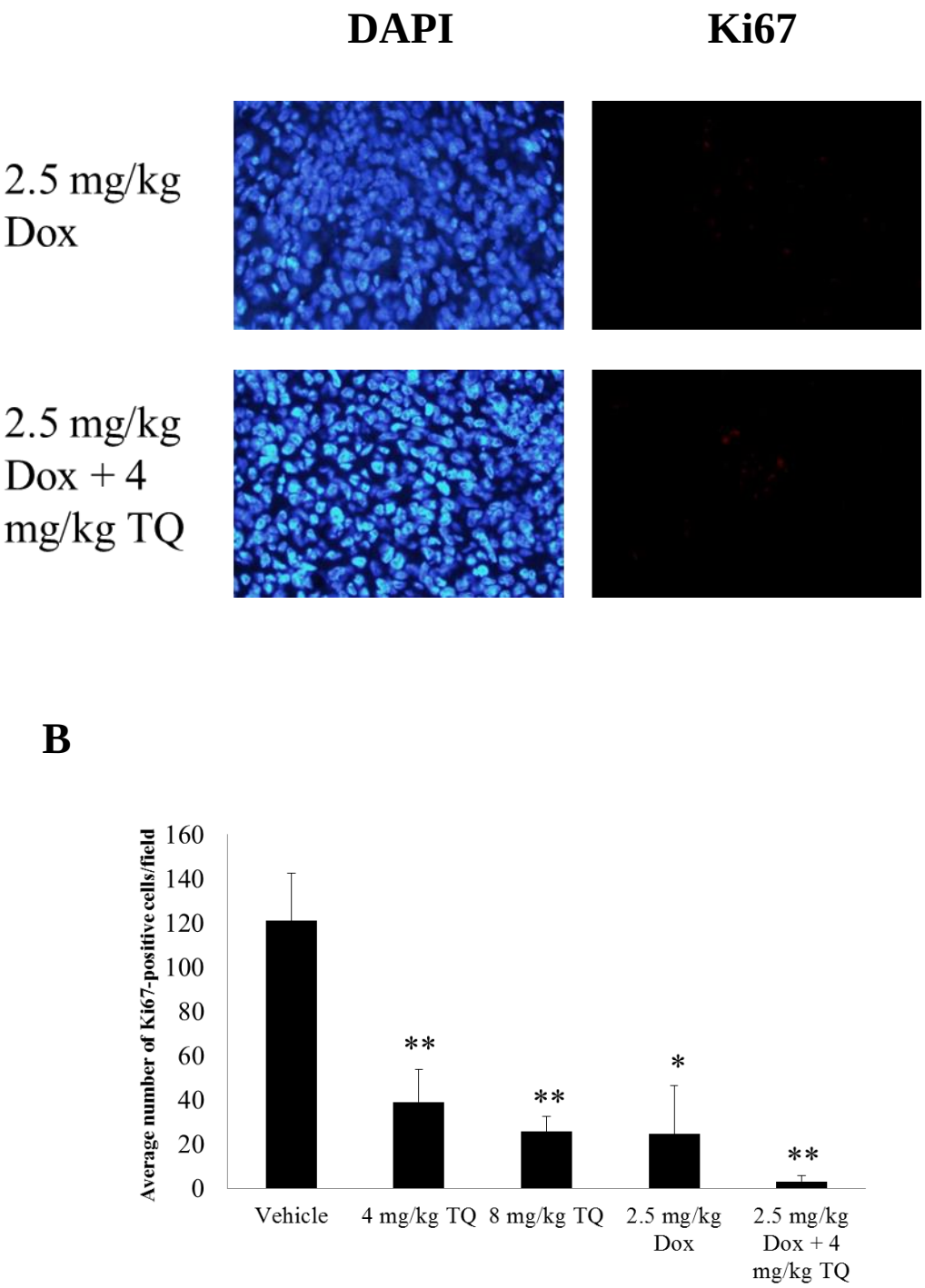
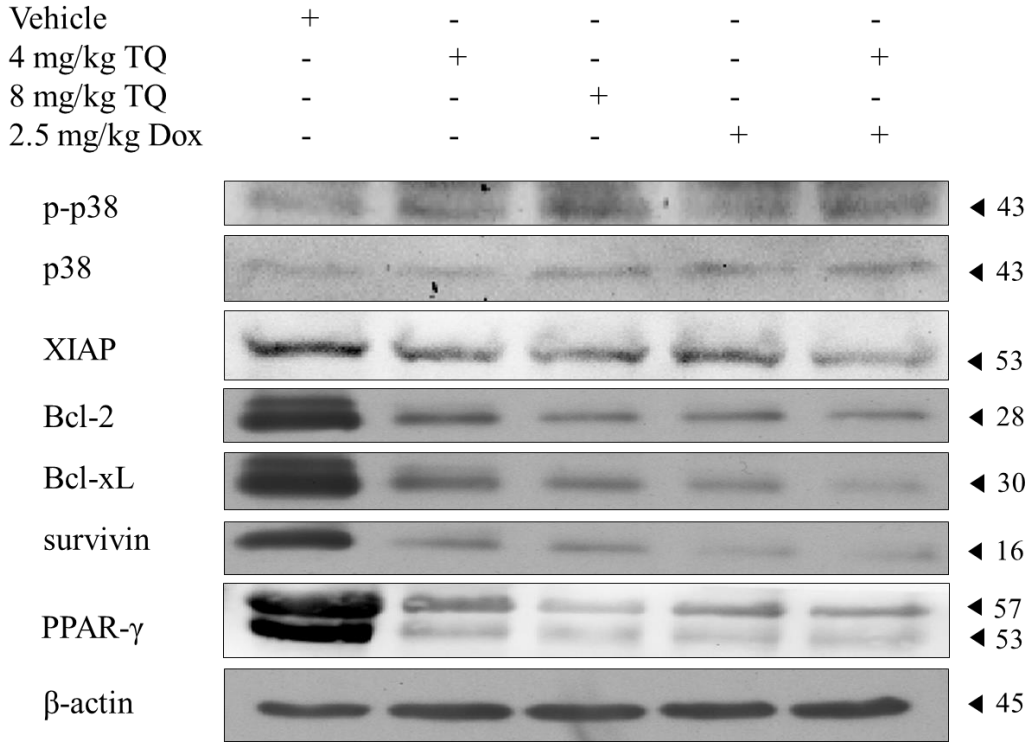


Figure 3.6.5 Ki67 immunohistochemical staining of tumor tissues from different treatment groups.

(A) Fluorescence microscopy observation of Ki67 and DAPI staining. (B) The average number of Ki67-positive cells per field. Values are means ± S.D. of three photographs. * $p<0.05$, ** $p<0.01$ vs. vehicle control group.

3.6.6 Effect of TQ on the expression of various genes in tumor tissues

The protein expression of various genes, such as p-p38/p38, PPAR-γ and anti-apoptotic genes, in TQ-treated tumor tissues was examined and these results were compared to those from *in vitro* studies. Figure 3.6.6 showed that TQ was able to increase p38 phosphorylation in the tumor tissues, which was in line with the cell line data. On the other hand, PPAR-γ was decreased in TQ-treated groups. Since these tumors were induced from MDA-MB-231 cells, and our previous study had shown that TQ could reduce PPAR-γ expression in MDA-MB-231 cells (in contrast to MCF-7), it was deduced that TQ treatment was able to suppress PPAR-γ expression both *in vitro* and *in vivo* of MDA-MB-231 cell origin. In addition, our results showed that TQ was able to suppress the protein expression of various anti-apoptotic genes including XIAP, Bcl-xL, Bcl-2 and survivin in the tumor tissues (Figure 3.6.6). These results showed that the apoptosis induced by TQ in the tumor tissues could be due to the suppression of these anti-apoptotic genes.



