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DOI 10.1055/s-0035-1558289
Planta Med 2016; 82: 312–321

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Rüdigerstraße 14
70469 Stuttgart
ISSN 0032-0943

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Thymoquinone from *Nigella sativa* Seeds Promotes the Antitumor Activity of Noncytotoxic Doses of Topotecan in Human Colorectal Cancer Cells *in Vitro*

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Key words
- *Nigella sativa*
- Ranunculaceae
- topotecan
- thymoquinone
- colorectal cancer
- apoptosis
- caspases

Abstract

Topotecan, a topoisomerase I inhibitor, is an anticancer drug widely used in the therapy of lung, ovarian, colorectal, and breast adenocarcinoma. Due to the primary dose-limiting toxicity of topotecan, which is myelosuppressive, it is necessary to identify other chemotherapeutic agents that can work synergistically with topotecan to increase its efficacy and limit its toxicity. Many studies have shown synergism upon the combination of topotecan with other chemotherapeutic agents such as gemcitabine. Other studies have demonstrated that pre-exposing cells to naturally occurring compounds such as thymoquinone, followed by gemcitabine or oxaliplatin, resulted in higher growth inhibition compared to treatment with gemcitabine or oxaliplatin alone. Our aim was to elucidate the underlying mechanism of action of topotecan in the survival and apoptotic pathways in human colon cancer cell lines in comparison to thymoquinone, to study the proapoptotic and antiproliferative effects of thymoquinone on the effectiveness of the chemotherapeutic agent topotecan, and to investigate the potential synergistic effect of thymoquinone with topotecan. Cells were incubated with different topotecan and thymoquinone concentrations for 24 and 48 hours in order to determine the IC_{50} for each drug. Combined therapy was then tested with ± 2 values for the IC_{50} of each drug. The reduction in proliferation was significantly dose- and time-dependent. After determining the best combination (40 µM thymoquinone and 0.6 µM topotecan), cell proteins were extracted after treatment, and the expression levels of B-cell lymphoma 2 and of its associated X protein, proteins p53 and p21, and caspase-9, caspase-3, and caspase-8 were studied by Western blot. In addition, cell cycle analysis and annexin/propidium iodide staining were performed. Both drugs induced apoptosis through a p53-independent mechanism, whereas the expression of p21 was only seen in thymoquinone treatment. Cell cycle arrest in the S phase was detected with each compound separately, while combined treatment only increased the production of fragmented DNA. Both compounds induced apoptosis through the extrinsic pathway after 24 hours; however, after 48 hours, the intrinsic pathway was activated by topotecan treatment only. In conclusion, thymoquinone increased the effectiveness of the chemotherapeutic reagent topotecan by inhibiting proliferation and lowering toxicity through p53- and Bax/Bcl2-independent mechanisms.

Abbreviations

ATCC:	American Type Culture Collection
Bax:	Bcl-2-associated X protein
Bcl-2:	B-cell lymphoma 2
DMEM:	Dulbecco's Modified Eagle Medium
DMSO:	dimethyl sulfoxide
ELISA:	enzyme-linked immunosorbent assay
G_0:	Gap 0
G_1:	Gap 1
IC_{50}:	inhibitory concentration 50
MTT:	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium
p21:	bromidecyclin-dependent kinase inhibitor of cyclin-CDK2,1,4 and 5, regulates cell progression at G1 and S phase
p53:	protein 53
PBS:	phosphate buffered saline
PI:	propidium iodide
SDS-PAGE:	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TP:	topotecan
TQ:	thymoquinone

received March 16, 2015
revised October 1, 2015
accepted October 20, 2015

Bibliography
DOI http://dx.doi.org/10.1055/s-0035-1558289
Published online February 5, 2016
Planta Med 2016; 82: 312–321

ISSN 0032-0943

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Introduction

Colorectal cancer is one of the most devastating malignancies in the world. This disease is the second most common cancer in females and the fourth most common reported cancer in males in Lebanon. An average of 630 individuals are diagnosed with colorectal cancer annually in Lebanon. In the West, colorectal cancer is the third most commonly occurring type of cancer, and the second most common cause of cancer-related deaths. Due to the prolonged series of neoplastic events required for clonal expansion from the time of initiation to the beginning of the invasive stage, adequate time and targets are available for preventive interventions [1,2].

Back in the 1960s, camptothecin derivatives were a promising group of chemotherapeutic agents due to their inhibition of topoisomerase I, which leads to DNA damage. However, the usage of camptothecin derivatives was limited due to their severe toxicity related to the instability of their chemical structure [3]. TP, or 10-hydroxy-9-dimethylaminomethyl-(S)-camptothecin, is a novel topoisomerase I inhibitor and a water-soluble camptothecin analog [4,5]. Topoisomerase I binds to super-coiled DNA at the 3′-end of the DNA phosphodiester backbone and causes single-stranded breaks during DNA replication, relieving the torsional stress that is introduced into DNA ahead of the replication complex or the moving replication fork. TP induces cell death by stabilizing the covalent complex of topoisomerase and strand-cleaved DNA, thus inducing breaks in the protein-associated DNA single strands [6,7]. TP is being evaluated in pediatric cancer patients for the treatment of leukemia, lymphoma, Ewing's sarcoma, rhabdomyosarcomas, gliomas, small cell and non-small cell bronchogenic, ovarian carcinoma, and small cell lung cancer [8,9]. TP is also the first-line therapy used for cisplatin-refractory ovarian cancer and the second-line therapy of small cell lung cancer [9]. This drug is considered a viable new second-line treatment for patients with advanced ovarian cancer who show resistance to platinum-based agents and/or paclitaxel [10]. Metronomic therapy with oral TP showed promising results in some clinical trials of metastatic colon cancer and is considered a beneficial long-term adjuvant for the maintenance therapy of colon cancer [11].

