

# Anti-neoplastic agent thymoquinone induces degradation of $\alpha$ and $\beta$ tubulin proteins in human cancer cells without affecting their level in normal human fibroblasts

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**Summary** The microtubule-targeting agents derived from natural products, such as vinca-*alkaloids* and taxanes are an important family of efficient anti-cancer drugs with therapeutic benefits in both haematological and solid tumors. These drugs interfere with the assembly of microtubules of  $\alpha/\beta$  tubulin heterodimers without altering their expression level. The aim of the present study was to investigate the effect of thymoquinone (TQ), a natural product present in black cumin seed oil known to exhibit putative anti-cancer activities, on  $\alpha/\beta$  tubulin expression in human astrocytoma cells (cell line U87, solid tumor model) and in Jurkat cells (T lymphoblastic leukaemia cells). TQ induced a concentration- and time-dependent degradation of  $\alpha/\beta$  tubulin in both cancer cell types. This degradation was associated with the up-regulation of the tumor suppressor p73 with subsequent induction of apoptosis. Interestingly, TQ had no effect on  $\alpha/\beta$  tubulin protein expression in normal human fibroblast cells, which were used as a non-cancerous cell model. These data indicate that TQ exerts a

selective effect towards  $\alpha/\beta$  tubulin in cancer cells. In conclusion, the present findings indicate that TQ is a novel anti-microtubule drug which targets the level of  $\alpha/\beta$  tubulin proteins in cancer cells. Furthermore, they highlight the interest of developing anti-cancer therapies that target directly tubulin rather than microtubules dynamics.

**Keywords** Microtubule · Thymoquinone · Apoptosis · Tumor suppressor gene p73 · Natural product

## Introduction

Microtubules, the major cytoskeleton components in cells, are composed of  $\alpha/\beta$  tubulin subunits. This structure is considered as one of the best targets in cancer chemotherapy [1]. Several natural compounds can target and inhibit successfully the normal function of microtubules [2]. The vinca-*alkaloids* and the taxanes are among the most important anti-microtubule drugs [2]. Indeed, microtubule-targeting drugs (MTDs) interfere with the assembly of microtubules of  $\alpha/\beta$  tubulin heterodimers without altering their expression level [3]. Although MTDs have clinical therapeutic benefits against haematological and solid tumors, the development of resistance to these drugs, their specificity towards tumors, and the induction of neutropenia, myeloid and neurological toxicities are the major problems observed during their clinical applications [4–10]. Deficiency of MTDs-induced apoptotic pathways in different cancers has been suggested as a mechanism of resistance to these drugs [11]. In this context, it has been suggested that p53 might be a guardian of microtubules composition and that mutations in p53 are likely to contribute to the development of resistance to MTDs [11]. Thus, increasing the tissue

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specificity, reducing the side effects, especially the peripheral neuropathy and leukopenia, are major objectives in the current research for new MTDs.

Many studies have shown that thymoquinone (TQ), a natural product present in black cumin seed oil with anti-inflammatory and anti-cancer activities, is a potent cytotoxic agent against many types of human cancer cells [12, 13]. It has been reported that TQ exerts its anti-cancer effects through the inhibition of cell growth, cell cycle arrest with subsequent apoptosis [14–17]. TQ can induce apoptosis in cancer cells by p53-dependent and -independent pathways [12, 16]. The TQ-induced apoptosis in p53-null myeloblastic leukemia and human osteosarcoma cells involves an increase in the Bax/Bcl2 ratio and the activation of caspases [18, 19]. Recently, we have shown that TQ induces apoptosis in acute lymphoblastic leukemia cell line (Jurkat cells), a p53-deficient cell line, through activation of the tumor suppressor p73, the structural and functional homolog of p53, which targets the anti-apoptotic protein UHRF1 (Ubiquitin-like PHD Ring Finger 1) [12]. Unlike p53, which is mutated in 50% of cancer cells, the mutations of the p73 gene are very rare (less than 1%), making it as an alternative strategy for anti-cancer treatment, especially for those where p53 is deficient or mutated [20, 21]. Indeed, several studies have proposed that p73 is a novel promising target for cancer therapy [22–26].

