

RESEARCH ARTICLE

In Vitro Anti-Neuroblastoma Activity of Thymoquinone Against Neuro-2a Cells via Cell-cycle Arrest

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Abstract

We have recently shown that thymoquinone (TQ) has a potent cytotoxic effect and induces apoptosis via caspase-3 activation with down-regulation of XIAP in mouse neuroblastoma (Neuro-2a) cells. Interestingly, our results showed that TQ was significantly more cytotoxic towards Neuro-2a cells when compared with primary normal neuronal cells. In this study, the effects of TQ on cell-cycle regulation and the mechanisms that contribute to this effect were investigated using Neuro-2a cells. Cell-cycle analysis performed by flow cytometry revealed cell-cycle arrest at G2/M phase and a significant increase in the accumulation of TQ-treated cells at sub-G1 phase, indicating induction of apoptosis by the compound. Moreover, TQ increased the expression of p53, p21 mRNA and protein levels, whereas it decreased the protein expression of PCNA, cyclin B1 and Cdc2 in a dose-dependent manner. Our finding suggests that TQ could suppress cell growth and cell survival via arresting the cell-cycle in the G2/M phase and inducing apoptosis of neuroblastoma cells.

Keywords: Neuroblastoma - thymoquinone - cell-cycle arrest - apoptosis - p53

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Introduction

Thymoquinone (TQ) is one of the bioactive component of *Nigella sativa* (Shoieb et al., 2003). Extracts prepared from black seeds of *N. sativa* have been used for medical purposes for centuries for a number of diseases (Ali and Blunden, 2003; Padhye et al., 2008). TQ is the active ingredient of black seed and has been shown to possess antitumor activity against a broad spectrum of cancer cells, including colon, ovarian, lung, osteosarcoma, and myeloblastic leukemia (Norwood et al., 2006; Gali-Muhtasib et al., 2008; Wilson-Simpson et al., 2007; Roepke et al., 2008; El-Mahdy et al., 2005). TQ has also been shown to inhibit chemically induced carcinogenesis in mice (Badary et al., 1999, 2001). More recently, it was demonstrated that TQ augments the antitumor activity of standard cancer chemotherapeutic agents in pancreatic cancer cells in vitro and in vivo (Banerjee et al., 2009). TQ has been shown to be safe on a wide variety of normal cells. The selective cytotoxicity of TQ for human cancer cells compared to primary mouse keratinocytes (Gali-Muhtasib et al., 2004), mouse normal kidney cells (Shoieb et al., 2003), non-malignant fibroblasts (Effenberger et al., 2010) and normal human lung fibroblasts (Gurung et al., 2010) has been reported in several studies.

For decades, TQ has been used as an anti-oxidant, anti-inflammatory and anti-neoplastic agent (Trang et

al., 1993; Hosseinzadeh and Parvardeh, 2004). Previous studies have shown that TQ exhibits inhibitory effects on cell proliferation of many types of cancer cells (Gali-Muhtasib et al., 2006) including non-Dox resistant breast cancer cells (MCF-7) (Shoieb et al., 2003; Ravindran et al., 2010; Effenberger-Neidnicht and Schobert, 2011). Studies had shown that TQ was able to induce apoptosis via p53-independent (Gali-Muhtasib et al., 2004) and p53-dependent pathways. Moreover, TQ induced apoptosis through activation of caspase pathway and mitochondrial events in p53-null myeloblastic leukemia HL-60 cells (El-Mahdy et al., 2005). TQ also induces cell death and inhibits tumor growth by suppressing NF- κ B, Akt activation and extracellular signal-regulated kinase signaling pathways as well as angiogenesis (Sethi et al., 2008; Yi et al., 2008).

p53, the tumor suppressor protein plays a key role in the regulation of the cell-cycle and cell death. Cell-cycle checkpoints and apoptosis play key roles in developmental biology and represent a new set of potential targets for chemotherapeutic agents. Quinones represent a class of drugs that induce both of these effects (Hartwell and Kastan, 1994; Karp and Broder, 1995). Quinones induce free radical-mediated DNA strand breaks, and also alkylate and cross-link DNA (Dusre et al., 1989; Begleiter and Leith, 1990). Other mechanisms of toxicity include interference with the cell-cycle (Morgan et al., 1992) due