The instability of the lactone E ring in the camptothecin molecule is the most critical structural feature leading to its toxicity [12]. The general toxicity symptoms of high doses of TP are mainly hematological, like neutropenia, thrombocytopenia, and anemia, combined with mild nonhematological side effects, such as alopecia and fatigue [9,13–16]. These observations have led to many attempts to modify the molecule in order to increase the efficacy of the treatment while decreasing its toxicity [12]. Tsang et al. stated that due to the primary dose-limiting toxicity of TP, other chemotherapeutic agents must be identified that will work synergistically with TP and that may potentially maintain or increase its efficacy while limiting its toxicity. They demonstrated synergistic effects of combining TP with bortezomin in neuroblastoma cell lines and in a preclinical mouse model; a significant decrease in tumor growth and tumor progression via the nuclear factor-κB pathway was observed compared to using the drugs separately [8]. Combining TP with irinotecan revealed moderate synergy in leukemic cells. By contrast, combining these camptothecin derivatives with CP-4055-gemcitabine, which is the elaidic acid ester of cytarabine, resulted in antagonistic interactions. This synergism was only found if the cells were pre-exposed to CP-4055 prior to TP treatment [17]. In addition, synergism has been reported in the combination of obatoclax mesylate with TP in a phase I study of patients with relapsed small cell lung cancer and other solid tumor malignancies [5]. By applying small doses of TP and irinotecan, partial responses were seen in patients with metastatic colon cancer, and this combination had only minor toxic effects [18].

The seeds of *Nigella sativa* L. (Ranunculaceae) have been mentioned in many historical and religious references. *N. sativa* is a spice that grows in the Mediterranean region and in Western Asian countries including India, Pakistan, and Afghanistan [19]. According to El-Dakhakhny, the bioactive constituents of the volatile oil of black seed (54%) were mainly TQ and a thymol and TQ dimer, also known as dithymoquinone [20].

TQ (2-isopropyl-5-methyle-1,4 benzoquinone, $C_{10}H_{12}O_2$, molecular weight: 164.2) has shown promising beneficial pharmacological effects for the treatment of dermatitis [21]. This compound also has antihistaminic, antihypertensive, hypoglycemic, antifungal, anti-inflammatory, and antineoplastic effects [22–27]. The antiproliferative effects of TQ have been demonstrated by Shoieb et al. in many types of cancer, such as osteocarcinoma and its cisplatin-resistant variant, human breast, and human ovarian adenocarcinoma. TQ did not exhibit any toxicity in normal kidney cells and human pancreatic ductal epithelial cells; hence it was considered an effective chemotherapeutic agent [28,29]. TQ also inhibited proliferation and induced apoptosis in small and non-small cell lung cancer cells, human osteosarcoma cell lines, colorectal HT29, leukemic cells lines, papilloma (SP-1), spindle (I7) carcinoma cells, and colon cancer cell line HCT-116 [30–34]. Jafri et al. demonstrated that the administration of TQ with cisplatin is an active therapeutic combination in small and non-small lung cancer cell lines *in vitro* and *in vivo*; no significant toxic effects were detected upon treatment with TQ alone or in combination with low doses of cisplatin in female SCID mice. The combined treatment was more efficient than administering each compound alone, whereby a 79% reduction in tumor volume was detected in mice without any toxic side effects. TQ also acts synergistically with cisplatin, and causes a decrease in cisplatin resistance by suppressing nuclear factor-κB [32]. Another *in vivo* study performed by Banerjee et al. showed that cells pre-exposed to TQ, prior to gemcitabine or oxaliplatin treatment, resulted in 60 to 80% growth inhibition compared to 15–25% inhibition when the drugs were used separately. In addition, TQ in combination with gemcitabine and/or oxaliplatin showed a higher effect *in vitro* compared to treatment with the drugs separately [29].

In order for a cell to undergo chemotherapy-induced apoptosis, the balance between proapoptotic and antiapoptotic signals must be altered. Thus, chemotherapeutic agents induce apoptosis not only by increasing the proapoptotic signal but by also decreasing the antiapoptotic signal. The extrinsic pathway can be triggered through death receptors that are members of the tumor necrosis factor receptor CD95 or tumor necrosis factor-related apoptosis-inducing ligand receptor families [35].

On the other hand, the intrinsic pathway is activated by the mitochondrial mediators of caspase-dependent apoptosis, such as cytochrome c; upon its release, cytochrome c activates caspase-3 through the formation of the cytochrome c/apaf-1/caspase-9-containing apoptosome complex [36].

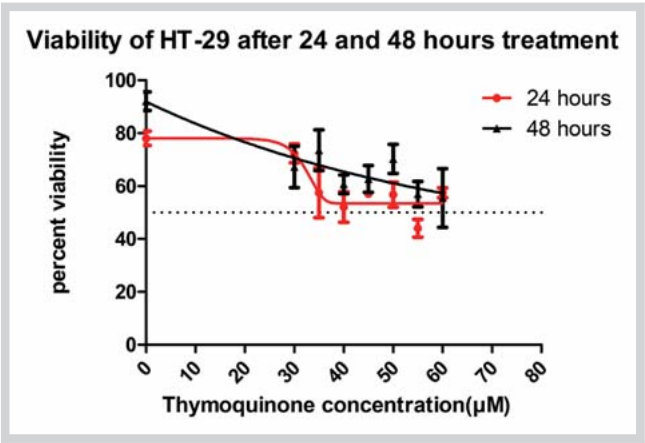

Fig. 1 Cell viability of HT-29 cells after 24 and 48 h of TQ treatment. Cells were treated with TQ at different concentrations. Cell viability was determined using the trypan blue exclusion method. The results are presented as the percent of total number of cells. Data is the mean ± SEM. TQ's IC_{50} s for HT-29 cells range between 59.2 μM and 68.4 μM at 24 and 48 h, respectively, with $p < 0.0001$. (Color figure available online only.)

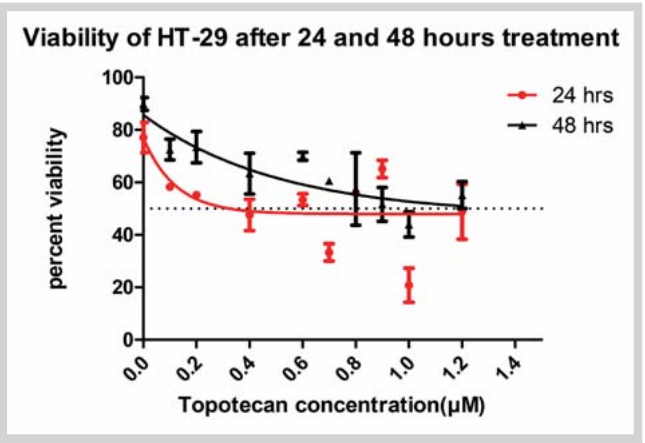

Fig. 2 Cell viability of HT-29 cells after 24 and 48 h of TP treatment. Cells were treated with TP at different concentrations. Cell viability was determined using the trypan blue exclusion method. The results are presented as the percent of total number of cells. Data is the mean ± SEM. The viability of the HT-29 cell line decreased significantly ($p \leq 0.0001$) in a dose- and time-dependent manner. TP's IC_{50} s for HT-29 cells varied from 0.77 to 1.06 μM at 24 and 48 h, respectively. (Color figure available online only.)

