Thymoquinone is a novel potential inhibitor of SIRT1 in cancers with p53 mutation: Role in the reactivation of tumor suppressor p73

MAHMOUD ALHOSIN[1,2]

[1]Department of Biochemistry, and [2]Cancer Metabolism and Epigenetic Unit, Faculty of Sciences, King Abdulaziz University, Jeddah 21589, Kingdom of Saudi Arabia

Abstract. The deacetylase sirtuin 1 (SIRT1) has been shown to act as a negative regulator of the function of tumor suppressor p73 through a process involving the inhibition of the acetyltransferase p300 with the subsequent inhibition of apoptosis. In cancer cells with p53 mutation, such as the human acute lymphoblastic leukemia cell line (Jurkat) and the human triple-negative breast cancer (MDA-MB-468 cells), the upregulation of p73 in response to anticancer agents, including thymoquinone (TQ), leads to the activation of several pro-apoptotic genes with the subsequent induction of apoptosis. The present study investigated the effects of TQ on SIRT1 expression in order to elucidate the mechanisms of the TQ-induced upregulation of p73 in cancers with p53 mutation. TQ induced a dose and time-dependent decrease in SIRT1 expression in the Jurkat cells associated with the upregulation of p73, the cleavage of caspase-3, and the inhibition of cell proliferation and the induction of apoptosis. The TQ-induced downregulation of SIRT1 mRNA expression in Jurkat cells was associated with an increase in the mRNA expression of p300. In MDA-MB-468 cells, TQ induced an inhibition of cell proliferation and an upregulation of p300 and SIRT1 mRNA expression. Overall, the findings of the present study suggest that p73 is activated and stabilized in Jurkat cells in response to TQ via the deacetylation/acetylation-dependent pathway involving the downregulation of SIRT1 and the upregulation of p300, respectively. These findings further suggest that the inhibition of SIRT1 by TQ may be a promising strategy for the treatment of cancers with p53 mutation.

Correspondence to: Dr Mahmoud Alhosin, Department of Biochemistry, Faculty of Sciences, King Abdulaziz University, Abdullah Suleiman Street, Jeddah 21589, Kingdom of Saudi Arabia
E-mail: malhaseen@kau.edu.sa

Introduction

The deacetylase sirtuin 1 (SIRT1), a class III histone deacetylase is overexpressed in several types of cancer, including in tumors with p53 mutation, such as acute lymphoblastic leukemia (ALL) (1,2) and triple-negative breast cancer (TNBC) (3,4). The tumor suppressor gene p53 is mutated in the acute lymphoblastic leukemia cell line, Jurkat (5,6), as well as in the TNBC cell line, MDA-MB-468 cells) (7,8), rendering these cancer cell lines as useful model systems which may be used to study the anticancer effects of novel drugs on tumors with p53 mutation. A high expression level of SIRT1 has been found in primary ALL cells from patients compared to peripheral blood mononuclear cells from healthy subjects (1). Of note, Tenovin-6, a selective inhibitor of SIRT1, has been shown to reduce the growth of primary ALL cells, sensitize ALL cells to etoposide and cytarabine and to activate the tumor suppressor p53 (1). In the same context, it has been shown that patients with TNBC express high levels of SIRT1 and that this overexpression is significantly associated with lymph node metastasis (3). The depletion of SIRT1 in TNBC cell lines using siRNA has also been shown to markedly suppress invasiveness, indicating that SIRT1 plays an oncogenic role in the invasiveness of TNBC (3).

SIRT1 can deacetylate histone proteins (9,10), as well as a number of non-histone proteins, including p53 (11) and p73 (12), which has a high degree of similarity with p53 (13,14). A number of human cancers express low levels of p73. p73 reactivation and stability in response to pharmacological tools enables cancer cells to undergo apoptosis via p53-independent pathways, which renders p73 a potent target for the anticancer therapy of tumors with p53 mutation, including ALL (15-17) and TNBC (e.g., MDA-MB-468 cells) (18,19). SIRT1 has been shown to interact with p73 and to suppress p73-dependent transcriptional activity, enabling cancer cells to escape apoptosis in response to chemotherapy (12). It has been shown that SIRT1 physically interacts with and suppresses the transactivation of the acetyltransferase p300 (20), known to acetylate p73 in cancer cells (21,22). Of note, p300 has been shown to acetylate and activate p73 in response to several anticancer drugs, such as doxorubicin and cisplatin (23). Thus, targeting SIRT1 in tumors with p53 mutation, including ALL and

TNBC (e.g., MDA-MB-468 cells) may be a promising tool for inducing apoptosis and decreasing tumor resistance to chemotherapy via the reactivation of p73 through an acetylation process involving p300.