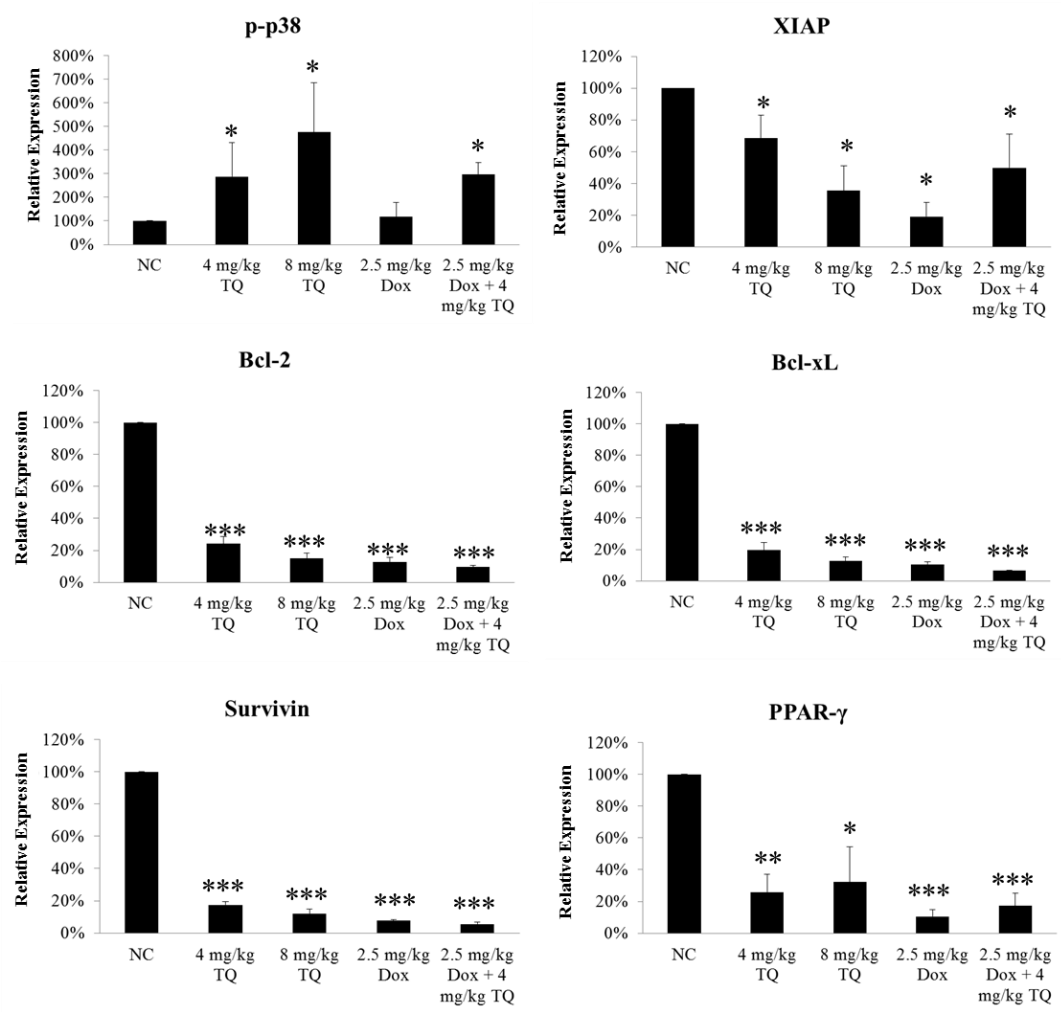


Figure 3.6.6 Effect of TQ on the protein expression of various genes in tumor tissues.

Tissue lysate was collected for Western blot analysis. Data are representative of at least three independent experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. vehicle control group.

3.6.7 Effect of TQ on the level of anti-oxidant enzymes/molecules in mouse liver tissues

The hepatic level of anti-oxidant enzymes/molecules was measured to investigate how ROS-inducing TQ interfered with the oxidation status in the liver. Our data showed that the catalase level was significantly increased in the drug treatment groups compared to the vehicle group (Figure 3.6.7A). The combined group had the highest catalase level among all treatment groups, and also significantly higher than either agent alone (Figure 3.6.7A). In addition, 8 mg/kg TQ and 2.5 mg/kg Dox treatment groups were able to increase the SOD level in the liver tissues compared to the vehicle group. 4 mg/kg TQ and the combined groups showed higher SOD level than the vehicle group, but these differences were not significant (Figure 3.6.7B). Moreover, TQ-alone treatment groups (4 mg/kg TQ, and 8 mg/kg TQ) showed significantly higher level of hepatic glutathione than the vehicle group (Figure 3.6.7C), while 2.5 mg/kg Dox group showed decreased level of glutathione. Interestingly, the combined group had lower glutathione level than the vehicle group, but higher than 2.5 mg/kg Dox group (Figure 3.6.7C).

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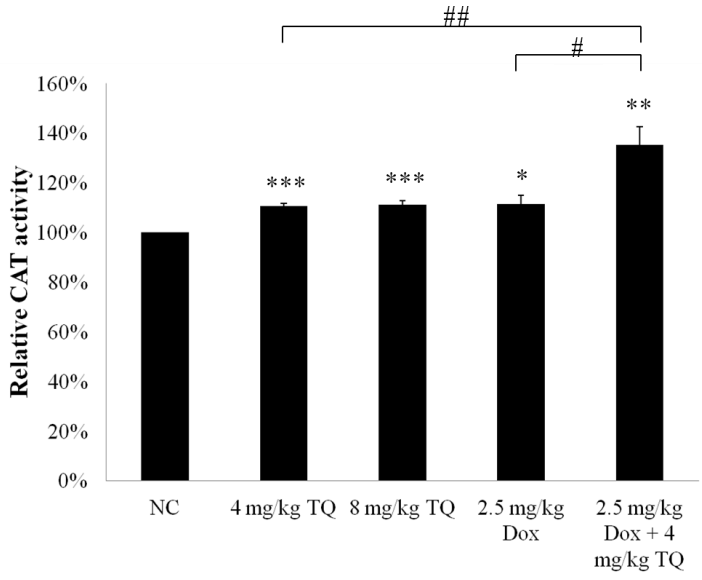

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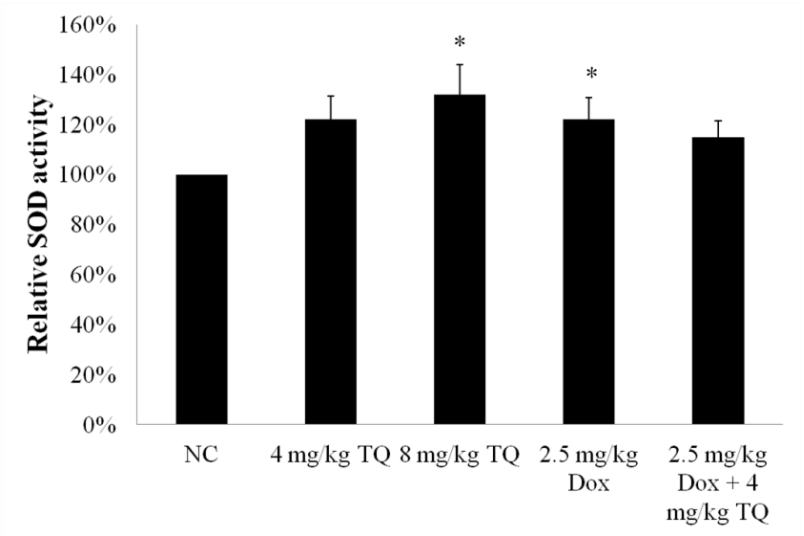