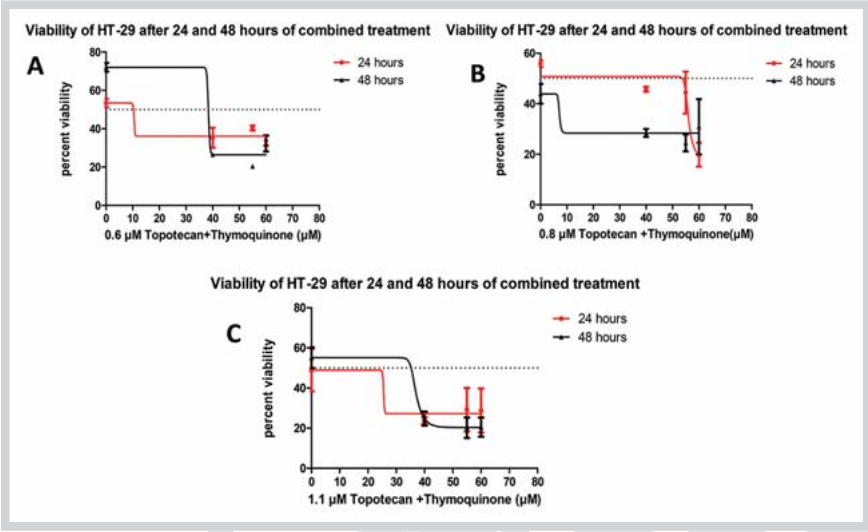

Fig. 3 Cell viability of HT-29 cells after 24 and 48 h of combined treatment. Cells were treated with 1.1 μM TP with different concentrations of TQ (0, 40, 55, 60 μM). Cell viability was determined using the trypan blue exclusion method. The results are presented as the percent of total number of cells. Data is the mean ± SEM (**A** $p < 0.0001$; **B** $p = 0.0286$; **C** $p = 0.0096$). The best combination was determined to be 40 μM TQ in addition to 0.6 μM TP, respectively, which has led to a decrease in cytotoxicity from 53% to 35% after 24 h and from 72.05% to 26% after 48 h. (Color figure available online only.)

Results

Viability was assessed using the trypan blue exclusion method, and the results are reported as the percentage of viable cells out of the total number of cells. The IC_{50} of TQ for HT-29 cells ranges between 59.2 μM and 68.4 μM at 24 and 48 h, respectively (**Fig. 1**). The viability of the HT-29 cell line decreased significantly ($p \leq 0.0001$) in a dose- and time-dependent manner.

The IC_{50} for TP in HT-29 cells varies from 0.77 to 1.06 μM at 24 and 48 h, respectively (**Fig. 2**). Cytotoxicity levels are dose- and time-dependent with $p < 0.0001$. The differences between the control and treated cells were determined by statistical analysis. Importantly, we could not differentiate whether the dead cells observed had died from necrosis or apoptosis. Following the determination of IC_{50}, several combinations of treatments were performed. The cell viability level decreased significantly when TP was combined with different concentrations of TQ with $p < 0.0001$, $p = 0.0286$, and $p = 0.0096$ for the combinations of 0.6 μM TP, 0.8 μM TP, and 1.1 μM TP with 40 μM, 55 μM, or 60 μM TQ, respectively (**Fig. 3**).

The best combination was determined to be 40 μM TQ with 0.6 μM TP, leading to a decrease in viability from 53% to 35% after 24 h and from 72.05% to 26% after 48 h.

To assess the effect on cell proliferation, the same concentrations of TQ and TP, which were used for the cytotoxicity assay, were applied to cells (TQ: 30 μM-60 μM and TP: 0.1 μM-1.2 μM). After 24 and 48 h, cell proliferation was assessed using the MTT assay. The results showed that TQ significantly inhibited the proliferation of HT-29 ($p \leq 0.0001$) in a dose- and time-dependent manner (**Fig. 4**). The percent of proliferation decreased with increasing TQ concentrations to reach its IC_{50} s, which were 58.08 549 μM at 24 h and 36.4889 μM at 48 h. The antiproliferative effect of TQ was greater than that of TP. HT-29 cells showed significant inhibition of proliferation after TP treatment only after 48 h, when the IC_{50} was reached at 1.4 μM with $p \leq 0.0001$ (**Fig. 5**).

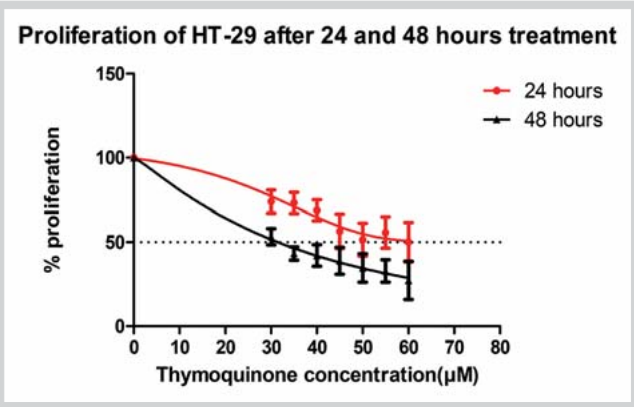

Fig. 4 Proliferation of HT-29 cells after 24 and 48 h of treatment with different concentrations of TQ. The absorbance was measured at 630 nm. Results were normalized to the untreated cells. Data is the mean ± SEM. TQ's IC_{50} s were reached at 58.08 549 µM at 24 h and 36.4889 µM at 48 h ($p \leq 0.0001$). (Color figure available online only.)