The present study has indicated that TQ induces the degradation of  $\alpha/\beta$  tubulin in human astrocytoma cells (cell line U87) and in the p53-deficient Jurkat cell line. This degradation is associated with p73 up-regulation in both cancer cell lines. Interestingly, TQ had no effect on  $\alpha/\beta$  tubulin protein level in normal human fibroblast cells. Altogether, the present findings suggest that TQ is a new MTD exhibiting specificity towards cancer cells.

## Material and methods

### Cell culture and treatment

The human astrocytoma cells (line U87, WHO grade IV) and human T lymphocyte cell line Jurkat were obtained from the America Type Culture Collection (Mannassa, VA, USA). Cell lines were maintained in a humidified incubator with 5% CO<sub>2</sub> at 37°C, and were grown in EMEM (Biowhitaker, Lonza, Belgium) for human astrocytoma cells and RPMI 1640 (Sigma-Aldrich, St-Louis, MO) for Jurkat cells and human fibroblast cells. Both media were supplemented with 15% (v/v) fetal calf serum (FCS, Biowhitaker), 2 mM glutamine, 100 U/ml penicillin and 50 mg/ml streptomycin (Sigma St. Louis, MO). For all treatments, a 100 mM solution of TQ (Sigma-Aldrich, St) was prepared in 100% DMSO (Dimethylsulfoxide; Millipore S.A.S.,

Molsheim, France) and appropriate working concentrations were prepared with cell culture medium. The final concentration of DMSO was less than 0.1% in both control and treated conditions.

### Western blot analysis

Cells were treated with TQ at different concentrations and incubated for the indicated time. Cells were then harvested and centrifuged at 200g for 5 min at room temperature. The pellets were resuspended in RIPA buffer (25 mM Tris pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) containing different protease inhibitors (Sigma-Aldrich) and incubated on ice for 20 min. The cell suspensions were then sonicated three times (30 s each) and the supernatants containing the proteins were collected after centrifugation at 10,000g for 16 min at 4°C. The protein concentration was determined by the Bradford assay (Bio-Rad, Marnes la Coquette, France). Proteins were separated on 10% SDS-polyacrylamide gels and transferred to PVDF membranes (Millipore, Saint Quentin en Yvelines, France), as previously described [27]. Immunoblot was performed using either a mouse monoclonal anti- $\beta$  tubulin antibody (Sigma-Aldrich), a purified monoclonal anti- $\alpha$  tubulin (Clone 10D8) antibody (Biolegend, San Diego, CA), a mouse monoclonal anti-p73 antibody (BD Biosciences Pharmingen), a mouse monoclonal anti-UHRF1 antibody (Proteogenix, Oberhausbergen, France), or a mouse monoclonal anti- $\beta$  actin antibody (Abcam, Paris, France), according to the manufacturer's instructions.

### Apoptosis assay

For detection of apoptosis, the annexin V-binding capacity of treated cells was examined using the annexin V Binding Guava Nexin<sup>®</sup> Assay by capillary cytometry (Guava Easy-cyte Plus HP system, with absolute cell count and six parameters) following the manufacturer's recommendations (Guava Technologies Inc, Hayward, CA, USA). Guava Nexin<sup>®</sup> Assay utilizes annexin V-Phycoerythrin (PE) to detect phosphatidylserine (PS) on the external membrane of apoptotic cells. The cell impermeant dye, 7-Amino-Actinomycin (7AAD), is used in the Guava Nexin<sup>®</sup> Assay as an indicator of cell membrane structural integrity. 7-AAD is excluded from viable cells as well as early apoptotic cells. Jurkat cells were seeded into 6-multiwell plates at a density of  $2 \times 10^6$  cells/well, grown for 24 h and exposed to TQ at different concentrations for 24 h. Jurkat cells were stained directly in the 96-well microplate with 100  $\mu$ l of Guava Nexin Reagent, a pre-made cocktail containing annexin V-PE and 7-AAD in buffer, in 200  $\mu$ l final volume. After 20 min incubation at room temperature, the samples were analysed using Guava<sup>®</sup> System.