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to increased expression of p21(waf1/cip1/sdi1) protein, an inhibitor of cyclin-dependent kinases and an important negative regulator involved in G1 cell-cycle control (Wu et al., 1998; Qiu et al., 1996). p21 also inhibits Cdc2/cyclin B1 during the G2/M transition (Harper et al., 1995). Thymoquinone has been shown to regulate cell-cycle proteins; inducing both G1/S and G2/M cell-cycle arrest in various cancer cell lines. It induces G1 cell-cycle arrest in HCT 116 (Gali-Muhtasib et al., 2004) and also induces cell-cycle arrest in human breast carcinoma cells (Velho-Pereira et al., 2011). The present study was conducted to resolve the molecular mechanisms underlying the cell-cycle arrest of TQ treated mouse neuroblastoma cells.

Neuroblastoma, being the most common solid tumor in children is one of the most challenging malignant tumors because of its heterogeneity, variety of clinical behavior and high recurrence. It is the second most frequent malignancy in infancy after lymphoblastic leukemia. Neuroblastoma arises from the neural crest cell precursors of the sympathetic nervous system failing to complete differentiation. At present, there is no effective or specific chemotherapy against neuroblastoma and new treatment strategies are urgently needed to improve the survival rate and the quality of life of children suffering from neuroblastoma (Berthold and Hero, 2000; Brodeur, 2003).

Materials and Methods

Chemicals and reagents

Thymoquinone (TQ), propidium iodide (PI), dimethyl sulfoxide (DMSO) and 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) were purchased from Sigma chemical Co., USA. TQ was dissolved in cell-culture tested, sterile DMSO at a stock concentration of 100 mM and was stored at -20°C until use. Trypsin-EDTA, fetal bovine serum (FBS), antibiotics-antimycotics, Dulbecco's modified Eagle's medium (DMEM) and phosphate buffered saline (PBS) were purchased from Gibco, Canada. Polyvinylidene difluoride (PVDF) membrane was purchased from Millipore, USA. Primary antibodies against p53, p21, PCNA, cyclin B1 and Cdc2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). β -actin was purchased from Sigma Aldrich Chemicals Pvt Ltd (USA). The secondary antibodies, horse radish peroxidase (HRP) conjugated goat anti-mouse IgG and goat anti-rabbit IgG were obtained from Banglore Genei, Bangalore. All the chemicals used were extra pure and of analytical grade.

Cell culture

Neuroblastoma (Neuro-2a) cell line was obtained from National Centre for Cell Science (NCCS), Pune, India. The cells were grown in T25 culture flasks containing DMEM supplemented with 10% FBS and 1% antibiotics (penicillin 100 U/ml and streptomycin 100 μ g/ml). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂.

Determination of cytotoxicity of TQ by 3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay

Cell cytotoxicity was assessed by MTT method as described earlier (Jana et al., 2004). For cell cytotoxicity assay, cells (approximately 5 X 10³ cells/well) were seeded into 96-well tissue culture plates and 24 h after seeding, the medium was changed and cells were treated with various concentrations of TQ, ranging from 0 to 70 μ M for 24 and 48 h. Cell viability was quantified by the ability of living cells to reduce the yellow color tetrazolium salt to a purple formazan product. This formazan product was then dissolved in DMSO and absorbance was measured at 570 nm. The cell viability was calculated using the formula:

$$\% \text{ Growth inhibition} = \left[\frac{A_{570} \text{ nm of treated cells}}{A_{570} \text{ nm of control cells}} \right] \times 100$$

Cell-cycle analysis

The effect of TQ on the cell-cycle was determined using flow cytometry analysis as described previously (Lo et al., 2007). Neuro-2a cells were seeded in 100 mm dishes at a density of 1 x 10⁶ cells. They were incubated and allowed to grow to 70-80% confluence after which they were treated with the indicated concentrations of TQ for 24 h. The cells were then harvested by trypsin and washed twice with phosphate buffered saline (PBS). The pellet was resuspended in an ice-cold solution of 70% ethanol in PBS (v/v). After two hours of fixation, cells were washed twice with PBS. Fixed cells were stained with staining solution (2 mg/ml propidium iodide and 10 mg/ml RNase in PBS) at 37°C for 30 min. Stained cells were analyzed by flow cytometry (FACS Calibur TM, Becton Dickinson, USA). The distribution of cells in G1, S and G2 was determined using Cell Quest software (Becton- Dickinson, San Jose, CA).