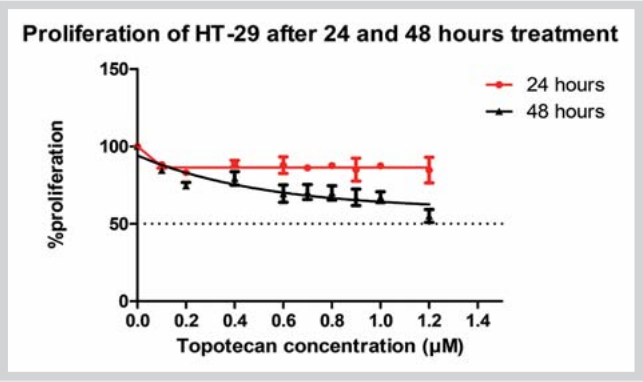

Fig. 5 Proliferation of HT-29 cells after 24 and 48 h of treatment with different concentrations of TP. The absorbance was measured at 630 nm. Results were normalized to the untreated cells. Data is the mean ± SEM. The IC_{50} of TP was reached only after 48 h (1.4 µM, $p \leq 0.0001$). (Color figure available online only.)

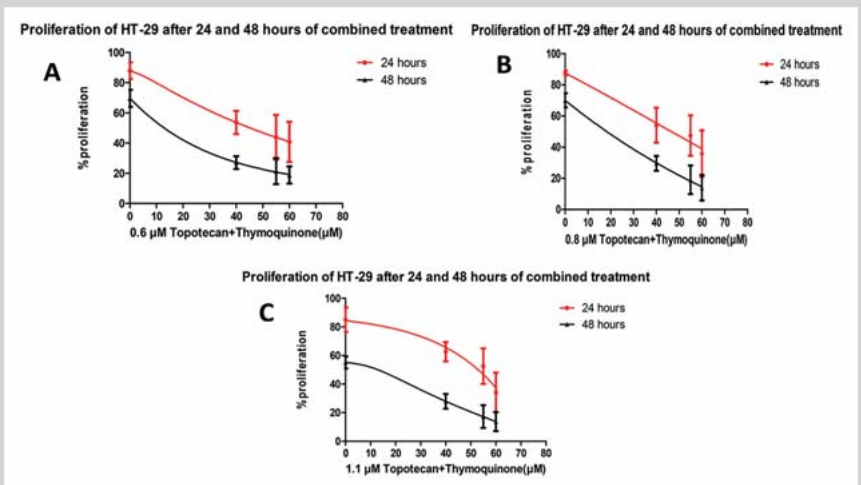

Fig. 6 Proliferation of HT-29 cells after 24 and 48 h of combined treatment with 0.6, 0.8, and 1.1 µM TP and different concentrations of TQ (0, 40, 55, 60 µM). The absorbance was measured at 630 nm after four incubations with the MTT labeling reagent and an overnight incubation with solubilization solution. Results were normalized to the untreated cells. Data is the mean ± SEM (**A** $p < 0.0002$; **B** $p = 0.0002$; **C** $p = 0.0006$). The best combination was found to be 40 µM and 0.6 µM for thymoquinone and topotecan respectively, which has led to the reduction in proliferation in a dose- and time-dependent manner, approximately from 87.94% to 53.64% after 24 h and from 69.57% to 27.03% after 48 h. (Color figure available online only.)

The same combinations that were used in the trypan blue exclusion method were also used to determine the effect of the combined treatment on proliferation. The combination of TP with different concentrations of TQ significantly decreased cell proliferation with $p < 0.0002$, $p = 0.0002$, and $p = 0.0006$ for the combinations of 0.6 µM, 0.8 µM, and 1.1 µM TP with 40 µM, 55 µM, and 60 µM TQ, respectively (**Fig. 6**). The best combination was found to be 40 µM TQ with 0.6 µM TP, leading to a dose- and time-dependent reduction in proliferation from approximately 87.94 to 53.64% after 24 h and from 69.57 to 27.03% after 48 h.

The effect of TQ, TP, and the combined treatment on the induction of apoptosis was then assessed using cell death detection ELISA^{PLUS}. In this technique, the enrichment factor is the ratio of the absorbance measured for each drug to that of the untreated controls. The absorbance reflects the quantity of anti-DNA peroxidase, which in turn reflects the level of DNA fragmentation generated by apoptosis. Both TQ and combination treatment showed an increase in the enrichment factors at 24 h. The results obtained at 48 h were contradictory, with no increase in the enrichment factor after applying the compounds separately (**Fig. 7**). However, the enrichment factor increased significantly when the two compounds were combined at 24 and 48 h. Overall, the results reveal the ability of the compounds to induce apoptosis in an HT-29 cell line, in accordance with the decrease in cell viability previously observed with the trypan blue exclusion method.

Another approach was used to assess the proapoptotic effects of TQ and TP on HT-29 cells: annexin V and PI were added to the cells after treatment, and the samples were then analyzed using a flow cytometer. Cells that stained positive for both annexin V-FITC and PI were considered to be in the end stage of apoptosis or already dead.

After 24 h, the percentage of dead cells increased from 8.9 to 17.8% and 17.5% upon TQ and TP treatment, respectively, whereas, upon combination, the percentage of dead cells reached 29.5% (**Fig. 8**). At 48 h, the percentage of apoptotic cells increased from 2.5% to 36% upon TQ treatment. The percentage upon TP treatment was 34.8% and increased to 39.07% upon combined treatment (**Fig. 9**). These results indicate a synergistic effect of combining TQ with TP on the induction of apoptosis in HT-29 cells.

After determining the best combination to inhibit cell proliferation in colorectal cancer cells, we aimed to evaluate whether this effect was through cell cycle arrest using flow cytometry. After analyzing the DNA content of cells treated with TQ, TP, and in

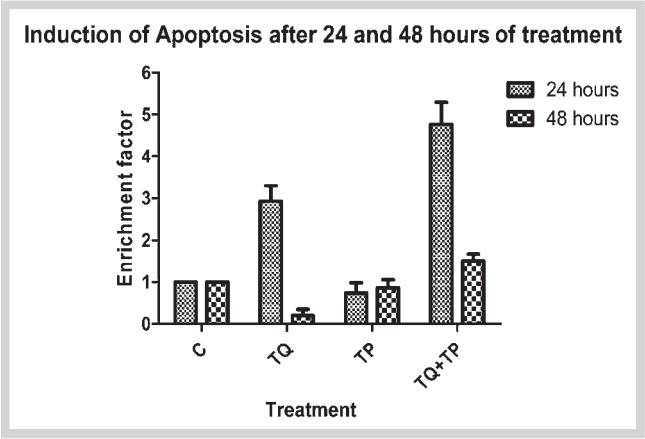

Fig. 7 Apoptosis was induced in the HT-29 cell lines after 24 and 48 h. Cells were treated with TQ (40 µM), TP (0.6 µM), and combined treatment. Absorbance was read at 405 nm after the addition of 2,2′-azino-di(3-ethyl-benzthiazolin-sulfonate) substrate using the cell death ELISA kit ($p = 0.0002$).