Thymoquinone (TQ), the bioactive compound of the volatile oil derived from the seeds of the *Nigella sativa* plant, has *in vitro* and *in vivo* potent pro-apoptotic activities against various cancer cells (24-29). Compared to cancer cells, TQ exerts mild cytotoxic effects on matched normal cells and tissues, such as normal keratinocytes and mouse fibroblasts (25,30,31). TQ has been shown to inhibit the proliferation and induce the apoptosis of the p53-mutant cell line, Jurkat, through p73 upregulation; however, the TQ-induced signaling pathways leading to p73 overexpression in ALL remain largely unknown (24,26). Consequently, the aim of the present study was to evaluate whether TQ can inhibit SIRT1 expression in cancer cells with p53 mutation, such as the human ALL cell line, Jurkat, and the human TNBC cell line, MDA-MB-468 cells, leading to the reactivation and stability of p73 with subsequent apoptosis.

Materials and methods

Cell culture and treatment. Human T lymphocyte cell line (Jurkat cells) and the human TNBC cell line (MDA-MB-468 cells) were obtained from the America Type Culture Collection (ATCC). The Jurkat cells were maintained in RPMI-1640 (Sigma-Aldrich; Merck KGaA) medium and MDA-MB-468 cells in Dulbecco's modified Eagle medium (DMEM; UFC-Biotech) supplemented with 15% (v/v) fetal calf serum (FCS, Lonza BioWhittaker), 2 mM glutamine, penicillin (100 IU/ml) and streptomycin (100 μg/ml) (Sigma-Aldrich; Merck KGaA). Both cell lines were maintained in a humidified incubator containing 5% CO_2 at 37˚C. For all treatments, a 10 mM solution of TQ (Sigma-Aldrich; Merck KGaA) was prepared in 10% dimethyl sulfoxide (DMSO; Merck Millipore) and appropriate working concentrations were prepared with cell culture medium. The final concentration of DMSO was always <0.1% in both the control and treatment conditions.

Cell proliferation assay. A colorimetric cell proliferation assay using the WST-1 Cell Proliferation Reagent kit (Sigma-Aldrich; Merck KGaA) was used to examine the effects of TQ on the proliferation of Jurkat cells and MDA-MB-468 cells. Briefly, the cells were seeded in 96-multi-well plates at a density of $4x10^4$ cells/well for the Jurkat cells and 10^4 cells/well for the MDA-MB-468 cells. Following 24 h of incubation at 37˚C, the cells were exposed to various concentrations of TQ for 24 h. The cell proliferation rate then was evaluated through a rapid WST-1 reagent. Following 24 h of incubation, 10 μl of the WST-1 solution were added followed by incubation for an additional 3 h at 37˚C. Finally, the absorbance was read at 450 nm using a microplate ELISA reader (ELx800™; BioTek Instruments, Inc.) and Gen5 software (BioTek Instruments, Inc.) was used to analyze the results. The reaction based on the cleavage of the tetrazolium salt WST-1 to formazan by cellular mitochondrial dehydrogenases. The quantity of formazan dye in the medium is directly proportional to the number of viable metabolically active cells. The percentage of cell viability was calculated by assuming control (untreated) samples as 100% viable.

Apoptosis assay. To examine apoptosis, the Jurkat cells were seeded in 96-well plates at a density of $4x10^4$ cells/well, grown for 24 h and exposed to various concentrations of TQ for 24 h or to 50 μM for 15 min, 30 min, 1 and 3 h. The cell apoptosis rate was evaluated using the Annexin V Binding Guava Nexin® assay by capillary cytometry (Guava Easycyte Plus HP system, with absolute cell count and 6 parameters) according to the manufacturer's recommendations (Guava Technologies Inc.). Guava Nexin® Assay utilizes Annexin V-PE.