Quantitative RT-PCR

mRNA expression levels of p53, and p21 were examined using real-time RT-PCR. The total RNA was isolated by using Tri Reagent (Invitrogen). Total RNA (1 μ g) from each sample was reverse transcribed using a commercial iScript cDNA synthesis kit according to the manufacturer's protocol, Bio-Rad, USA. Real time-PCR was carried out in MX3000p PCR system (Stratagene, Europe) using IQ SYBR Green PCR master mix kit. The following primers were used for p53 Forward Primer: 5'- GGAAATTTGTATCCCGAGTATCTG-3', Reverse Primer: 5'- GTCTTCCAGTGTGATGATG GAA-3' (183 bp); p21 Forward Primer: 5'- GAGAA CGGTGGAACCTTTGAC-3', Reverse Primer: 5'- GTGCAAGACAGCGACAAGG-3' (214bp) and 18S rRNA Forward Primer: 5'- CGCTTCCTTACCTGGTTGAT-3', Reverse Primer: 5'-GAGCGACCAAAGAACCATA-3' (136 bp). Real time PCR was performed as follows: 95°C for 10 minutes, followed by 40 cycles (94°C for 30 sec; 55°C for 30 sec). A melting point dissociation curve generated by the instrument was used to confirm product specificity. Relative RNA levels expressed in arbitrary units were calculated using comparative method based on Δ Ct values. Reactions were carried out in duplicate and the mRNA expression was normalized against 18S rRNA as an internal control.

Western blot analysis

After 24h treatment period the cells were lysed using RIPA buffer containing 1X protease inhibitor cocktail and then the protein concentrations were measured using Lowry's method (Lowry et al., 1951). Cell lysates (20-50µg) were electrophoresed in 12% SDS polyacrylamide gel and then transferred into PVDF membranes. The membranes were incubated with primary antibodies against p53, p21, PCNA, cyclin B1 and Cdc2 (1: 1000) in Tris-buffered saline. After washing, the membranes were incubated with HRP conjugated goat-anti mouse IgG (1:5000) or HRP conjugated goat-anti rabbit IgG (1:5000). Protein bands were detected using chemiluminescence system (ECL Kit) and quantified in Chemi Doc XRS Imaging System, Bio-Rad (USA).

Statistical analysis

Data were expressed as mean \pm SEM. Statistical analyses were performed using one-way ANOVA followed by Student-Newman-Keul's (SNK) tests for comparison between treatment values and control values using Statistical package for student version 7.5 (SPSS) software. P values $<$ 0.05 were considered to be statistically significant.

Results

The anti-proliferative effect of TQ in Neuro-2a cells

In the previous study, we showed that the cytotoxic effects of TQ in Neuro-2a cells and primary normal neuronal cells were measured by MTT assay. Neuro-2a cells showed a significant decrease in the percentage of viable ($p < 0.05$) cells treated with different concentrations of TQ for 24 h and 48 h. TQ was significantly less cytotoxic towards the primary normal neuronal cells. The greatest difference in survival between the primary normal neuronal cells and Neuro-2a cells following TQ treatment occurred at concentration of 40 μ M. The cytotoxic effects of TQ in Neuro-2a cells were significantly higher than primary normal neuronal cells. IC_{50} values of TQ in Neuro-2a cells after 24 and 48 h of exposure were determined as 40 and 36 μ M, respectively. Based on the viability trend obtained, 20 and 40 μ M for 24 h were chosen for subsequent assays.