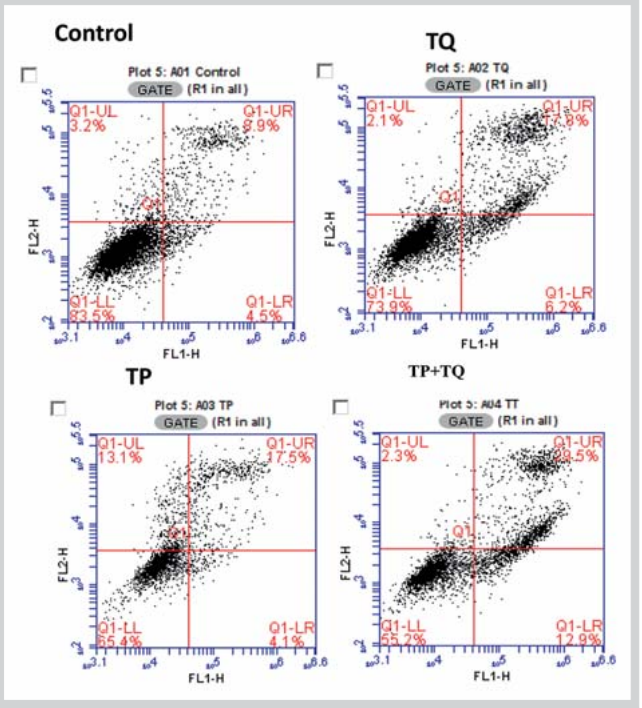

Fig. 8 Effect of TQ and TP on apoptosis after 24 h. Cells were treated with TQ (40 µM) and TP (0.6 µM) separately and combined for 24 h, stained with annexin/PI, then analyzed using a flow cytometer. (Color figure available online only.)

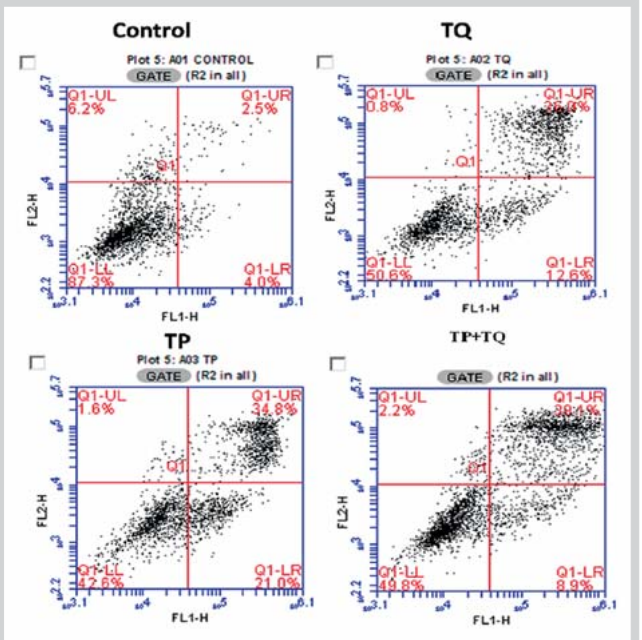

Fig. 9 Effect of TQ and TP on apoptosis after 48 h. Cells were treated with TQ (40 µM) and TP (0.6 µM) separately and combined for 48 h, stained with annexin/PI, then analyzed using a flow cytometer. (Color figure available online only.)

combination, cells were assigned to their respective phases: cells in Pre-G_1 contain less than 2 n, those in G_0/G_1 contain 2 n, those in S contain > 2 n but < 4 n, and those in the M phase contain 4 n. In all of the treatments performed, an increase in the Pre-G_1 phase was detected, reflecting the presence of fragmented DNA and confirming the apoptotic effect of both compounds, separately and in combination.

Upon TP treatment, the percentage of cells in the S phase increased from 32% to 38% and 51% after 24 and 48 h, respectively, indicating an arrest in the S phase. On the other hand, TQ had no effect on cell cycle arrest after 24 h, but showed an increase in the percentage of cells in the S phase after 48 h (**Fig. 10** and **11**). Furthermore, combined treatment slightly increased the percentage of cells in G_0/G_1 after 24 h and those arrested in the S phase after 48 h (**Fig. 10** and **Fig. 11**). Also, the addition of TQ to TP treatment increased the percentage of cells in the G_0/G_1 and G_2/M phases after 24 and 48 h compared to treatment with TP alone. These results are consistent with the results obtained using the MTT proliferation assay.

To identify the pathway by which TQ and TP inhibit cell proliferation and promote apoptosis, the expression of p53 was determined using Western blot. The results revealed that both treatments exerted their antiproliferative effect through p53-independent pathways because no significant change was detected in the expression of p53. Only cells treated with TQ alone expressed an upregulation of p21 protein.

The expression of p21 upon combined treatment was lower in comparison to exposure to TQ or TP alone. The proapoptotic effect of both compounds was assessed by measuring the expression of Bax and Bcl-2. The results revealed an upregulation of Bax, especially in cells treated with TP alone and a downregulation of Bcl-2 (**Fig. 12**). However, the difference in the ratio of Bax/Bcl-2 was not significant, revealing that the pathway by which cells were undergoing apoptosis was not Bax/Bcl-2-dependent.