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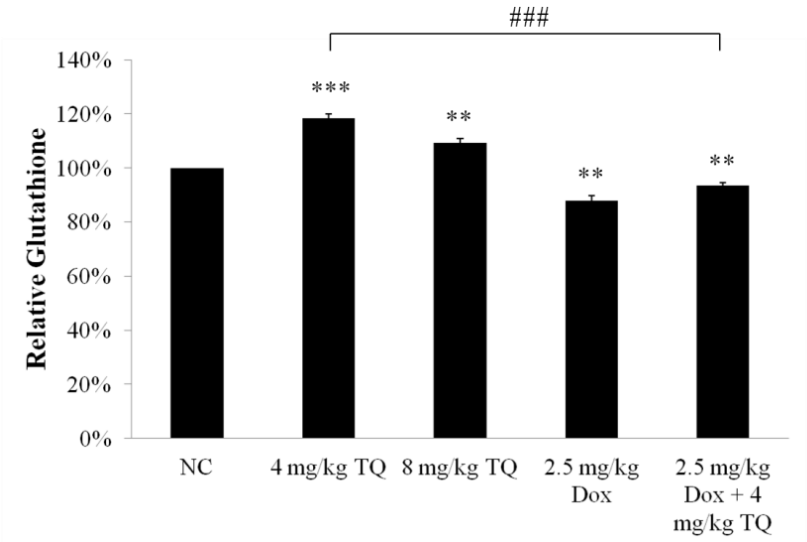

Figure 3.6.7 The level of hepatic anti-oxidant enzymes/molecules in each treatment group.

(A) Effect of TQ on the level of catalase in mouse liver tissues. Liver tissues were harvested for catalase assay. Values are means ± S.E.M. of at least three independent experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. vehicle control group. # $p<0.05$, ## $p<0.01$. (B) Effect of TQ on the level of SOD in mouse liver tissues. Liver tissues were harvested for SOD assay. Values are means ± S.E.M. of at least three independent experiments. * $p<0.05$ vs. vehicle control group. (C) Effect of TQ on the level of glutathione in mouse liver tissues. Liver tissues were harvested for glutathione assay. Values are means ± S.E.M. of at least three independent experiments. ** $p<0.01$, *** $p<0.001$ vs. vehicle control group. ### $p<0.001$.

4 DISCUSSION

4.1 General discussion

Due to increasing incidence and death rate from cancer in the United States, US President Richard Nixon declared the "War on Cancer" in 1971 in an effort to find cures for cancer disease through extensive research. Despite massive development in the last several decades, little has changed with regard to the incidence and mortality rate of this disease. Why has the discovery of anticancer drug been so difficult? The reason could lie with the disease itself. Cancer is a multi-complex disease arising from multiple signaling pathways that affect cell survival and development. Following the identification of the targets for an anticancer drug, the most important consideration is to make distinction on whether the targets have essential or non-essential functions (Kamb et al., 2007). Inhibition of essential functions can be fatal to the cancer cells, but also resulting in narrow therapeutic window owing to the requirement of these functions in normal cells. Inhibitors of non-essential functions have limited efficacy but are usually well tolerated in the human body. The issue on single-targeted or multi-targeted drugs is also widely discussed in drug discovery. Although single-targeted drugs are specific, however, when the dose is increased, off-target toxicity can arise due to the inhibition of unintended or unknown functions (Kamb et al., 2007). Kamb et al. also suggested that clinicians prefer multi-targeted drugs for maximal antitumor effect because they can manage toxicities through other interventions (Kamb et al., 2007). With cancer cells carrying mutation of about 300 genes and deregulation of 500 gene products, how single-targeted drugs treat a multi-complex disease like cancer is still a question (Aggarwal et al., 2007).

Natural products or traditional medicine have a long history in treating various kinds of illness including cancer. Because these medicines have been used in human body for many generations, any possible adverse effects/toxicities have been well identified and likely manageable. Generally, these medicines target multiple signaling pathways. As such, many researchers take an advantage on this property to discover potential anticancer drug. For example, curcumin,

luteolin and ursolic acid are natural products that have received extensive attention for their potential inhibitory effects in various types of carcinoma. Thus, in this project, we focused on the anticancer activities of thymoquinone, a natural product isolated from *Nigella sativa*, in breast carcinoma and tried to elucidate its mechanism of action in multiple signaling pathways.

4.2 Cytotoxic and pro-apoptotic effects of TQ

TQ has been shown to exhibit cytotoxic effect in many different kinds of cancer cells such as leukemia (HL-60 and Jurkat) (El-Mahdy et al., 2005; Alhosin et al., 2010), lung (NCI-H460 and A549) (Jafri et al., 2010; Rooney et al., 2010), colorectal (HT-29, HCT-116, DLD-1, Lovo and Caco-2) (El-Najjar et al., 2010; Rooney et al., 2010), pancreatic (MIA PaCa-2, HPAC and BxPC-3) (Banerjee et al., 2009; Rooney et al., 2010) and prostate cancer (LNCaP, C4-2B, DU145 and PC-3) (Kaseb et al., 2007; Koka et al., 2010). However, the cytotoxicity of TQ in breast cancer cells was not explained until we began this project. Our results showed that TQ treatment was able to suppress the growth of MCF-7, MDA-MB-231 and BT-474 breast cancer cells in a dose- and time-dependent manner. MCF-10A normal breast cells were also vulnerable to the toxicity of TQ, however this cell line was less sensitive to TQ than MDA-MB-231 and BT-474 cell lines. Thus, by determining the optimal dose, TQ can be used to target certain breast cancer cells with little or no toxicity to normal cells. Indeed, TQ was reported to cause little or no toxicity in several non-cancerous cell lines such as BPH-1 prostate epithelial cells (Kaseb et al., 2007), IMR90 human normal lung fibroblast cells (Gurung et al., 2010) and FHs74Int human normal intestinal cells (El-Najjar et al., 2010). Moreover, it has been shown that human body could tolerate a dose of TQ up to 2600 mg/day (Al-Amri et al., 2009). However, whether TQ is safe for long term use in human body is still remain to be studied.

MTT assay was used in this project to access the viability of treated cells. The principle of this assay is based on the ability of NAD(P)H-dependent cellular

oxidoreductase enzymes in live cells which capable of reducing the tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to its insoluble formazan crystals, which produce purple color after dissolving with DMSO. The properties of this assay are rapid, quantitative, and highly reproducible with a low intra-test variation between data points. However, under certain conditions, MTT assay may not accurately access cell viability (Wang et al., 2010). For example, rapidly replicating cells have higher rate of MTT reduction due to high metabolic rate. Moreover, some resting cells such as thymocytes and splenocytes, are viable but not necessarily display high metabolic activity. Alternative markers of cell dead should be used, for example cell membrane damage/leakage. As such, we also investigated the cytotoxicity of TQ via other assays such as propidium iodide staining and Annexin V assay.

In the present, there are a few studies reporting the anticancer effect of TQ on breast cancer cells. It was shown that TQ inhibited Akt, which in turn, suppressed cyclin D1 leading to G1 arrest in MDA-MB-468 and T-47D cells (Rajput et al., 2013). TQ was found to up-regulate PTEN for apoptotic cell death in doxorubicin-resistant MCF-7 cells (Arafa et al., 2011). Interestingly, there is a study reported that TQ was not able to kill MCF-7 cells due to high expression of NADPH quinone oxidoreductase 1, whereby inhibition of NADPH quinone oxidoreductase 1 with dicoumarol could render MCF-7 cells to TQ-sensitive (Sutton et al., 2012).