The effect of TQ on the G2/M cell-cycle distribution and apoptosis in Neuro-2a cells

The cytotoxicity caused by TQ may be due to anti-proliferative and proapoptotic effects. To test whether the anti-proliferative effect of TQ was related to cell-cycle arrest, Neuro-2a cells were treated with TQ and the cycle progression was examined by the flow cytometry. TQ suppressed the proliferation of Neuro-2a cells after treating with different concentrations (0, 20 and 40 μ M). At 24 h, cells in the G2/M phase of the cell-cycle increased from 26.96% to 34.82% when treated with 40 μ M of TQ (Figure 1A), this was accompanied by a progressive decrease in the percentage of cells in the G0/G1 phase. All these findings indicated that TQ caused cell-cycle arrest at G2/M phase. The block in the cell-cycle progression, therefore, contributes to TQ induced anti-proliferative

effects. In addition, there was a significant increase in the sub-G0 phase, resulting in an increase in TQ-induced apoptotic cell death in a concentration dependent manner

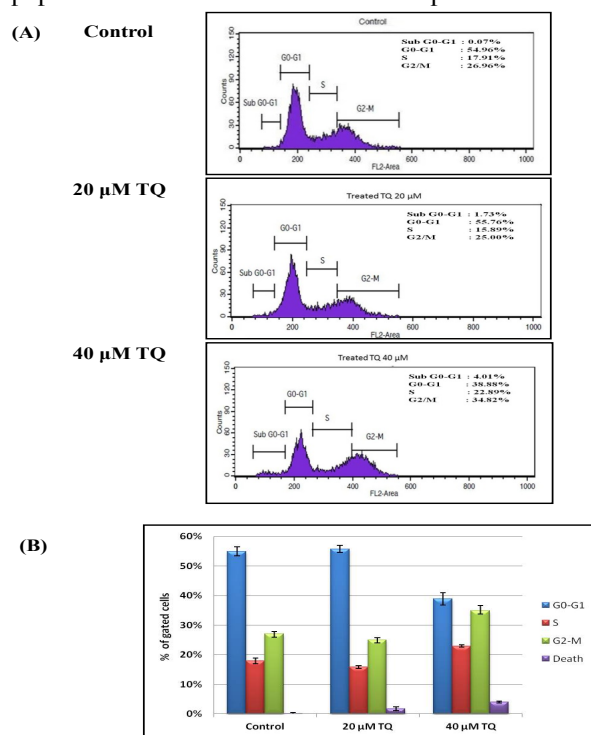


Figure 1. The Cell Cycle Distribution of Neuro-2a Cells Treated with 0, 20 and 40 μ M of TQ for 24 h was Determined using flow Cytometry (A). The percentage of cells at different stages of cell cycle (B)

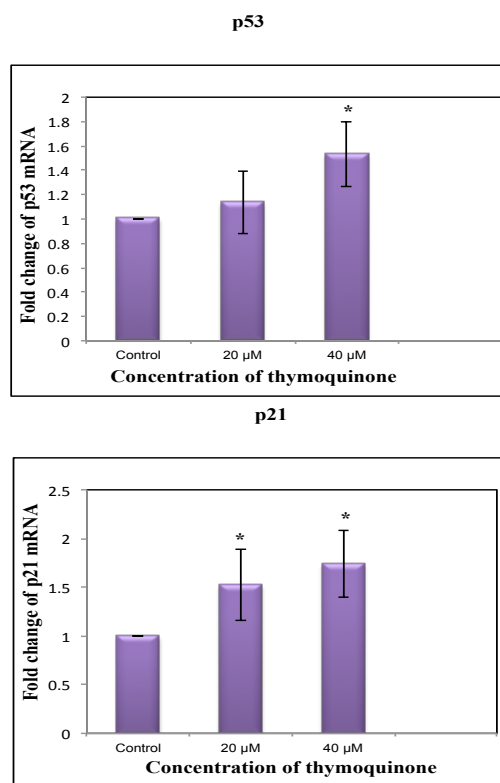


Figure 2. Expression of p53 and p21 mRNA in Neuro-2a Cells Treated with 0, 20 and 40 μ M of TQ for 24 h Results are Expressed as fold Change from Control. “ * ” denotes statistical significance at $p < 0.05$ when compared with control

(Figure 1A). Figure 1B shows the percentage of cells at different stages of cell cycle.