TQ induced an increase in the caspase-8 precursor and a downregulation of procaspase-3, whereas TP induced the expression of these proteins only at 24 h. After 48 h, an increase in procaspase-9 was observed upon TP treatment. Apoptosis appears to

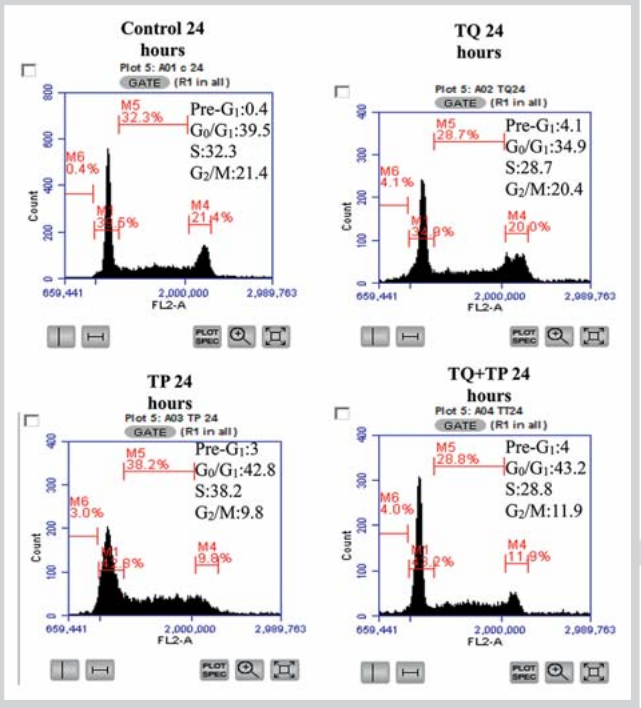

Fig. 10 Effect of TQ, TP, and combined treatment on the cell cycle and apoptosis by flow cytometry on HT-29 after 24 h treatment. Cells were treated with TQ (40 µM) or TP (0.6 µM) for 24 h and the control cells were left untreated. Cells were incubated for 40 min with PI after fixation with absolute ethanol at − 80 °C and analyzed using a C6 flow cytometer. Based on their DNA content, cells were distributed accordingly: Pre-G1 cells were less than 2 n, G_0/G_1 cells were 2 n, and S/M phase cells were > 2 n. (Color figure available online only.)

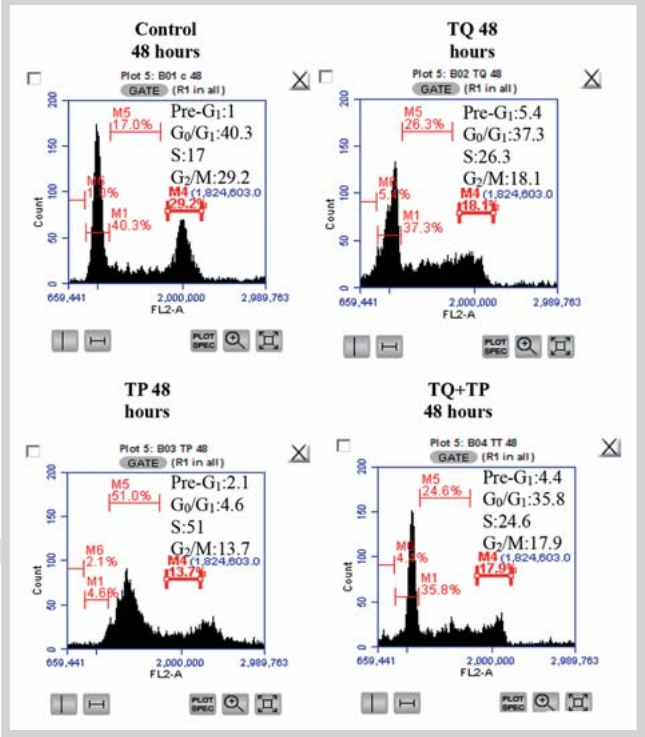

Fig. 11 Effect of TQ, TP and combined treatment on the cell cycle and apoptosis by flow cytometry on Ht-29 after 48 h treatment. Cells were treated with TQ (40 µM) or TP (0.6 µM) for 48 h, and the control cells were left untreated. Cells were incubated 40 min with PI after fixation with absolute ethanol at − 80 °C and analyzed using a C6 flow cytometer. Based on their DNA content, cells were distributed accordingly: Pre-G1 cells were less than 2 n, G_0/G_1 cells were 2 n, and S/M phase cells were > 2 n. (Color figure available online only.)

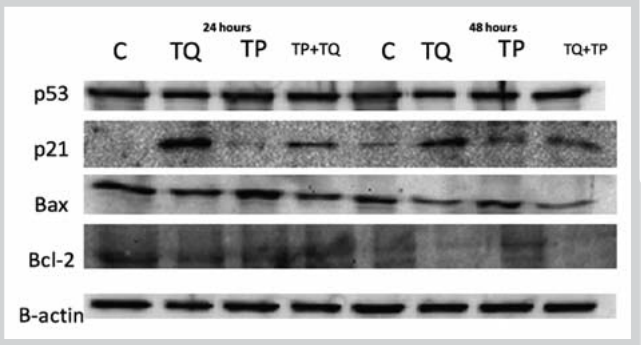

Fig. 12 Western blot analysis for the apoptotic and proliferative related proteins. HT-29 cells were treated with 40 µM TQ, 0.6 µM TP, and combined treatment for 24 and 48 h.

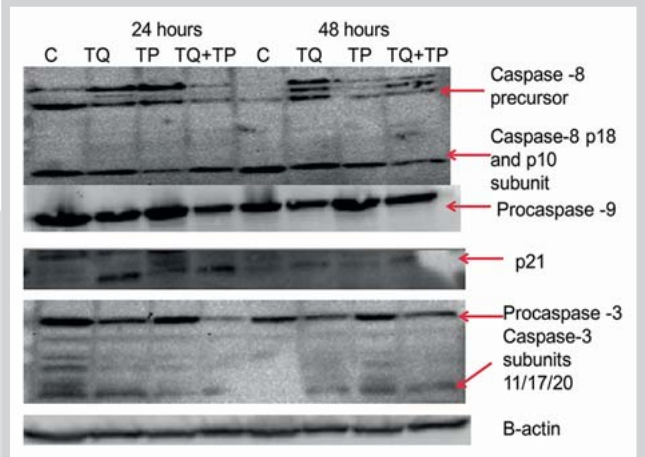

Fig. 13 Western blot analysis for caspases. HT-29 cells were treated with 40 µM TQ, 0.6 µM TP, and combined treatment for 24 and 48 h. (Color figure available online only.)

have been induced through the extrinsic pathway at 24 h, whereas, at 48 h, the intrinsic pathway was triggered (▶ **Fig. 13**).