We showed that TQ did not cause cell cycle arrest in MCF-7 cells. This result was further explained by no significant change in the protein expression of cell cycle genes, including cyclin D1 and cyclin E, in TQ-treated MCF-7 cells. Interestingly, there was an increase in G2/M phase at 24 h time point even in the negative control group. Since these cells were synchronized with serum-free media before used in the experiment, the increase of G2/M phase might be due to cell cycle progression when the cells were released from starvation. On the other hand, there are studies reported that TQ was able to induce G0/G1 arrest or G2/M arrest in different types of cancer cells. For example, TQ was shown to induce G0/G1 arrest in SP-1 mouse papilloma carcinoma cells possibly through the increase of p16 and decrease of cyclin D1 (Gali-

Muhtasib et al., 2004b). In addition, TQ was found to induce G0/G1 arrest in acute lymphoblastic leukemia Jurkat cells through p73-mediated pathway, as confirmed by p73 siRNA gene silencing which could reactivate the cell cycle progression (Alhosin et al., 2010). On the other hand, TQ induced G2/M arrest in I7 mouse spindle carcinoma cells with increased p53 and decreased cyclin B (Gali-Muhtasib et al., 2004b). Moreover, G2/M arrest was also observed in MNNG/HOS human osteosarcoma cells after TQ treatment together with the increased expression of $p21^{WAF1}$ (Roepke et al., 2007). Together, these results demonstrate that the cell cycle arrest induced by TQ, whether it is G0/G1 or G2/M arrest, is highly dependent on the cell lines used.

In this study, TQ was found to induce apoptosis in MCF-7 cells as shown by increased percentage of Annexin V positive cells and increased PARP-cleavage. In addition of increased Bax/Bcl-2 ratio, TQ could decrease the expression of pro-caspases 8, 9 and 7. Thus, we suggest that TQ-induced apoptosis in MCF-7 cells might involve both intrinsic and extrinsic apoptosis pathways. TQ has been shown to induce apoptosis in different kinds of cancer cells such as liver, lung, colon and prostate cancer (Woo et al., 2012). There are studies reported that TQ-induced apoptosis was mediated possibly through p53, p73, STAT3, PTEN and NF-κB pathways (Woo et al., 2012). For example, TQ-induced apoptosis in HCT116 human colorectal carcinoma cells could be blocked by p53-specific inhibitor, pifithrin-α (Gali-Muhtasib et al., 2004a). This was further explained by another study showing that p53-null HCT116 cells were resistance to TQ-induced apoptosis (Gali-Muhtasib et al., 2008a). In contrast, apoptosis was observed in TQ-treated p53-null myeloblastic leukemia HL-60 cells, suggesting a p53-independent pathway (El-Mahdy et al., 2005). p73, a protein homologue to p53, is also involved in TQ-induced apoptosis. For example, TQ was shown to induce apoptosis in p53-null acute lymphoblastic leukemia Jurkat cells through the activation of p73 pathway, whereby p73 siRNA gene silencing could prevent TQ-induced cell death (Alhosin et al., 2010). In addition, TQ was reported to inhibit both constitutive and IL-6-inducible STAT3 phosphorylation in U266 multiple myeloma cells (Li et al., 2010). Interestingly, STAT3 over-expression or deletion could rescue U266 cells from TQ-induced apoptosis (Li et al., 2010).

Moreover, TQ was shown to activate PTEN for apoptotic cell death, whereby PTEN knockdown by siRNA gene silencing could abrogate TQ-induced apoptosis (Arafa et al., 2011). TQ was found to suppress TNF-induced NF-κB activation and NF-κB-regulated anti-apoptotic gene products such as IAP1, IAP2, XIAP, Bcl-2, Bcl-xL, and survivin (Sethi et al., 2008). Moreover, TQ was found to inhibit NF-κB in SaOS-2 human osteosarcoma cells and SaOS-2-induced tumors (Peng et al., 2013). Taken together, these results suggest that TQ is a multi-targeted, pro-apoptotic anticancer drug.

Moreover, our data showed that the combination of TQ with chemotherapeutic agents, such as doxorubicin and 5-fluorouracil, could result in enhanced cytotoxicity in MCF-7 cells. This enhanced inhibitory effect was significantly higher than either agent alone. This kind of synergistic effect has been reported in NCI-H460 non-small cell lung cancer cells by combining TQ with cisplatin (Jafri et al., 2010). Moreover, pre-treating TQ to human pancreatic cells (HPAC and BxPC-3) could sensitize these cells to the growth inhibition effect of gemcitabine and oxaliplatin (Banerjee et al., 2009). In addition, TQ was found to potentiate the apoptosis induced by bortezomib and thalidomide in U266 multiple myeloma cells (Li et al., 2010). Moreover, TQ could potentiate MCF-7 and T47D human breast adenocarcinoma cells to the cytotoxic effect of radiation (Velho-Pereira et al., 2011), which make it a potential drug for neoadjuvant chemotherapy. The synergism of the combination of TQ and chemotherapeutic drugs on cytotoxicity could be explained by down-regulation of NF-κB, Bcl-2 family, and NF-κB–dependent anti-apoptotic signaling (Banerjee et al., 2009). This is further supported by another study showing that down-regulation of NF-κB by TQ could sensitize cancer cells to the action of platinum drugs (Nessa et al., 2011). Together, these reports demonstrate that TQ is effective in suppressing cancer cells, while it also can combine with chemotherapeutic agents for greater therapeutic effect. Our data expanded the list by explaining the cytotoxic effect of TQ alone or in combination with chemotherapeutic agents on breast cancer cells.

In addition, there are a number of studies reported the analogs of TQ which could produce greater efficacy. The chemical structure of TQ was modified to achieve greater efficacy and higher cellular uptake (Woo et al., 2012). 6-

hencosahexaenyl conjugate of TQ (fatty acid conjugate) was reported to exhibit greater cytotoxic and pro-apoptotic effects than its parent compound in 518A2 melanoma and HL-60 leukemia cells possibly through increased ROS generation (Breyer et al., 2009). TQ-loaded poly-(D, L lactide-co-glycolide) nanoparticles were found to be more potent on anti-oxidant activity and growth inhibitory effect in MDA-MB-231 breast cancer cells (Ganea et al., 2010). Moreover, TQ poly-(lactide-co-glycolide) nanoparticles were more potent in inhibiting NF-kB, cyclin D1, MMP-9 and VEGF compared to TQ (Ravindran et a., 2010). The same study also showed that TQ poly-(lactide-co-glycolide) nanoparticles were able to produce greater cytotoxicity in MCF-7 breast cancer, KBM-5 myeloid leukemia, PC-3 prostate cancer, HCT116 colon cancer and U266 multiple melanoma cells than its parent compound (Ravindran et a., 2010). Modification at the carbonyl or benzenoid sites using single pot synthesis was able to produce TQ analog that exhibited greater cytotoxic and pro-apoptotic effects in MiaPaCa-2 (gemcitabine-resistant) pancreatic cancer cells (Banerjee et al., 2010). The same study also showed that this kind of TQ analog could sensitize MiaPaCa-2 cells to the cytotoxicity and apoptosis induced by gemcitabine or oxaliplatin (Banerjee et al., 2010). TQ analogs appended with gallate and fluorogallate pharmacophores showed greater cytotoxicity in pancreatic cancer cells as well as combined with gemcitabine for enhanced cytotoxic effect (Yusufi et al., 2013). Together, these studies demonstrate the potential of TQ analogs in inhibiting cancer cells. However, the efficacy of these analogs is yet to be explained in animal models.

4.3 Anti-metastatic effect of TQ

In addition to cytotoxic and pro-apoptotic effects, TQ was also found to exhibit anti-metastatic effect in breast cancer cells. In the present study, our data showed that TQ treatment could inhibit MCF-7 and MDA-MB-231 cell migration, as well as the invasion of MDA-MB-231 cells in a dose-dependent manner. The suppressive effect of TQ in MDA-MB-231 cell invasion was also reported by Attoub et al. together with LNM35 lung cancer cells (Attoub et al.,

2012). Moreover, there are studies reported that TQ was able to suppress C26 colon (Gali-Muhtasib et al., 2008b) and NCI-H460 lung (Jafri et al., 2010) cancer cell invasion. The suppressive effect of TQ was also reported in human umbilical vein endothelial cell migration, invasion, and tube formation (Yi et al., 2008). The inhibition on human umbilical vein endothelial cell tube formation by TQ was in a dose-dependent manner (Peng et al., 2013). TQ was found to inhibit CXCL12-mediated chemotaxis in multiple myeloma cell lines and primary cells isolated from multiple myeloma patients (Badr et al., 2011). Also, Sethi et al. reported that TQ treatment inhibited the protein expression of MMP-9, an enzyme involved in the breakdown of extracellular matrix, in KBM-5 human myeloid cells (Sethi et al., 2008). Together, these results suggest the potential inhibitory effect of TQ in cancer cell metastasis, however, the molecular mechanisms involved are not well explained and more results from *in vivo* study are needed to draw a more convincing conclusion.