Effect of TQ on p53 and p21 gene expression in Neuro-2a cells

p53 functions as a transcription factor by binding to a p53-specific DNA sequence in responsive genes, which would increase the synthesis of p21, the most important checkpoint proteins involved in cell arrest at both G1 and G2/M. Previous reports have described that TQ arrested MCF-7/DOX cells at G2/M phase and increased cellular levels of p53 and p21 proteins (El-Shaimaa et al., 2011). In this study, TQ-treated Neuro-2a cells showed a significant increase in the expression of p53 and p21 mRNA level (Figure 2). These results suggest that TQ induced cell-

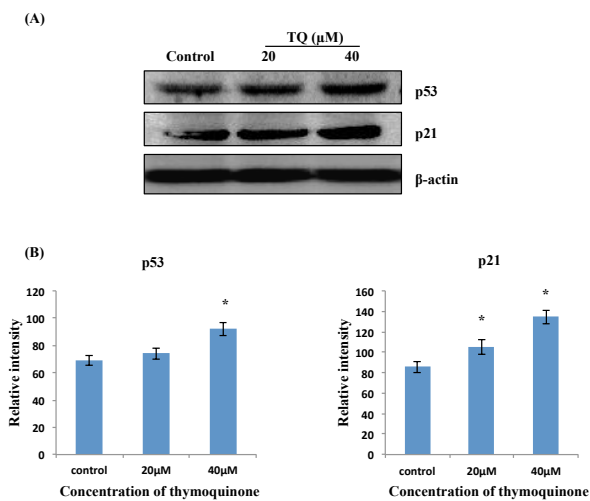


Figure 3. Expression of p53 and p21 Proteins in Neuro-2a Cells Treated with 0, 20 and 40 μM of TQ for 24 h. (A) Western blots showing levels of p53, p21 proteins and β-actin used as loading control. (B) densitometry analysis of the western blot. “*” denotes statistical significance at $p < 0.05$ when compared with control

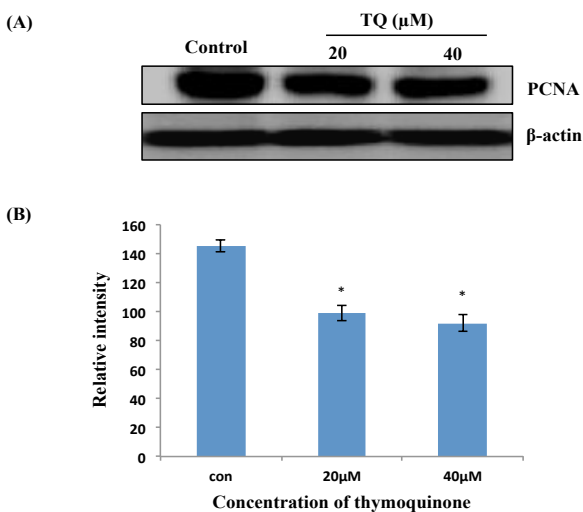


Figure 4. Expression of PCNA Protein in Neuro-2a Cells Treated with 0, 20 and 40 μM of TQ for 24 h. (A) Western blot showing level of PCNA protein and β-actin used as loading control. (B) Densitometry analysis of the western blots. “*” denotes statistical significance at $p < 0.05$ when compared with control

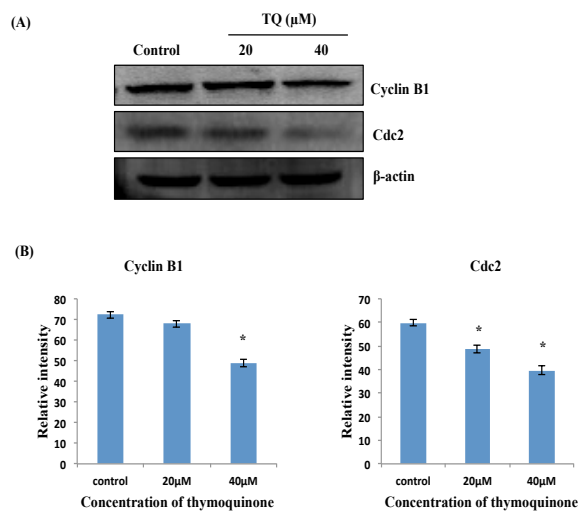


Figure 5. Expression of Cyclin B1 and Cdc2 Proteins in Neuro-2a Cells Treated with 0, 20 and 40 μM of TQ for 24 h. (A) Western blots showing levels of Cyclin B1, Cdc2 proteins and β-actin used as loading control. (B) Densitometry analysis of the western blots. “*” denotes statistical significance at $p < 0.05$ when compared with control

cycle arrest at G2/M phase via alteration of the p53 and p21 mRNA expression in Neuro-2a cells.