Discussion

TP or camptothecin derivatives have shown antitumor effects against many types of cancer, such as leukemia, lymphoma, sarcoma, gliomas, small cell and non-small cell bronchogenic carcinoma, ovarian carcinoma, and small cell lung cancer [8,9]. The lack of stability of its lactone E ring, which can be partially hydrolyzed before clinical administration in the parental solution to form an inactive carboxylate, made it toxic at higher doses, with hematological side effects such as neutropenia and thrombocyto-

penia [9, 13–16]. Several studies have investigated the effect of combining TP with other chemotherapeutic agents to increase its efficacy while limiting its toxicity by using lower doses [8, 17]. Moreover, other researchers focused on combination therapies with naturally occurring anticancer drugs that have the potential to overcome resistance to chemotherapeutic drugs and to lower their toxicity [29, 32].

In our study, we aimed to investigate the mechanism of action of TP in the survival and apoptotic pathways in cancer cell lines in comparison with TQ, a naturally occurring anticancer compound, and to demonstrate a potential synergistic effect of these two compounds.

First, we determined the IC_{50} of each drug, then, using the data obtained, many combinations of the two drugs were applied to the colon cells, and the best combination was found to be 40 µM TQ with 0.6 µM TP. This combination led to a decreased viability from 53 to 35% after 24 h and from 72.05 to 26% after 48 h, and reduced proliferation in a dose- and time-dependent manner from 87.94 to 53.64% after 24 h and from 69.57 to 27.03% after 48 h.

Our second aim was to determine the mechanism by which these two compounds inhibited cell proliferation and to investigate their effect on apoptosis because sensitivity to chemotherapeutic agents is determined by the balance between the survival and apoptotic pathways in cells. By examining the antiproliferative effect through analyzing the cell cycle progression, we showed that TQ had no effect on the cell cycle after 24 h. This finding is in accordance with the results obtained by Norsharina et al. in 2011 in HT-29 cells [33]. However, S arrest was detected after 48 h in addition to the presence of fragmented DNA, as was revealed in the increased percentage of cells in the Pre-G_1 phase. S/G_2 arrest in p53+/+ HCT 116 was also observed in a previous study [37]. These results are different from many studies that have reported TQ arresting progression from G_1 to the S phase in prostate cancer cells, colon cancer, and cisplatin-resistant cells [28, 30, 38]. Petak et al. showed that TQ inhibited the G2-M phase transition in TS human colon carcinoma and delayed apoptosis by a Fas-independent pathway and caspase-dependent activation [39]. Another study also reported that TQ, by inhibiting survivin, stopped cell cycle progression through M phase arrest, leading to apoptosis in pancreatic cells [29]. TQ treatment induced a G_0/G_1 cell-cycle arrest in papilloma cells, and a G_2/M cell-cycle arrest in spindle carcinoma cells, associated with an increase in p53 [31].

Our results demonstrate the efficacy of TP at increasing the number of cells in the S phase, confirming that the cytotoxic mechanism of camptothecin is S phase-dependent due to the collision between the replication fork and the camptothecin-stabilized cleavable complex, causing breaks in DNA double strands [7, 40–42].

However, if camptothecins are applied at higher concentrations, apoptosis is induced by an unknown mechanism, killing all the cells, including the non-S phase cells [43]. Many studies have shown an increase in the apoptotic sub-G1 fraction of A549 cells [44], and p53-deficient cells were unable to leave the S phase upon TP treatment. However, p53 wild-type cells were only transiently blocked in the S phase, and they recovered to progress into G1 [45].

According to the results reported in this study, combining TQ with TP caused an increase in the number of cells in the Pre-G_1 phase and increased the amount of fragmented DNA, which was detected by annexin/PI staining and by cell death ELISA, confirming the death of cells by apoptosis rather than necrosis.

Western blot analysis revealed that both TP and TQ induce apoptosis through p53-independent mechanisms, contradicting many reports observed in HCT-116 [31]. On the other hand, p53-deficient mouse embryonic fibroblasts, upon treatment with TP, were significantly more sensitive than wild-type cells, inducing a higher frequency of apoptosis and DNA strand breaks, accompanied with the degradation of topoisomerase I. These data suggest that p53 causes resistance of cells to topoisomerase I inhibitors through the stimulation of TP-triggered topoisomerase I degradation, which may impact TP-based cancer therapy [45]. Although p53 promotes apoptosis [46, 47], the protein also has antiapoptotic effects in some cell types, including MEFs [48–50]. Thus, scientists have suggested that for efficient TP treatment, pharmacologic inhibition of p53 might be beneficial.

Even though p21 is associated with S and G_1 arrest, TQ induced the expression of p21, and this increase was associated only with S rather than G_1 arrest at 48 h. p21 also promotes apoptosis through both p53-dependent and p53-independent mechanisms under certain cellular stresses [51].

The Bax/Bcl2 ratio did not significantly vary, although in many studies TQ induced the downregulation of the Bcl-2 family [29–31]. TQ and TP induced apoptosis through the extrinsic pathway after 24 h, whereas, after 48 h, the intrinsic pathway was triggered during TP treatment only. This observation could be explained by the fact that apoptosis induced by chemotherapy is generally thought to be dependent on a pathway headed by caspase-9; however, Ferreira et al. proved that in the non-small cell lung cancer cell line H460, a novel apoptotic pathway exits with activation of caspase-8 instead of caspase-9. They demonstrated that apoptosis is activated by some anticancer drugs, leading to the activation of mitochondria and caspase-8 (in a caspase-9-independent manner) and finally the activation of the effectors of caspases [52].

Tomicic et al. showed that the intrinsic apoptotic pathway is activated via caspase-3-mediated cleavage in p53-deficient cells that are hypersensitive to TP. They suggested that for improvement of TP-based therapy in glioma cells (wild-type p53), the downregulation of XIAP and survivin should be triggered. They also showed that caspase-2 is more activated in p53-deficient cells upon TP treatment, thus indicating that caspase-2 cleavage is p53- and PIDD-independent and that caspase-3 upregulates caspase-2, which is involved in G2/M arrest [45].

To provide information about the mechanisms of cancer resistance, the study of topoisomerase I proteolysis after TP treatment will be vital because protein stability seems to be essential for the initiation and maintenance of apoptosis.