Western blot analysis. The Jurkat cells were treated with various concentrations of TQ for 24 h or to 50 μM for 15 min, 30 min, 1 and 3 h. The cells were then harvested, centrifuged at 201 x g for 10 min at room temperature to discard the RPMI medium, washed with cold phosphate-buffered saline (PBS) and resuspended in RIPA buffer (25 mM Tris, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate and 0.1% SDS; Sigma-Aldrich; Merck KGaA) containing protease inhibitors. Equal amounts (20 μg) of total protein were separated on 10-15% polyacrylamide gels and electrophoretically transferred to a nitrocellulose membrane. After blocking with 5% non-fat dry milk and Tween-20 in PBS, the nitrocellulose membranes were incubated, at 4˚C overnight, with either mouse monoclonal anti-SIRT1 antibody (B-10: cat. no. sc-74504; Santa Cruz Biotechnology, Inc.; diluted at 1:200), mouse monoclonal anti-p73 antibody (cat. no. 558785; BD Biosciences; diluted at 1 μg/ml), rabbit polyclonal anti-cleaved caspase-3 (Asp175) antibody (cat. no. 9661; Cell Signaling Technology, Inc.; diluted at 1:1,000) or mouse monoclonal anti-β-actin antibody (cat. no. ab8227; Abcam; diluted at 1:25,000), according to the manufacturer's instructions. The membranes were then washed 3 times with PBS for 10 min. The membranes were, thereafter, incubated with the appropriate horseradish peroxidase-conjugated secondary antibody [Cell Signaling Technology, Inc.; diluted to 1:10,000 for anti-mouse antibody cat. no. 7076 and 2:10,000 for anti-rabbit antibody cat. no. 7074 at room temperature for 1 h and 30 min. The membranes were then washed with PBS 5 times. Signals were detected by chemiluminescence using the ECL Plus detection system (Amersham; GE Healthcare Life Sciences). For the quantification of SIRT1, p73 and cleaved caspase-3 proteins, images of the western blots were processed using NIH ImageJ software (Java 8).

Reverse transcription-quantitative PCR (RT-qPCR). Cells were treated with various concentrations of TQ for 24 h. Total RNA was purified and subjected to reverse transcription using Oligo(dt) (Sigma-Aldrich; Merck KGaA) and Superscript II reverse transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.). Quantitative PCR was performed using the LightCycler 480 SYBR-Green I Master kit (Roche Diagnostics) and the Mastercycler Realplex apparatus (Eppendorf). The results were normalized to ribosomal protein L11 (RPL11) mRNA. PCR was performed with 30 cycles of denaturation for 30 sec at 95˚C; annealing for 45 sec at 60˚C; and extension for 60 sec at 72˚C. The sequences of the primers for PCR amplification are listed in Table I. Amplicons were size-controlled on

Table I. Sequences of the primers used for PCR amplification.

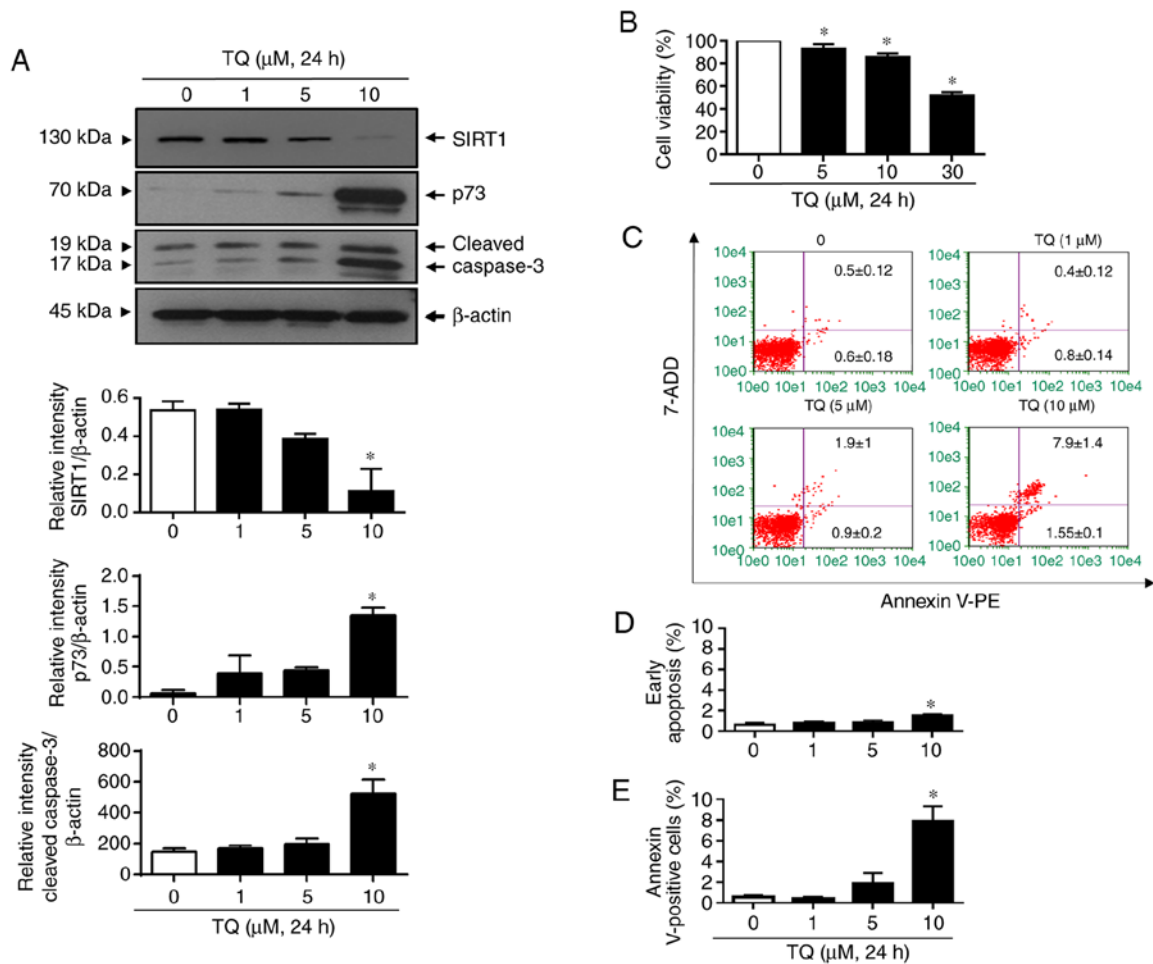| Target gene	Sense sequence (5'-3')	Antisense sequence (3'-5')
SIRT1	CCGCTTGCTATCATGAAACCA	TCACAGTCTCCAAGAAGCTCT
p300	TCCTGGACAGCAGATTGGAG	CTTGCGCTTCTCTGGATCAG
RPL11	ATCCTTTGGCATCCGGAGAA	GTCCAGGCCGTAGATACCAA