Effect of TQ on expression of cell-cycle associated proteins in Neuro-2a cells

The cyclin-dependent kinase (CDK) inhibitor p21 is involved in p53-mediated growth arrest. Induction of p21 protein expression stops cell-cycle progression by inhibiting the activity of CDKs and by interacting with the proliferation cell nuclear antigen and thereby preventing DNA synthesis (Li et al., 1994; Li et al., 1996). To elucidate the molecular mechanism by which TQ induces cell-cycle arrest at the G2/M phase, the cells were treated with different concentration (0, 20 and 40 μM) of TQ, whole cell extracts were prepared and analyzed for the protein expression of p53, p21, PCNA, cyclin B1 and Cdc2 by western blotting with β-actin as the control. The results have shown that TQ-treated Neuro-2a cells showed a significant increase in the expression of p53 and p21 (Figure 3), whereas decreased expression of PCNA (Figure 4), cyclin B1 and Cdc2 (Figure 5) levels were observed, and there is no change in the expression of p53, Cyclin B1 and Cdc2 proteins after treating with low concentration (20 μM) of TQ. The TQ treated G2/M arrested Neuro-2a cells showed the accumulation of the p53 and p21 at both the mRNA and protein levels in a dose-dependent manner, as proven by performing quantitative real time PCR and western blotting respectively. These results clearly showed that TQ induced cell-cycle arrest at G2/M phase in Neuro-2a cells.

Discussion

Numerous cancer research studies have been conducted using traditional medicinal plants in an effort to discover new therapeutic agents that lack toxic side effects associated with current chemotherapeutic agents. Recently various natural compounds have been shown

to be promising chemotherapeutic agents with lesser cytotoxicity to the normal cells (Vuorelaa et al., 2004). Thymoquinone (TQ) is the main active constituent found in the crude extracts of the seeds of *Nigella sativa* and studies have shown that TQ possesses anti-neoplastic properties (Shoieb et al., 2003). TQ has been demonstrated as a cytotoxic agent in several multi-drug resistant human tumour cell lines with minimal toxicity to normal cells (Worthen et al., 1998). Other studies have also shown lack of TQ toxicity to normal cells as well as its selective growth inhibitory and apoptotic effects in cancer cells (Gali-Muhtasib et al., 2004; 2004). It has further been demonstrated to inhibit B(a)P-induced clastogenicity in mice (Badary et al., 2007). Although TQ has been shown to suppress the growth of many different types of tumor cells (Roepke et al., 2007; El-Mahdy et al., 2005; Gali-Muhtasib et al., 2004; Kaseb et al., 2007; Ivankovic et al., 2006) the inhibitory effect of this compound in neuroblastoma is not well documented. We have recently shown that TQ inhibits angiogenesis *in vivo* using zebrafish embryos (Paramasivam et al., 2012a), we have also found that TQ has a potent cytotoxic effect and induces apoptosis through caspase-3 activation with down-regulation of XIAP in Neuro-2a cells (Paramasivam et al., 2012b). In this study, reduction in cell viability was attributed to the induction of apoptosis by TQ in Neuro-2a cells as evidenced by higher percentage cells in sub-G1 phase of the cell-cycle in TQ treated cells in a dose dependent manner.

Cell-cycle abnormality is one of the specific characteristics of malignant tumors, which results in continuous proliferation of tumor cells. Currently, cancer treatment focuses on how to intervene the cell-cycle of tumor cells, in order to slow down cell proliferation or induce apoptosis. Studies have revealed that many anti-tumor drugs can specifically inhibit cell-cycle (Singh et al., 2002). Previous studies have documented that TQ induces cell-cycle arrest at the G0/G1 and G2/M phase in different cancer cells (Gali-Muhtasib et al., 2004; Gurung et al., 2010). Recent studies have also shown that TQ induces cell-cycle arrest at the G0/G1 and G2/M phase (Alhosin et al., 2010; El-Shaimaa et al., 2011). Our results indicated that TQ significantly inhibited cell proliferation of Neuro-2a cells and induced G2/M cell arrest in a dose-dependent manner. We propose that TQ may prolong the cell-cycle by inducing G2/M arrest to inhibit proliferation of Neuro-2a cells.