"Wound-healing" assay, or also known as scratch assay, is a type of cell migration assay. There are other examples of cell migration assay such as Boyden chamber assay and cell-exclusion zone assay. "Wound-healing" assay is a rapid, convenient and low cost assay that commonly used in research to study the movement of cells under various conditions. However, there are several drawbacks in this assay, for example it is a 2-D surface and endpoint assay, and it is not suitable for non-adherent cells. Moreover, the gap closure in this assay is a consequence of a mixture of cell growth and cell migration. Perhaps the use of an anti-cell proliferation drug (but not affecting cell migration) in this assay could be an approach to explain the gap closure is due to cell migration but not cell growth. In this project, the ability of TQ on inhibiting gap closure might be a consequence of a mixture of cell death and inhibition of cell migration. Therefore, the use of a caspase inhibitor could be a solution to rule out the cytotoxicity of TQ.

4.4 The role of the PPAR-γ pathway in the anticancer effects of TQ

Generally, the risk factors of breast cancer can be divided into two types. The first type is the risk factors that cannot be changed, such as age, gender, gene mutation (e.g. BRCA1/2), family history, ethnicity and menstrual periods (Key et al., 2001; Higa, 2009). The second type of risk factors is amenable to change, such as changing in life style and diet, including childbearing, breastfeeding, alcohol intake and obesity. Since PPAR-γ is involved in the regulation of adipogenesis and lipid metabolism, this receptor has received extensive attention for its potential role in tumorigenesis. It was shown that the decrease of PPAR-γ expression is associated to the colorectal cancer metastatic progression (Pancione et al., 2009).

A number of studies reported that PPAR-γ activation was able to inhibit tumor cell growth. For example, Kumar et al. reported that PPAR-γ activation by 15d-PGJ2 treatment could inhibit the growth of MCF-7 cells via down-regulation of NHE1 (Kumar et al., 2009). It has been shown that troglitazone, a ligand of PPAR-γ, inhibited MCF-7 cells through G1 arrest by decreasing cyclin D1, Cdk2, Cdk4 and Cdk6 (Yin et al., 2001). In this project, we were interested on whether the anticancer effects of TQ involved the PPAR-γ pathway. Indeed, our results are the first to show that TQ was able to specifically activate PPAR-γ, but not other PPARs, in MCF-7 cells. This activation was partially involved in the apoptotic effect of TQ, as it could be abrogated by pre-treatment with a PPAR-γ-specific inhibitor, GW9662. Moreover, the decrease of survivin induced by TQ could be reversed by both GW9662 and PPAR-γ dominant negative. These results suggested that the suppression of survivin could be due to TQ-induced PPAR-γ activation.

Molecular docking study of TQ showed that TQ was able to form interactions with 7 polar residues and 6 non-polar residues of PPAR-γ molecule, with interaction energy of -18.06 kcal/mol (Woo et al., 2011). Among these interactions, Ser289, His323, His449, Tyr473 and Phe363 are important for PPAR-γ binding and activity (Nolte et al., 1998; Gampe et al., 2000; Salam et al., 2008). This suggest that TQ might directly interact with PPAR-γ for downstream signaling, in addition of indirect pathway via ROS.

In contrast, TQ treatment was shown to down-regulate the PPAR-γ expression in MDA-MB-231 cells. We also found that the protein expression of PPAR-γ in the breast tumor xenograft induced by MDA-MB-231 cells was also decreased in the TQ-treated groups. However, we did not further study whether the reduction of PPAR-γ in MDA-MB-231 cells was playing a role in the anticancer activities of TQ. There are increasing studies reported the reduction of PPAR-γ as a strategy in cancer therapy. Combined treatment of γ-tocotrienol and PPAR-γ antagonist (GW9662 or T0070907) synergistically suppressed the growth of MCF-7 and MDA-MB-231 cells (Malaviya and Sylvester, 2013). Moreover, PPAR-γ inhibition via dominant negative PPAR-γ mutant (Δ 462) was able to suppress cellular proliferation and induce apoptosis in MCF-7 (Zaytseva et al., 2008) and MDA-MB-231 cells (Zaytseva et al., 2011). T0070907, a PPAR-γ antagonist, could inhibit breast cancer cell proliferation and motility (Zaytseva et al., 2011). In addition, PPAR-γ immuno-reactivity was detected in 42% of 238 human breast carcinoma tissues (Suzuki et al., 2006). Increase in PPAR-γ activation might not able to initiate malignant transformation, however, once an initiating event has taken place, aberrant PPAR-γ could contribute to a more aggressive phenotype (Zaytseva et al., 2011).

On the other hand, a recent study reported that PPAR-γ activation was required for the apoptotic effect of thiazolidinedione and hydralazine in MDA-MB-231 cells (Jiang et al., 2011). The treatment of troglitazone could induce apoptosis and G1 arrest in MDA-MB-231 cells together with the decrease in pRb, cyclin D1, cyclin D2, cyclin D3, Cdk2, Cdk4 and Cdk6 expressions (Yu et al., 2008). However, these contradicted with another report which showed that delta2-troglitazone, which is devoid of PPAR-γ agonist activity, was able to suppress both MCF-7 and MDA-MB-231 cells (Colin et al., 2010). Perhaps better pharmacological and molecular approaches are needed to more clearly elucidate the role of the PPAR-γ pathway in breast cancer cells, as well as to explain the difference in PPAR-γ expression in both MCF-7 and MDA-MB-231 cell lines after TQ treatment. Currently, there is no study reporting the role of PPAR-γ based on the difference between ER+ and ER- breast cancer cells.

4.5 The involvement of ROS in the anticancer effects of TQ

Although excessive ROS can damage human tissues during inflammation, they can also kill malignant cells. Thus, if this activity can be fully utilized at the tumor-specific site, it can serve as a good tool to target tumor cells. In this project, we showed that the cytotoxic and pro-apoptotic effects of TQ in MCF-7 and MDA-MB-231 breast cancer cells were mediated via increase in ROS production. In the later experiments, we also showed that the suppression of anti-apoptotic gene products, such as XIAP, survivin, Bcl-2 and Bcl-xL, in MCF-7 cells was due to TQ-induced ROS production, as shown by the reversal of these suppressions by NAC pre-treatment. Although NAC is a strong anti-oxidant drug and it could effectively block the effect of TQ, additional antioxidant drugs, such as tocotrienol and selenium, can also be used to confirm the specificity of TQ on ROS induction.

Furthermore, TQ-induced ROS production was found to activate p38 for apoptotic cell death. Several types of ROS such as singlet oxygen and NO have been found to activate the p38 pathway for oxidant-induced apoptosis (Matsuzawa and Ichijo, 2005). For example, arsenic trioxide was found to induce ROS production, which in turn, activating p38 for apoptosis in HeLa human cervical cancer cells (Kang and Lee, 2008). In addition, Butein was found to induce ROS production for apoptotic cell death in MDA-MB-231 cells with decreased ERK and increased p38 (Yang et al., 2012). Chicoric acid, a compound found in various edible vegetables, was found to induce apoptosis in 3T3-L1 preadipocytes through ROS-mediated p38 activation (Xiao et al., 2013). On the other hand, there is a report showed that TQ was able to induce ROS production for ERK and JNK activations but not p38 in DLD-1 colon cancer cells (El-Najjar et al., 2010). This suggested that TQ-induced p38 activation might be cell line specific. A recent study reported that chlorpyrifos, a pesticide, was able to induce cell death and ROS production as well as activating MAPKs, including ERK1/2, JNK and p38, in SH-SY5Y human neuroblastoma cells; however, it was shown that treatment with MAPK inhibitors could inhibit cell death and ROS production (Ki et al., 2013). This suggested that p38 could also regulate ROS level under certain conditions.