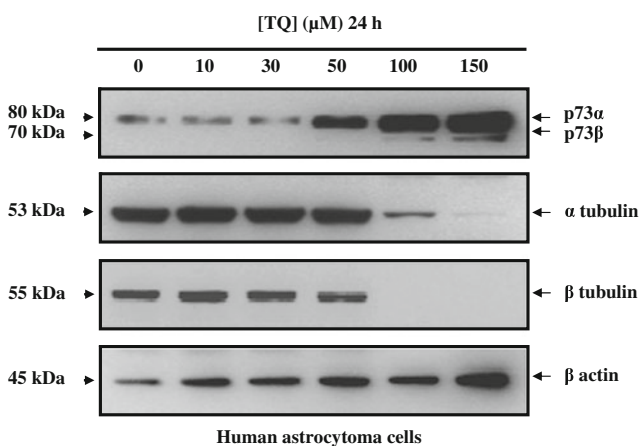
## Statistical analysis

Data are presented in a bar graph form, expressed as means  $\pm$ SEM from at least three independent experiments. Statistical analyses were performed using the one-way ANOVA. Significance levels were defined as \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  (versus the corresponding untreated group).

## Results

TQ induces a concentration-dependent degradation of  $\alpha/\beta$  tubulin associated with p73 up-regulation in human astrocytoma cells (line U87)

We first investigated the effect of TQ on  $\alpha/\beta$  tubulin and p73 proteins in human astrocytoma cells (line U87 MG, Fig. 1). For this purpose, human astrocytoma cells were exposed to different concentrations of TQ for 24 h. We observed that TQ induces a concentration-dependent  $\alpha/\beta$  tubulin down-regulation in U87 MG cells (Fig. 1). Indeed, TQ at 100  $\mu$ M caused a pronounced decrease in  $\alpha$  tubulin level, while  $\beta$  tubulin was undetectable (Fig. 1). TQ-induced  $\alpha/\beta$  tubulin degradation in astrocytoma cells was associated with a concentration-dependent p73 up-regulation which started at 50  $\mu$ M suggesting that  $\alpha/\beta$  tubulin degradation is a downstream effect of p73 up-regulation in the mechanism of action of TQ. These results show that TQ induced  $\alpha/\beta$  tubulin down-regulation associated with the up-regulation of p73 in human astrocytoma cells (line U87).