SIRT1, sirtuin 1; RPL11, ribosomal protein L11.

Figure 1. Dose-dependent effect of TQ on the protein expression of SIRT1, p73 and cleaved caspase-3, and on the proliferation and apoptosis of Jurkat cells. Cells were exposed to increasing concentrations of TQ for 24 h. (A) Representative blots of SIRT1, p73 and cleaved caspase-3 proteins, as assessed by western blot analysis (top panel), and corresponding cumulative data (bottom panels). (B) Cell proliferation rate was assessed by WST-1 assay in Jurkat cells. (C) Apoptosis was assessed by flow cytometry using the Annexin V/7AAD staining apoptosis assay and events in each of the 4 quadrants. The numbers of apoptotic cells in the (D) early stage and (E) those in late apoptosis and dead cells are expressed as a percentage relative to the total cell number. Values are shown as the means ± SEM of 3 different experiments. *$P<0.05$ vs. control. TQ, thymoquinone; SIRT1, sirtuin 1.

an agarose gel and the purity was assessed by analysis of the melting curves at the end of the RT-PCR reaction. The expression level of the target gene in the treated cells was measured relative to the level observed in the untreated cells and was quantified using the formula: $2^{-\Delta\Delta Cq}$ (32).

Statistical analysis. All data are presented as the means ± SEM of triplicates performed for the same experiment or an average of at least 3 separate experiments. Statistical analysis was performed using one-way ANOVA followed by Tukey's

post hoc test using GraphPad Prism 6 software (GraphPad Software) and significant differences were indicated as with values of $P<0.05$.