In addition, our data showed that TQ-induced ROS production might play an important role in PPAR-γ expression. The increase or decrease of PPAR-γ expression in MCF-7 and MDA-MB-231 cells, respectively, after TQ treatment could be reversed by NAC pre-treatment. On the other hand, pre-treatment with GW9662 did not make any significant change to TQ-induced ROS production. As such, we suggested that the effect of TQ on ROS was upstream of PPAR-γ. Indeed, it was reported that beta-eleostearic acid was able to induce apoptotic cell death in T24 human bladder cancer cells possibly through ROS-mediated PPAR-γ activation, as shown by the reversal of PPAR-γ activation by NAC (Sun et al., 2012). This was supported by another study showing that NAC could inhibit PPAR-γ expression in 3T3-L1 adipocytes (Calzadilla et al., 2011). However, the relationship of ROS and PPAR-γ is not always one way. For example, 5,7-dimethoxyflavone, an activator of PPAR-α/γ, was shown to increase catalase expression, which resulting in the decrease of ultraviolet B-induced ROS production (Kim et al., 2012a). Moreover, pioglitazone, a PPAR-γ agonist, was found to reduce COX expression and ROS production in vascular smooth muscle cells from hypertensive rats (Mart ń et al., 2012). These studies suggested that PPAR-γ could modulate anti-oxidant enzymes to regulate the level of ROS. However, in different cases, the activation of PPAR-γ can increase ROS level. Troglitazone, a PPAR-γ agonist, induced apoptosis and ROS production in HT29 human colon cancer cells, which could both be reversed by GW9662 (Wang et al., 2011a). Moreover, troglitazone-induced cell death could not be prevented by NAC in 786-O, Caki-2 and ACHN human renal cell carcinoma cells (Fujita et al., 2011). Together, these findings suggest the complexity of the relationship between ROS and PPAR-γ pathway. While our results suggesting the interaction between ROS and PPAR-γ pathway in the anticancer activities of TQ, more experiments are needed to describe the importance of this relationship in a greater detail. For example, TQ can be exposed to stable PPAR-γ overexpressed/knockdown cell line to study the level of ROS and apoptosis.

In addition to MCF-7 cells, TQ has been shown to induce ROS production as a mechanism of apoptosis in other carcinoma cells. Recently, a study reported

that TQ could induce apoptosis in Jurkat cells via ROS production and depletion of glutathione (Dergarabetian et al., 2013). Hussain et al. reported that TQ was able to induce ROS production in activated B-cell lymphoma cells, which in turn, inhibited NF-κB activation by dephosphorylating IκBα and reduced nucleus translocation of p65 subunit (Hussain et al., 2013). Moreover, TQ-induced ROS production was found to inhibit Akt activation in primary effusion lymphoma cells (Hussain et al., 2011). The role of ROS is highly dependent on their levels, with low to modest levels promote tumor initiation, while high level suppresses tumor growth (Gupta et al. 2012). Since normal cells have higher capacity to cope with ROS before get killed, this makes cancer cells to preferentially be targeted by ROS accumulation and die selectively (Trachootham et al., 2009). Though ROS are beneficial in suppressing tumor cells, a persistent state of oxidative stress can cause cancer cells to adapt and become resistant through up-regulation of redox-sensitive transcription factors such as NF-κB (Tiligada, 2006; Sullivan and Graham, 2008). Perhaps, the combination of ROS-generating drug with an inhibitor to suppress redox-adaptation could be a better approach to the management of cancer malignancy. Despite various lines of evidence from studies in cancer cells, little is known about the role of TQ-induced ROS in the animal model. Further studies in animals are needed to elucidate the effect of TQ on ROS production in the body system, as well as to examine its role on tumor suppression.

4.6 The role of p38 MAPK in the anticancer activities of TQ

The family of MAPKs plays important roles in many cell functions, such as cell proliferation, apoptosis and cell survival (Johnson and Lapadat, 2002). Our data showed that TQ could increase the phosphorylation of ERK, JNK and p38 MAPK in MCF-7 cells. TQ was also shown to specifically increase the phosphorylation of p38 in MCF-7 and MDA-MB-231 cells, whereby these increases could be reversed by p38-specific inhibitor, SB203580. At the present, there are a few papers reporting the effect of TQ on MAPKs pathway.

TQ was shown to induce the phosphorylation of ERK and JNK, but not p38, in DLD-1 colon cancer cells (El-Najjar et al., 2010). In contrast, there is a report showed that TQ could induce both JNK and p38 phosphorylation in FG/COLO357 pancreatic cancer cells (Torres et al., 2010). Although the same paper elucidated that the JNK and p38 activations were mediated via TQ-induced Mucin-4 down-regulation, the role of JNK and p38 activations in the anticancer activities of TQ was not explained (Torres et al., 2010).

Our results showed that p38 activation induced by TQ involved in the cytotoxic and pro-apoptotic effects of TQ, as these effects could be abrogated by p38-specific inhibitor and p38 siRNA gene silencing. In contrast, TQ could induce ERK and JNK phosphorylation in DLD-1 cells, and the inhibition of these MAPKs by the treatment of ERK (PD98059) and JNK (SP600125) specific inhibitors could sensitize the cancer cells to TQ-induced apoptosis (El-Najjar et al., 2010). This was supported by another study showing that although TQ could induce JNK phosphorylation in PC-3 prostate cancer cells, pre-treatment with JNK-specific inhibitor (SP600125) could not prevent cell death from TQ. Therefore, even though TQ was shown to induce the phosphorylation of ERK and JNK, the results from these papers suggested that these activations might serve as a survival mechanism in response to the cytotoxicity of TQ. Nevertheless, more experiments should be conducted on different cell lines to explain this point in a greater detail.

The role of p38 in apoptosis has been reported in many cancer cell types, thus making it a possible tumor suppressor. The p38 protein can bind to p53 to form a complex, which in turn, enhancing the phosphorylation of p53 (She et al., 2000). Pharmacological inhibition and gene knockdown of p38 could prevent p53-mediated apoptosis (She et al., 2001). Interestingly, despite the role of p38 in apoptosis, there are data showed that p38 activation might involve in cancer cell invasion (Yong et al., 2009). This is largely due to the regulatory role of p38 in MMP protein family which is involved in tumor invasion and metastasis. For example, p38 was found to phosphorylate heat-shock protein 27, which in turn, activating MMP-2 for PC3 prostate cancer cell invasion (Xu et al., 2006). The relationship of p38 and MMP-2 was also reported in other carcinomas including human melanoma cells (Denkert et al.,

2002) and patient metastatic ovarian carcinoma (Davidson et al., 2003). In addition to MMP-2, p38 pathway was also found to regulate the expression of MMP-9 in human ovarian epithelial carcinoma cells (SKOV-3 and CaOV-3) (Zhou et al., 2007) and human squamous carcinoma cells (UM-SCC-1) (Simon et al., 2001). Moreover, MMP-1 and MMP-13 were found to be regulated by p38 in transformed human epidermal keratinocytes (Johansson et al., 2000).