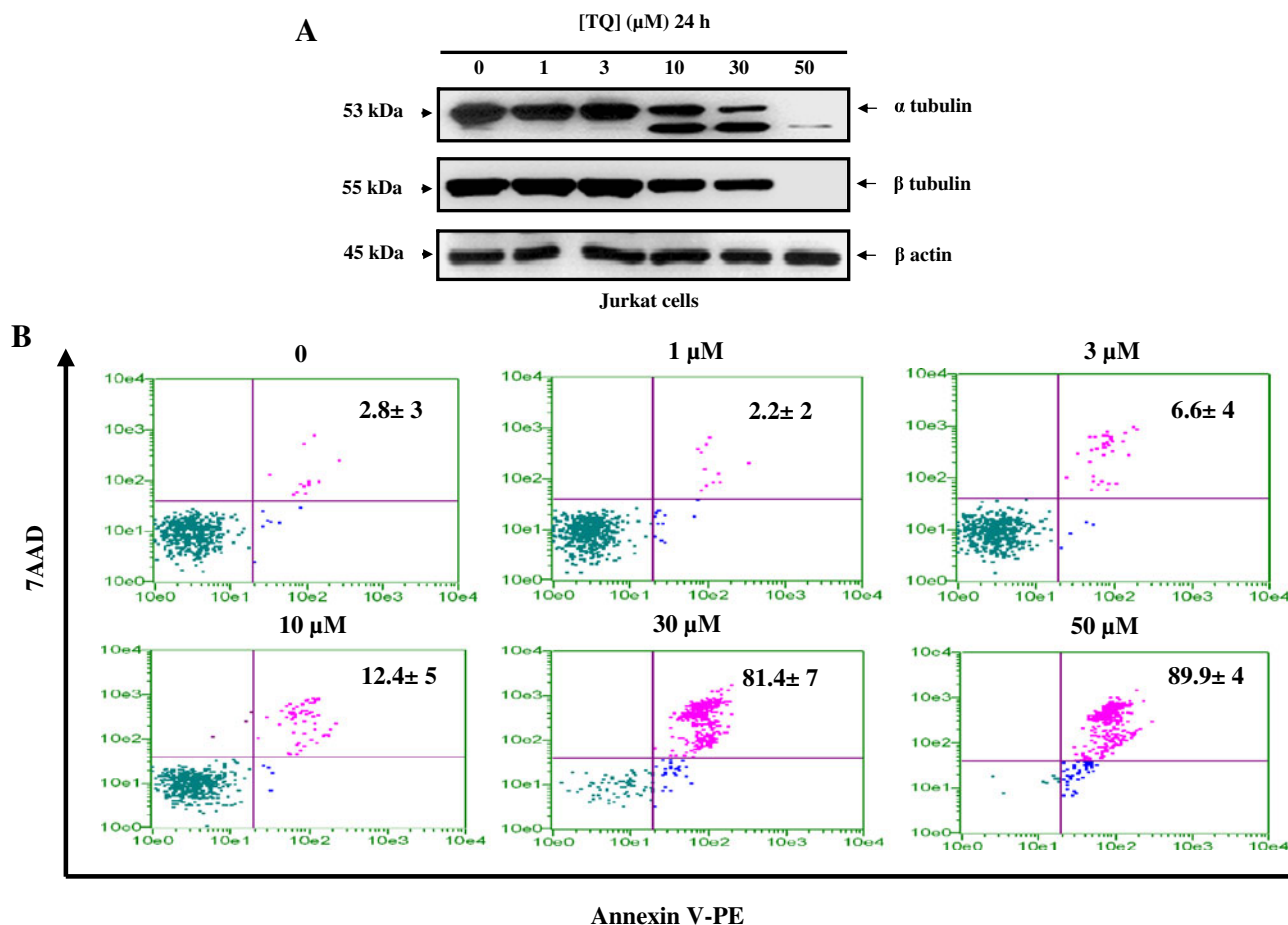
**Fig. 1** Effects of TQ on  $\alpha/\beta$  tubulin and p73 expression in human astrocytoma cells (cell line U87). Cells were exposed to TQ at the indicated concentration and incubated for 24 h. Immunoblot analyses were performed as described in “Material and methods” with different antibodies. Specific bands were detected with their expected apparent molecular weight. All membranes were reblotted with an anti  $\beta$  actin probe to ensure equal protein loading and transfer. The data are representative of at least three independent experiments

TQ induces a concentration- and time-dependent degradation of  $\alpha/\beta$  tubulin in acute lymphoblastic leukaemia cells (Jurkat cell line)

We have investigated the effect of TQ on the expression of  $\alpha/\beta$  tubulin in the p53-deficient Jurkat cell line. Figure 2a shows that TQ induced  $\beta$  tubulin down-regulation and the appearance of a lower molecular weight form of  $\alpha$  tubulin starting at 10  $\mu$ M. Interestingly, at 50  $\mu$ M of TQ,  $\alpha/\beta$  tubulin protein level became undetectable (Fig. 2a). The apoptotic effect of TQ in Jurkat cells was also studied under the same experimental conditions. Cells were exposed to different concentration of TQ for 24 h and then apoptosis was assessed as described in “Material and methods”. As shown in Fig. 2b, increasing concentrations of TQ induced an increasing number of apoptotic cells. TQ began to trigger apoptosis at 10  $\mu$ M, a concentration where  $\alpha/\beta$  tubulin proteins were down-regulated. TQ at 50  $\mu$ M, induced approximately apoptosis of 90% of cells (Fig. 2b), a response which was associated with a total loss of  $\alpha/\beta$  tubulin proteins (Fig. 2a). In our previous study we have shown that p73 is expressed at a low level in untreated Jurkat cells and that TQ induced a marked up-regulation of its expression starting at concentrations of or greater than 10  $\mu$ M [12]. Taken together, these results indicate that TQ like in human astrocytoma cells induced  $\alpha/\beta$  tubulin down-regulation in acute lymphoblastic leukaemia cells and that this effect is associated with activation of the tumor suppressor p73 leading subsequently to apoptosis.