Results

TQ induces a concentration-dependent degradation of SIRT1 associated with p73 upregulation in Jurkat cells. Firstly, the effects of TQ on SIRT1 and p73 protein expression levels in Jurkat cells were evaluated (Fig. 1A). For this
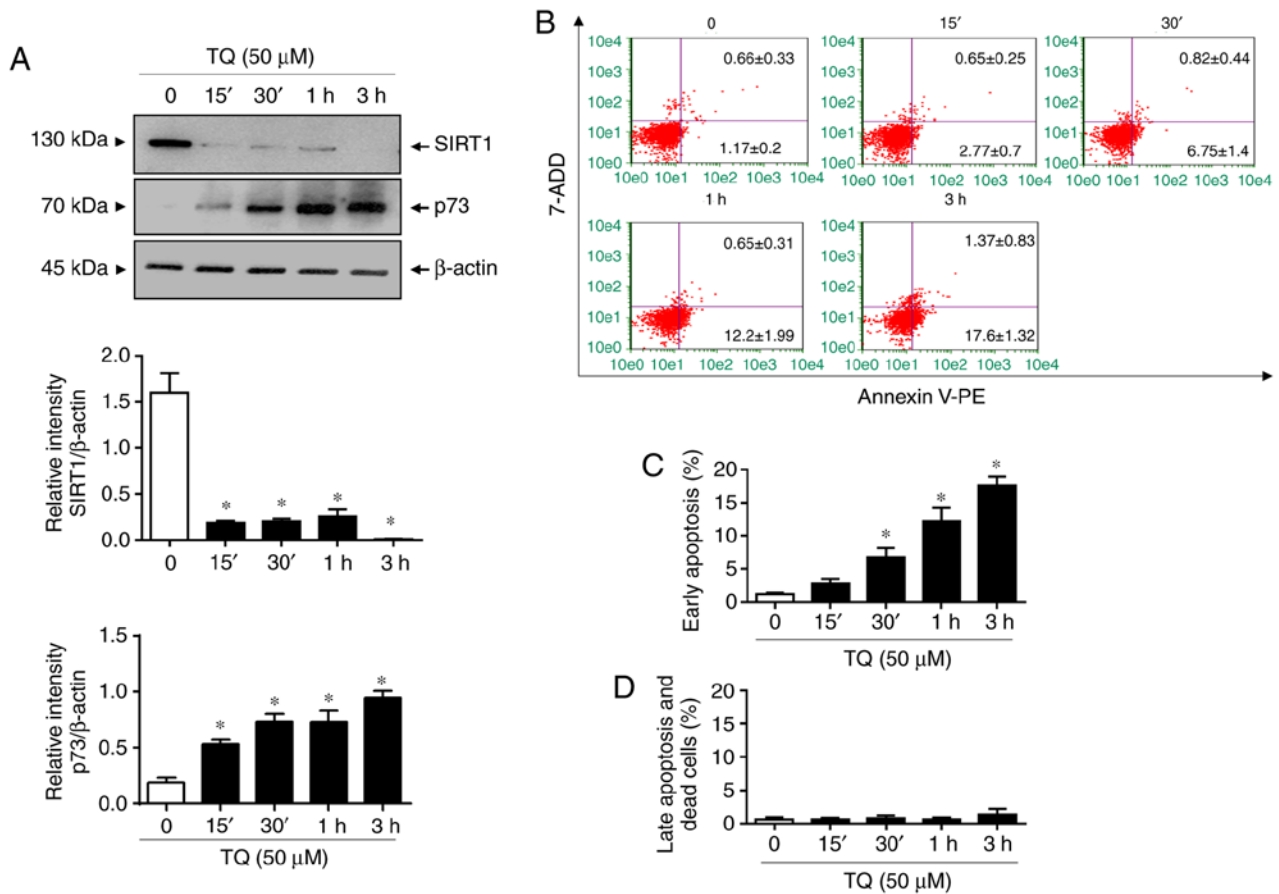
Figure 2. Time-course of the effects of TQ on the protein expression of SIRT1 and p73 and apoptosis in Jurkat cells. Cells were exposed to 50 μM of TQ for the indicated periods of time. (A) Representative blots of SIRT1 and p73 proteins as assessed by western blot analysis (top panel), and corresponding cumulative data (bottom panels). (B) Apoptosis was assessed by flow cytometry using the Annexin V/7AAD staining apoptosis assay and events in each of the 4 quadrants. The numbers of apoptotic cells in the (C) early stage and (D) those in late apoptosis and dead cells are expressed as a percentage relative to the total cell number. Values are shown as the mean ± SEM of 3 different experiments. *P<0.05 vs. control. TQ, thymoquinone; SIRT1, sirtuin 1.

purpose, the cells were incubated with increasing concentrations of TQ for 24 h. The results revealed that TQ induced the downregulation of SIRT1 in a concentration-dependent manner in the Jurkat cells (Fig. 1A). Indeed, treatment with TQ at 5 μM induced a slight decrease in the SIRT1 level and a significant decrease was detected at 10 μM. The TQ-induced SIRT1 degradation was associated with the upregulation of p73 and cleaved caspase-3. Indeed, TQ induced a significant increase in the expression levels of p73 and cleaved caspase 3 at the concentration of 10 μM (Fig. 1A). The anti-proliferative and pro-apoptotic effects of TQ on the Jurkat cells were then analyzed under the same experimental conditions. Cell proliferation in response to TQ was decreased in a concentration-dependent manner (Fig. 1B). TQ significantly inhibited cell proliferation from the concentration of 5 μM and the inhibition rate reached approximately 10 and 50% in the Jurkat cells treated with 10 and 30 μM TQ, respectively (Fig. 1B). Under the same conditions, TQ was found to induce apoptosis in a dose-dependent manner, which became significant at the concentration of 10 μM (Fig. 1C-E). Indeed, at the concentration of 10 μM, approximately 2% of Jurkat cells were in the early apoptotic stage (Fig. 1C and D) and 8% were in the late apoptotic stage (Fig. 1C and E). Taken together, these results indicated that TQ induced the downregulation of SIRT1 in Jurkat cells,

which could lead to an upregulation of p73 and cleaved caspase-3, with subsequent cell proliferation inhibition and apoptosis induction.