Our data showed that GW9662 was able to enhance TQ-induced p38 activation in MCF-7 cells, but not MDA-MB-231 cells. On the other hand, SB203580 treatment was found to increase the protein expression of PPAR-γ in MCF-7 cells. These results suggested that TQ-induced activation of p38 and PPAR-γ might antagonize each other. A recent study reported that rosiglitazone, a PPAR-γ agonist, could inhibit p38 phosphorylation in microglia (He et al., 2012). This was supported by another paper showing that T33, a novel PPAR-γ/α agonist, was able to reduce TNF-α, COX-2, and p-p38 levels, whereby these reductions could be reversed by the GW9662 (Wang et al., 2011c). In addition, KR-62980, a novel PPAR-γ ligand, was shown to reduce ERK1/2 and p38 phosphorylation in endothelial cells (Kim et al., 2011). Pre-treatment with BADGE, a PPAR-γ antagonist, could reverse the effects of KR-62980 (Kim et al., 2011). Although they are PPAR-γ agonists in the same family, rosiglitazone, but not pioglitazone, was found to deactivate various MAPKs including ERK1/2, JNK and p38 in renal podocytes. However, PPAR-γ activation does not always lead to the suppression of p38. For example, troglitazone was found to increase the level of p-p38 in 786-O, Caki-2 and ACHN human renal cell carcinoma cells, whereby these increases could be blocked by the p38-specific inhibitor, SB202190 (Fujita et al., 2011). Thus, depending on the cell types and ligands used, the activation of MAPKs might work differently (Gardner et al., 2005). As such more data are needed to explain the interaction between PPAR-γ and p38 in the mechanism of action of TQ. For example, conditional p38 knockdown can be used to study the activity of PPAR-γ with or without TQ treatment.

4.7 The antitumor effect of TQ in animal models

Animal models provide better extrapolation and estimation to the effects of a drug candidate in the human body, and thus, the data from animal works are always a pre-requisite to bring a drug to future clinical setting. In the present, different administrations of TQ in mouse model were reported, including 5 mg/kg i.p. (Gali-Muhtasib et al., 2008b), 20 mg/kg i.p. (Kaseb et al., 2007), 20 mg/kg s.c. (Kaseb et al., 2007; Jafri et al., 2010), 5 mg/kg intratumoral (Ivankovic et al., 2006), 3 mg/mouse intragastric (Banerjee et al., 2009), and 100 μg/ml in drinking water (Badary et al., 1999). Since no pharmacokinetic study of TQ is available, the dose of TQ used in our animal model was determined after justification of several TQ's reports. We were interested to investigate the systemic therapeutic effect of TQ, thus intraperitoneal injection was selected. Two doses of TQ, 4 mg/kg and 8 mg/kg, were selected after justification from two papers in the literature (Gali-Muhtasib et al., 2008b; Kaseb et al., 2007).

In this project, our data showed that TQ was able to suppress the growth of breast tumor xenograft and, when combined with doxorubicin, produced greater inhibitory effect. TQ was found to suppress cell proliferation and induce apoptosis in breast tumor tissues as shown by decreased Ki67-positive and increased TUNEL-positive cells, respectively. The expression of anti-apoptotic genes such as survivin, XIAP, Bcl-xL and Bcl-2 in tumor tissues were inhibited in TQ treatment groups. Moreover, p-p38 expression in TQ treatment groups was increased with no change in p38 expression, which was in line with the findings in cell line. PPAR-γ expression was suppressed in TQ treatment groups, in line with the findings in MDA-MB-231 cell line.

The antitumor effect of TQ and its possible molecular mechanisms have been shown in other types of tumor xenograft such as lung (Jafri et al., 2010), colon (Gali-Muhtasib et al., 2008b), prostate (Kaseb et al., 2007) and pancreas cancer (Banerjee et al., 2009). For example, NF-κB, a transcription factor which might be responsible for cisplatin resistance, was down-regulated by TQ treatment in NCI-H460 cells-induced lung tumor xenograft (Jafri et al., 2010). This is supported by another study showing that TQ was able to

suppress NF-κB and its-regulated molecules (such as XIAP, survivin and VEGF) in SaOS-2-induced osteosarcoma tumors (Peng et al., 2013). In addition, TQ treatment was found to down-regulate androgen receptor, E2F-1 and cyclin A in C4-2B cells-induced prostate tumor xenograft (Kaseb et al., 2007). On the other hand, by drinking TQ-added water after benzo(a)pyrene induction, forestomach tumor incidence and multiplicity could be reduced (Badary et al., 1999). A recent study showed that TQ could prevent 1,2-dimethyl-hydrazine-induced oxidative stress and colon tumor development (Jrah-Harzallah et al., 2013). The combination of TQ and chemotherapeutic agents has been reported to cause greater inhibitory effect not only in cancer cells but also in animal models, for example combined with cisplatin in lung tumor xenograft (Jafri et al., 2010), as well as combined with gemcitabine or oxaliplatin in pancreatic tumor xenograft (Banerjee et al., 2009).

In addition to antitumor effect, TQ is able to protect against the toxicity caused by various chemotherapeutic agents. For example, TQ was found to ameliorate the nephrotoxicity caused by cisplatin in mice and rats (Badary et al., 1997). In addition, doxorubicin-induced nephropathy in rats could be reduced by TQ treatment (Badary et al., 2000). Moreover, cardio-toxicity, the major concern of doxorubicin regimen, could be ameliorated by TQ treatment without compromising its therapeutic efficacy (Al-Shabanah et al., 1998). The similar protective activity was also observed in cyclophosphamide-induced cardiotoxicity in rats (Nagi et al., 2010). The increase in quinone reductase and glutathione transferase in mice liver after TQ treatment might explain the protective effect of TQ against drug toxicities (Nagi and Almakki, 2009). If the toxicities caused by chemotherapeutic agents are able to be ameliorated by TQ, clinicians are more willing to increase the dose of these agents for greater efficacy. These results suggest the possible complementary role of TQ in combination regimen to target resistant cancer.

The effect of TQ on anti-oxidant enzymes/molecules was investigated in this project. Our results showed that TQ treatment increased catalase, SOD and glutathione levels in mouse liver tissues. However, we are not certain whether the increase of these anti-oxidant enzymes/molecules was due to the direct effect of TQ or in response to the ROS-inducing effect of TQ. Although this

study and many other papers reported the induction effect of TQ treatment on ROS production together with its role in apoptosis, there are studies reporting the efficacy of TQ's antioxidant effect. For example, TQ could act as a potent superoxide radical and free radical scavenger at micromolar and nanomolar range of concentrations, respectively (Mansour et al., 2002). This was supported by another report showing that TQ could act as a potent superoxide anion scavenger and inhibited iron-dependent microsomal lipid peroxidation in a dose-dependent manner (Badary et al., 2003). On the other hand, TQ was shown to reverse the decrease of catalase, glutathione-S-transferase, glutathione peroxidase and reduced glutathione in the kidney and liver tissues of streptozotocin-diabetic rats (Sankaranarayanan and Pari, 2011). This was supported by another study showing that TQ was able to reverse the decrease in the activity and mRNA expression of catalase, glutathione peroxidase and glutathione-S-transferase in the liver tissues of diethylnitrosamine-induced rats (Sayed-Ahmed et al., 2010). The increase of glutathione after TQ treatment could improve the experimental allergic encephalomyelitis in female Lewis rat (Mohamed et al., 2003). However, these studies did not explain whether the changes in the level of anti-oxidant enzymes/molecules were the direct effect of TQ. Interestingly, there is a report showed that TQ did not cause any significant change in the glutathione level in mouse hepatic, cardiac and kidney tissues (Mansour et al., 2002). Taken together, the role of TQ as an anti-oxidant or pro-oxidant drug needs further evaluation especially in the animal model. Thus, well-designed animal models are needed to elucidate the role of TQ in different diseases such as inflammatory disorder and cancer. For example, the oxidation status in TQ-treated tumor tissues can be studied to investigate whether the pro-oxidant or anti-oxidant effect of TQ is responsible for its antitumor action. Moreover, tumor-induced mice can be co-treated with TQ and anti-oxidant drug to investigate whether ROS induction is playing a role in TQ's antitumor effect.