It is well established that p21 consists of at least two functional domains that bind to proliferating cell nuclear antigen (PCNA) and Cdk/cyclins (Xiong et al., 1992; Zhang et al., 1993; Goubin and Ducommun, 1995). The role of PCNA in cell-cycle regulation is suggested by the fact that polymerase δ is regulated by cell-cycle proteins (Prosperi, 1997). In fact, PCNA was shown to interact with various Cdk-cyclin complexes (Xiong et al., 1992; Hindges and Hubscher, 1997; Szepesi et al., 1994). Thus, PCNA may act as a platform for multiple protein-protein interactions involved in replication, repair, recombination and cell-cycle regulation. Interestingly, the PCNA protein levels increased steadily through the entire cell-cycle period and remained high at G2/M (Zhang et al., 1994). Thus, PCNA may also be involved in G2 cell-

cycle control.

p53, the first tumor suppressor gene linked to apoptosis functions as a checkpoint protein involved in cell-cycle arrest, DNA repair and apoptosis. As a sensor of cellular stress, p53 is activated by a variety of stimuli such as anticancer drugs or irradiation, leading to cell-cycle arrest and/or apoptosis. Mutations of p53 occur in the majority of human cancers and are often associated with advanced disease and poor prognosis (Vogelstein et al., 2000). In contrast, mutations of p53 are rare in primary neuroblastoma (Maris and Matthay, 1999). p53 is able to block cell-cycle progression by inducing p21. p21 is the downstream gene of p53, which belongs to the cyclin-dependent kinase inhibitor (CDKI) family. It can inhibit CDK1, CDK2, CDK4 and CDK6 to induce cell-cycle arrest. After binding to PCNA, p21WAF1 inhibits PCNA dependent DNA synthesis to suppress cell proliferation (Gehen et al., 2007). Recent studies indicate that p21WAF1 participates in regulation at G2/M phase (Prasad et al., 2007; Kim et al., 2007). PCNA could interact with several CDK-cyclin complexes and form a tetramer with Cdc25C and CDK1/cyclin B1, which would promote cells to enter M phase from G2. When it is induced, p21WAF1 competes with Cdc25C to bind PCNA, so that CDK1/cyclin B1 could not be activated by Cdc25C, thus lead to G2/M cell-cycle arrest (Kontopidis et al., 2005; Ando et al., 2001). We found that after Neuro-2a cells were treated with TQ, the expression of p53 and p21 was up-regulated, while that of PCNA was down-regulated, which may be associated with TQ induced G2/M arrest in Neuro-2a cells.

Cyclin B1 is a key regulatory factor promoting cells to enter M phase from G2 phase. When cells enter G2 phase, the content of cyclin B1 is dramatically increased. During mitotic metaphase, the expression and activity of cyclin B1 reach the peak and are decreased quickly in the anaphase (Kakino et al., 1996). The expression of cyclin B1 directly influences the activity of CDK1. Combination of cyclin B1 and CDK1 is essential to initiate and facilitate cells to go into M phase (Obaya and Sedivy, 2002). Cyclin B1 is found to be closely associated with G2/M cell-cycle arrest as down-regulated expression of cyclin B1 would induce G2/M cell-cycle arrest (Brown et al., 2008). In the present study, TQ treated Neuro-2a cells showed that G2/M cell-cycle arrest was accompanied by a significant decrease in cyclin B1 and Cdc2 protein level in a dose dependent manner.

In this study, TQ suppressed cell survival and induced G2/M phase cell-cycle arrest of cultured Neuro-2a cells, which then up-regulated p53 and p21 and down-regulated PCNA, cyclin B1 and Cdc2 protein expression in a dose-dependent manner as evidenced by western blot analysis. Together, these results suggest that TQ can be used as a complement to conventional chemotherapeutic drugs for the purpose of enhancing the anti-tumor effect.

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