TQ-induced p73 upregulation and apoptosis are associated with the rapid downregulation of SIRT1. In the next step, a kinetic analysis of TQ on SIRT1 expression in Jurkat cells was performed in order to determine the chronology of the molecular events induced by TQ leading to the upregulation of p73 and apoptosis. For this objective, cells were exposed to 50 μM of TQ, the concentration at which SIRT1 protein expression was undetectable after 24 h (data not shown). The time-course effects of TQ on SIRT1 expression in Jurkat cells at 50 μM revealed that SIRT1 expression began to significantly decrease after 15 min and the loss was almost complete after 3 h of treatment (Fig. 2A). A significant decrease in SIRT1 expression observed after 15 min of TQ treatment and was associated with a significant increase in the expression of p73 (Fig. 2A). Notably, TQ induced the apoptosis of Jurkat cells in a time-dependent manner (Fig. 2B-D) following the TQ-induced deregulation of the SIRT1 and p73 expression levels (Fig. 2A). Indeed, TQ caused a significant increase in the number of apoptotic cells in the early stage after 30 min (Fig. 2B and C). The percentage of apoptotic cells in the early stage reached approximately 18% after 3 h (Fig. 2B and C).
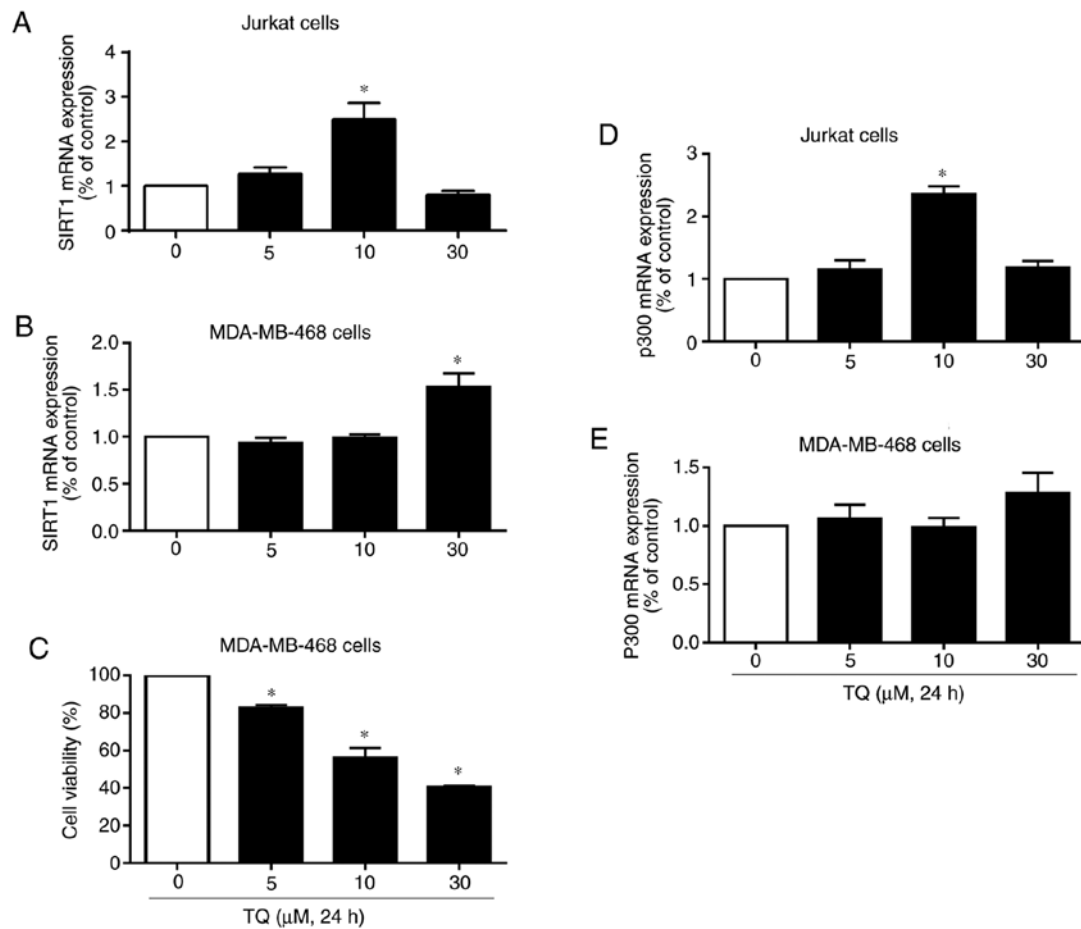
Figure 3. Effects of TQ on the mRNA expression levels of SIRT1 and p300, and the proliferation of Jurkat cells and MDA-MB-468 cells. Cells were treated with increasing concentrations of TQ for 24 h. Histograms show the quantification data of mRNA expression levels of SIRT1 in (A) Jurkat cells and (B) MDA-MB-468 cells. (C) Cell proliferation rate of MDA-MB-468 cells, as assessed by WST-1 assay. Histograms show the quantification data of the mRNA expression levels of p300 in (D) Jurkat cells and (E) MDA-MB-468 cells, as assessed by RT-qPCR. Results are the means of 3 separate experiments performed in triplicate. Values are shown as the means ± SEM of 3 different experiments. *P<0.05 vs. control. TQ, thymoquinone; SIRT1, sirtuin 1.