TQ has no effect on  $\alpha/\beta$  tubulin protein expression in normal human fibroblast cells as compared to Jurkat cells

In order to determine the chronology of the molecular events induced by TQ leading to down-regulation of  $\alpha/\beta$  tubulin proteins, we analyzed the time-course effect of TQ in Jurkat cells at 50  $\mu$ M (concentration at which  $\alpha/\beta$  tubulin proteins were completely depleted, Fig. 2a). Figure 3a shows that TQ induced a rapid  $\alpha/\beta$  tubulin down-regulation in Jurkat cells at 3 h associated with a p73 up-regulation, this decrease in the  $\alpha/\beta$  tubulin level was more pronounced at 6 h. In the next step, we have investigated the effect of TQ on  $\alpha/\beta$  tubulin protein in normal human fibroblast cells as a non-cancerous cell model (Fig. 3b). Interestingly, in the same conditions, TQ had no effect on  $\alpha/\beta$  tubulin protein expression in normal human fibroblast cells (Fig. 3b), even after 12 h of treatment (data not shown). All these findings indicate that TQ degrades  $\alpha/\beta$  tubulin proteins in Jurkat cells without affecting their level in normal human fibroblast cells.



**Fig. 2** Effects of TQ on  $\alpha/\beta$  tubulin expression and apoptosis in Jurkat cells. **a** Cells were exposed to TQ at different concentrations and incubated for 24 h. Immunoblot analyses were performed as described in “Material and methods” with different antibodies. The data are representative of at least three independent experiments. **b** Cell apoptosis rate was assessed by capillary cytometry using the annexin V-Phycoerythrin (PE) and 7-Amino-Actinomycin (7AAD) staining assay. Events in each of the four quadrants were: Lower-left quadrant: viable cells, not undergoing detectable apoptosis (Annexin

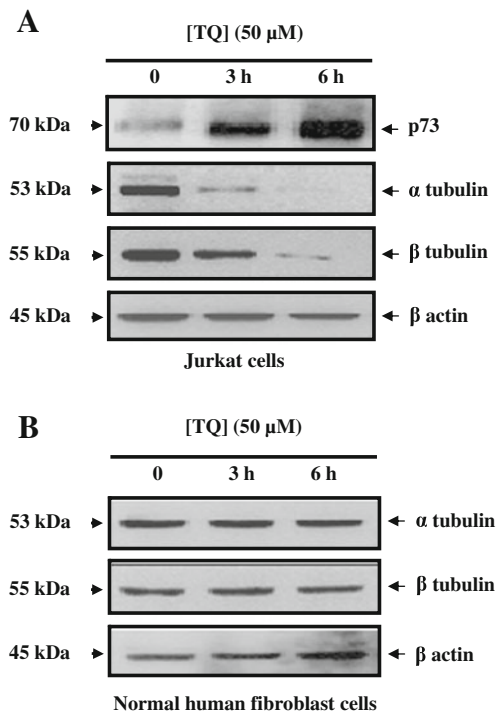
V-PE negative and 7-AAD negative cells). Lower-right quadrant: cells in the early stage of apoptosis (Annexin V-PE positive and 7-AAD negative cells). Upper right quadrant: cells in the late apoptotic or dead (Annexin V-PE positive and 7-AAD positive cells, their number, expressed as percentage relative to the total cell number, is indicated on the right). Upper-left quadrant: mostly nuclear debris (Annexin V-PE negative and 7-AAD positive). The data are representative of at least three independent experiments

## Discussion

Because of their essential role in cell proliferation, microtubules are considered as one of the best targets for the development of anti-cancer drugs [2, 28]. It has been suggested that targeting tubulin level directly without manipulating microtubules dynamics could be an additional approach to induce apoptosis in cancer cells [28]. In the present study, we evaluated the effect of TQ on  $\alpha/\beta$  tubulin protein level in human astrocytoma cells (line U87) and in p53-deficient acute lymphoblastic leukaemia cell line (Jurkat cells). Our results showed that TQ induces a concentration- and time-dependent degradation of  $\alpha/\beta$  tubulin associated with the up-regulation of tumor suppressor p73 in both cancer cell lines. In human astrocytoma cells, TQ induced  $\alpha/\beta$  tubulin down-regulation at 100  $\mu\text{M}$