5 CONCLUSION

The studies in breast cancer cells and in the breast tumor xenograft mouse model demonstrated that TQ is a potential anticancer agent. In addition to growth inhibition and pro-apoptotic effects, TQ was found to inhibit the migration and invasion of breast cancer cells. Moreover, the inhibitory effect of TQ might be mediated via PPAR-γ, ROS and p38 MAPK pathways. TQ was able to induce PPAR-γ activity specifically. This was later found to be responsible for the suppression of survivin. The effect of TQ on ROS induction was also noted. By using NAC, an antioxidant agent, to block ROS induction, the anticancer effects of TQ could be abrogated. Moreover, TQ-induced suppression of anti-apoptotic genes, such as survivin, XIAP, Bcl-2 and Bcl-xL, were able to be reversed by NAC. On the other hand, TQ was found to induce p38 activation which could be reversed by p38-specific inhibitor. The TQ-induced p38 activation was later found to be responsible for TQ's cytotoxic and pro-apoptotic effects. Moreover, ROS production was found to be the upstream regulator of PPAR-γ and p38 in the mechanistic action of TQ. TQ was also found to suppress breast tumor growth in nude mice and this suppression was greater when combined with doxorubicin. Decreased cell proliferation and increased apoptosis were observed in breast tumor xenograft after TQ treatment. TQ was found to increase the hepatic level of catalase, SOD and glutathione, which might explain its ROS-inducing effect. Overall, the effects of TQ in breast carcinoma are summarized in Figure 5.1.

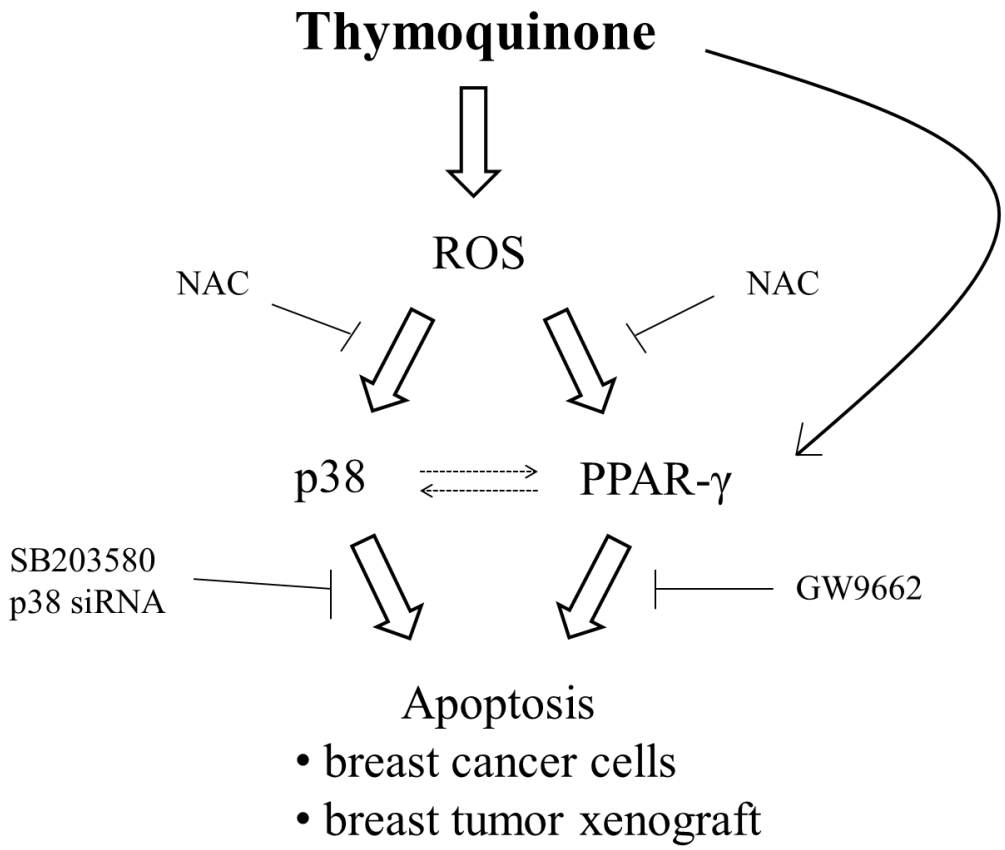

Figure 5.1 Proposed mechanism of action of TQ in breast cancer

6 FUTURE DIRECTIONS

Collectively, other studies and our data suggest that TQ is a multi-targeted anticancer drug. The concern regarding off-target effect which may result in side effect(s) need to be addressed. A well-designed toxicological study should be conducted to evaluate the safety of this drug in humans. Moreover, the studies on the bioavailability and pharmacokinetic of TQ are also important to determine how the body handles TQ. TQ has low solubility due to high hydrophobicity, whereby this will resulting in low bioavailability. There is a study showed that TQ could be loaded in liposomes to increase bioavailability (Odeh et al., 2012). This strategy however require more data from animal models.

This project has identified the interesting effects of TQ, whereby it induced the expression of PPAR-γ in MCF-7 cells, but reduced the expression of PPAR-γ in MDA-MB-231 cells. This difference should be explained in a greater detail, possibly by genetic approaches such as PPAR-γ overexpression or PPAR-γ shRNA gene knockdown in MCF-7 and MDA-MB-231 cell lines follow by experiments with TQ. In addition, co-treatment of TQ with PPAR-γ ligand/inhibitor in MDA-MB-231 cells can be carried out to study the role of PPAR-γ in the antitumor effect of TQ.

Several studies have shown that the anticancer activities of TQ in various cancer cell lines were mediated via the increase in ROS level. However, this mechanism of action still remains unexplained in the *in vivo* model. Since ROS induction could be the upstream regulator in the anticancer mechanism of TQ, it is important to evaluate the regulatory role of ROS on proteins such as p53, p73, p38, PPAR-γ, NF-κB, STAT3 and PTEN, which had been reported to be involved in TQ-induced apoptosis.

The combination of TQ and chemotherapeutic agents has been shown to produce greater efficacy in several tumor xenograft models, including lung and pancreas (Woo et al., 2012). However, more studies are needed to examine the effects of different combination with TQ on various tumor types,

such as liver and colon cancer. In addition, toxicological studies are warranted to evaluate the safety of such combinations.

Numerous studies have demonstrated the anti-inflammatory effect of TQ in different disease models including asthma (El Gazzar et al., 2006a; El Gazzar et al., 2006b), arthritis (Budancamanak et al., 2006; Tekeoglu et al., 2006; Vaillancourt et al., 2011) and diabetes (Pari and Sankaranarayanan, 2009). TQ was shown to attenuate allergic airways by suppressing several Th1 cytokines, including IL-4, IL-5 and IL-13, and infiltration of eosinophil into the airways (El Gazzar et al., 2006b). Moreover, TQ could inhibit 5-lipoxygenase and leukotriene C4 synthase in human blood cells (Mansour and Tornhamre, 2004). It was also shown that TQ could inhibit cyclooxygenase-2 protein expression and prostaglandin E2 production in HPAC pancreatic cancer cells (Banerjee et al., 2009). Since excessive inflammation lead to the initiation of carcinogenesis, TQ as an anti-inflammatory agent may protect against cancer development. Further studies can be carried out to investigate whether TQ is able to prevent carcinogenesis by regulating body immune system.

The anticancer activities of TQ have been reported in many pre-clinical studies mainly in cancer cell lines and tumor xenograft mice. There are limited numbers of clinical trial conducted with TQ to date. A phase I trial reported that TQ did not cause significant systemic toxicity in adult patients with solid tumors or hematological malignancies, and the human body could tolerate a dose of TQ up to 2600 mg per day (Al-Amri and Bamosa, 2009). However, the same study also reported that there was no anticancer effect observed in these patients. Since the pharmacokinetic of TQ remains unknown, the effect of TQ in the human body is unpredictable because we do not know how the body absorbs and handles TQ. On the other hand, a double blinded crossover clinical trial has shown that TQ was able to exhibit antiepileptic effects in children with epilepsy (Akhondian et al., 2011).

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