These findings suggest that the TQ-induced downregulation of SIRT1 expression is a main event in the reactivation and stability of the tumor suppressor, p73, with the subsequent induction of apoptosis.

TQ induces the transcription-dependent downregulation of SIRT1 in Jurkat cells with p53 mutation. To investigate whether TQ affects SIRT1 expression also at the transcriptional level, Jurkat cells were exposed to TQ under the same conditions. As shown in Fig. 3A, the exposure of Jurkat cells to 10 μM of TQ significantly increased SIRT1 mRNA expression. At the concentration of 30 μM, TQ induced a decrease in SIRT1 mRNA expression (Fig. 3A), indicating that the TQ-induced downregulation of SIRT1 expression in Jurkat cells results from its effects at both the transcriptional and protein level. The mRNA expression levels of SIRT1 were also investigated in the human breast cancer cell line, MDA-MB-468 (Fig. 3B). TQ had no effect on SIRT1 mRNA expression at the concentrations of 5 and 10 μM, whereas its expression significantly increased following treatment of the cells with 30 μM TQ (Fig. 3B). The effects of TQ on the proliferation of MDA-MB-468 cells were also investigated. TQ significantly inhibited cell proliferation from the concentration of 5 μM and the inhibition rate reached approximately 45 and 60%

in the MDA-MB-468 cells treated with 10 and 30 μM TQ, respectively (Fig. 3C). Taken together, these findings indicate that the TQ-induced downregulation of SIRT1 expression in Jurkat cells results from its effects on both the transcriptional and protein levels, and that TQ inhibits the proliferation of MDA-MB-468 cells via a mechanism independent of SIRT1 mRNA expression.

TQ induces the transcriptional upregulation of the histone acetyltransferase p300 in cancer cells with p53 mutation. SIRT1 has been shown to physically interact with and suppress the transactivation of the acetyltransferase, p300 (20). Of note, p300 has been shown to acetylate and activate p73 in response to treatment with several anticancer drugs, such as doxorubicin and cisplatin (23). In the present study, in order to investigate the mechanisms underlying p73 upregulation following the decrease in the expression of the deacetylase SIRT1 in response to TQ treatment, the effects of TQ on the expression of p300 were evaluated in Jurkat and MDA-MB-468 cells. As shown in Fig. 3D, treatment of the Jurkat cells with 10 μM TQ significantly increased the transcriptional levels of p300, as detected by RT-qPCR (Fig. 3D). Indeed, at the concentration of 10 μM, an approximately 2-fold increase in p300 expression was observed (Fig. 3D), in parallel with a significant decrease

in SIRT1 protein expression and a significant increase in p73 protein expression, as shown in Fig. 1A. At 30 μM, only a slight increase in the level of p300 was found compared to the control (Fig. 3D). In the MDA-MB-468 cells, TQ had no significant effect on p300 mRNA expression at the concentrations of 5 and 10 μM, while p300 expression levels began to increase at the concentration of 30 μM TQ (Fig. 3E). These results indicate that p73 is activated and stabilized in response to TQ in cancer cells with p53 mutation through the deacetylation/acetylation-dependent pathway involving the downregulation of SIRT1 protein and the upregulation of p300, respectively.