while in Jurkat cells this effect started at 10  $\mu\text{M}$  suggesting that leukaemia cells are more sensitive to TQ than astrocytoma cells. Interestingly, TQ had no effect on the expression of  $\alpha/\beta$  tubulin protein in normal human fibroblast cells as compared to Jurkat cells. The human astrocytoma cell line (U87 MG) is a high-grade invasive form of astrocytic brain tumors with a mean survival time of less than 1 year. As microtubules are essential components for cell division and intracellular transport, it is reasonable to suggest that  $\alpha/\beta$  tubulin degradation which was observed in TQ-treated human astrocytoma cells is likely to limit tumor cells’ invasiveness. Indeed, previous studies have shown that TQ reduced colon tumor cell invasion as well as significantly inhibited the invasion of lung cancer cell [29, 30]. Presently, we don’t have an explanation why TQ selectively induced  $\alpha/\beta$  tubulin





**Fig. 3** Time-course of the effects of TQ on  $\alpha/\beta$  tubulin and p73 expression in Jurkat cells and in normal human fibroblast cells. Jurkat cells (a) and normal human fibroblast cells (b) were exposed to 50  $\mu$ M of TQ for the indicated times. a, b show representative immunoblots. Similar observations were made in three independent experiments

degradation in cancer cells without affecting their level in normal cells. Since  $\alpha/\beta$  tubulin proteins are essential for cell proliferation, it is possible that because cancer cells multiply rapidly they are more sensitive to TQ compared to normal-growing cells. In agreement with such a hypothesis, we observed that TQ induced a dramatic decrease in the expression of UHRF1, an anti-apoptotic protein, which is over-expressed in numerous cancers and essential for cell proliferation [12, 13] in Jurkat cells without affecting its level in normal human fibroblast cells (Fig. 1A-B, supplementary data). Since previous studies have shown that TQ induces p73 up-regulation-dependent UHRF1 down-regulation [12, 14] and that UHRF1 down-regulation induces cell cycle arrest in G2/M and apoptosis [31], it is likely that TQ-induced p73 up-regulation in the present study leads to UHRF1 and  $\alpha/\beta$  tubulin degradation resulting ultimately in extensive apoptosis. Although the present study indicates that  $\alpha/\beta$  tubulin proteins are selectively degraded in human astrocytoma cells and leukaemia cells, the mechanism underlying the inhibitory effect of TQ remains to be clarified. Some studies have shown that MTDs such as paclitaxel (taxol) can induce caspase-independent mitotic death (CIMD) by up-regulating of p73 but not its homolog p53 [32–35]. In addition, high

concentrations of TQ also induced the up-regulation of p73 with the subsequent induction of apoptosis independently of caspase 3 activation in Jurkat cells [12]. Thus, TQ might be able to induce the degradation of  $\alpha/\beta$  tubulin proteins through a caspase-independent pathway, which is activated by the up-regulation of p73. Furthermore, isothiocyanates (ITCs), one of the best chemopreventive small molecules, have been shown to induce a selective degradation of  $\alpha/\beta$  tubulin and that this degradation is initiated by their interaction resulting in the ubiquitination of  $\alpha/\beta$  tubulin and their subsequent degradation by the proteasome [36]. It has also been shown that the small molecule T0070907 (a PPAR inhibitor) is able to decrease the protein level of  $\alpha/\beta$  tubulin by activation of the proteasome pathway [37]. Since TQ is a small drug-like molecule [38, 39], it is likely that TQ decreases  $\alpha/\beta$  tubulin level in cancer cells by increasing the rate of proteasomal degradation rather than by interfering with its synthesis. The present findings suggest that TQ is an attractive MTD with potent anticancer-activity, associated to low cytotoxic effects on normal human cells and which also has anti-inflammatory and neuroprotective activities as well as a sensitizing effect on cancer cells resistant to chemotherapy [15, 40–44]. In conclusion, we have shown that TQ causes in cancer cells  $\alpha/\beta$  tubulin degradation associated with p73 up-regulation. These data support the idea that small molecules can be used to decrease  $\alpha/\beta$  tubulin levels, and suggest that TQ might act as a novel and potent antimicrotubule drug.

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