Discussion

The deacetylase SIRT1 has been shown to act as a negative regulator of the function of the tumor suppressors, p53 (33) and p73 (12), leading to the inhibition of apoptosis. SIRT1 has been found to directly bind to p73, reducing its transcriptional activity via a deacetylation process with the subsequent inhibition of apoptosis (12). p53 and p73 proteins have a high degree of similarity in both structure and function (13,14). Of note, in tumors with p53 mutation, including ALL (1,2,24) and TNBC (3,4), the upregulation of p73 in response to anticancer agents leads to the activation of several pro-apoptotic genes with the subsequent induction of apoptosis. Thus, it is of interest to identify novel natural compounds that can target SIRT1/p73 interaction, highlighting new strategies for cancer therapy. The present study demonstrated that treatment of Jurkat cells with TQ induced a decrease in SIRT1 protein expression in a concentration- and time-dependent manner, and that this effect was associated with an increase in p73 protein expression. The TQ-induced downregulation of SIRT1 expression was associated with an increase in cleaved caspase-3 expression and apoptosis. TQ also induced an increase in the expression of the acetyltransferase, p300, in Jurkat cells and the human breast cancer cell line, MDA-MB-468.

SIRT1 can act as either an oncogene or a tumor suppressor, depending on its cellular targets or specific cancers (34-36). Considering the fact that SIRT1 is overexpressed in several tumors with p53 mutation, including ALL (1) and TNBC (3,4,37), SIRT1 inhibition holds promise as a novel approach for cancer therapy in these tumors. The present study demonstrated that SIRT1 protein expression was downregulated in Jurkat cells treated with TQ in parallel with an increase in p73 protein expression, indicating that the low expression levels of p73 found in Jurkat cells may be a result of its degradation through the SIRT1-mediated deacetylation process; this suggests that TQ may be a novel potential inhibitor of SIRT1 in cancers with p53 mutation. This conclusion is supported by ample evidence. A recent study indicated that the pre-treatment of cancer cells with nicotinamide, an inhibitor of SIRT1, was able to increase the expression of p73 and that of pro-apoptotic, Bax (38). In the same context, it has been shown that the anti-leukemic drug, arsenic trioxide, induced an upregulation of p73 expression, leading to the apoptosis of acute promyelocytic leukemia cells via the inhibition of several oncogenes, including SIRT1 (39). The results of the present study are also in line with previous results, highlighting SIRT1 as an inhibitor of p73 activity through the deacetylation-mediated process (12). Indeed, the knockdown of SIRT1 in HeLa cells

using SIRT1 antisense was previously shown to result in the upregulation of p73 protein and the induction of apoptosis, while the overexpression of SIRT1 counteracted p73-induced apoptosis, indicating that SIRT1 negatively regulated the expression of p73 and apoptosis (12). This indicates that the knockdown of SIRT1 mimics the effects of TQ on the expression of p73 and apoptosis observed in the present study. The present study also demonstrated that TQ increased the expression of the acetyltransferase, p300, in Jurkat cells and MDA-MB-468 cells, suggesting that the upregulation of p73 in response to TQ involves its acetylation by p300. Of note, the significant increase in the levels of p300 mRNA in Jurkat cells detected following treatment with 10 μM TQ was inversely associated with SIRT1 protein expression and positively with p73 protein expression under the same conditions, indicating that p73 is activated and stabilized in response to TQ in Jurkat cells through the deacetylation/acetylation-dependent pathway involving the downregulation of SIRT1 and the upregulation of p300, respectively. This hypothesis is supported by the findings of several previous studies. Indeed, SIRT1 has been shown to physically interact with and suppress p300 transactivation (20). Moreover, it has been shown that the anticancer drug, doxorubicin, increases the expression of p300, leading to the acetylation of p73 in the human colon cancer cell line, HCT116 (23). This indicates that the activation and stability of the tumor suppressor, p73, through the deacetylation/acetylation-dependent pathway, is the main target in tumors with p53 mutation for natural compounds exhibiting anticancer activities, including TQ.

In conclusion, the present study demonstrates that TQ induces the downregulation of the deacetylase SIRT1, with a coordinated upregulation of the tumor suppressor, p73, most likely through the acetylation-mediated process. p300 may be the most likely acetyltransferase associated with the TQ-induced p73 upregulation with the subsequent induction of apoptosis. However, TQ-induced SIRT1/p300/p73 deregulation warrants further investigation in order to decipher the chronology of the molecular events involved, namely SIRT1 downregulation, which triggers the upregulation of p300, leading to the stability and the reactivation of p73 followed by apoptosis. The findings of the present study provide new insight into the regulation of SIRT1/P73 expression upon treatment with natural anticancer drugs, as it suggests that the inhibition of SIRT1 by TQ may be a promising tool for cancer therapy in cancers with p53 mutation.

Availability of data and materials

All data generated or analyzed during this study are included in this published article or are available from the corresponding author on reasonable request.

Author contributions

MA designed the study, performed the research and analyzed the data, and wrote the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The author declares that there are no competing interests.

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