Devy et al. showed that the poor effectiveness of TP treatment could be due to low levels of topoisomerase I expression and its downregulation in each cell line, which could explain why, in HT-29 and A-549 cell lines, TP treatment was successful only when it was used in combination with other drugs [53].

In the current study, the exact mechanism by which apoptosis is induced by the combination treatment is still unknown. We were able to determine the most significant combination that led to decreased cytotoxicity of TP and increased inhibition of cell proliferation through the promotion of apoptosis. Future studies will focus on studying the effects of these compounds on other pathways.

In conclusion, TQ, when combined with TP in noncytotoxic doses, caused synergistic effects on cytotoxicity and proliferation in HT-

29 cells. Both compounds were able to induce apoptosis by p53-independent mechanisms, with TP and TQ both inhibiting the S phase and the combination being efficient in increasing the percentage of fragmented DNA. However, this synergism was not found at the molecular level when the effects of the drug combination on the expression of apoptotic and antiproliferative proteins were examined. Thus, the combination of TQ with TP appears to be therapeutically active in colon cancer, but the anticancerous effect at the molecular level requires further investigation.

Materials and Methods

Cell culture

A human colon cancer cell line (HT-29) was obtained from ATCC. The cell line was cultured in DMEM supplemented with 10% fetal bovine serum and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin) in a humidified atmosphere containing 5% CO_2 at 37 °C. The cells were grown in a monolayer (70–80% confluence) before being incubated with various concentrations of TQ and TP. The cells were detached using trypsin and split every 3 days at a ratio of 1 : 4. Before plating, 10 µL of the cells were mixed with 10 µL of trypan blue and counted using a hemocytometer to check the cell viability.

Preparation of drugs

Stock solutions of 1000 µM TQ (Aldrich 274 666, 99%) and TP (Sigma T2705, TP hydrochloride hydrate ≥ 98%, 10 mg) were prepared by dissolving these compounds in DMSO. On the day of the treatment, several concentrations of TQ (30 µM-60 µM) and TP (0.1 µM-1.2 µM) were prepared by dissolving the stock in media (DMEM) and applied on seeded cells. After determining the IC_{50} of each drug separately, the following combinations were prepared and added to the seeded cells: TQ (40 µM, 55 µM and 60 µM) ± TP (0.6 µM, 0.8 µM and 1.1 µM).

Cytotoxicity: trypan blue exclusion method

HT-29 cells were seeded into 96-well plates at a density of 1×10^5. After 24 h of plating, the cells were treated with different concentrations of TQ and TP for 24 and 48 h. Control cells were treated with DMEM. Cytotoxicity was then measured by the trypan blue exclusion method: 10 µL of the cells and 10 µL of trypan blue were mixed. The dead and living cells were counted in both the supernatant and the pellet by using a hemocytometer under the microscope. Dead cells were stained blue and the living cells appeared bright. The percent viability was determined by dividing the number of living cells by the total number of cells and multiplying by 100.

Proliferation: cell proliferation kit

To determine the effect of the compounds on the proliferation of HT-29 cells after 24 and 48 h, an MTT-based assay (Roche) was used. This assay is based on the ability of the mitochondria of metabolically active cells to cleave the yellow tetrazolium salt MTT to purple formazan crystals that can be quantified spectrophotometrically. HT-29 cells were seeded into 96-well plates at a density of 1×10^5. After 24 h of plating, the cells were treated with different concentrations of TQ and TP for 24 and 48 h. Control cells were treated with DMEM. After treatment, 10 µM of the MTT labeling reagent [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] were added to each well, and the plate was left in the dark for 4 h in a humidified atmosphere (37 °C, 5% CO_2). After this incubation period, purple insoluble formazan crystals were formed due to the cleavage of MTT upon reduction. The crystals were then rendered soluble by adding the solubilization solution (100 µL/well), and the plates were left overnight in the incubator. The solubilized formazan product was spectrophotometrically measured using an ELISA reader (Biotek ELx808) at 630 nm.

Cell death detection ELISA kit

The cell death detection ELISA (Roche) kit was used following the manufacturer's instructions. The procedure is based on a quantitative sandwich-enzyme-immunoassay principle using mouse monoclonal antibodies directed against DNA and histones. This allows the specific detection and quantification of mono- and oligonucleosomes that are released into the cytoplasm of cells undergoing apoptosis. 2,2′-Azino-di(3-ethylbenzthiazolin-sulfonate) substrate tablets were used to measure changes in the peroxidase activity spectrophotometrically at 405 nm.

Cell cycle analysis: flow cytometry

The cells were seeded at a density of 0.5×10^6 cells/mL and were treated as above. The cells were collected after 24 and 48 h and then centrifuged at 1500 rpm at 4 °C for 5 min. The pellet was suspended in 0.6 mL ice-cold PBS, and the cells were fixed by adding 1.4 mL of ice-cold 96% ethanol and stored at − 80 °C for several days. On the day of the analysis, RNase and propidium iodide (Sigma LifeScience) were added, incubated for 40 min, and analyzed by an Accuri C6 flow cytometer using BD Accuri C6 software. The distribution of the cells into their respective cell cycle phases was based on their DNA content: sub-G_0/G_1 (Pre-G) cells were < 2 n, G_0/G_1 cells were 2 n, and S cells were > 2 n but < 4 n, whereas M phase cells were 4 n. Cell death was determined by an increase in the percentage of cells in the pre-G phase compared to the control.

Detection of apoptosis by annexin V/PI

HT-29 cells were cultured in 6-well plates at a density of 10^6 cells/mL. After plating for 24 h, TQ (40 µM), TP (0.6 µM), or a combination of the two was applied. Cells were collected after 24 and 48 h and centrifuged at 1500 rpm at 4 °C for 5 min. The pellet was suspended in 500 µL suspension buffer, 5 µL annexin V, and 5 µL propidium iodide (annexin V-FITC apoptosis detection kit, Abcam) and immediately analyzed by a flow cytometer. Annexin V is a Ca^{2+}-dependent phospholipid-binding protein that has a high affinity for phosphatidylserine, which is translocated from the cytoplasmic surface to the outer leaflet of the cell membrane upon apoptosis. The cell membrane is impermeable to PI, and hence PI is excluded from living cells. Cells that stain negative for annexin V-FITC and are negative for PI are considered living cells. Cells that stain positive for both annexin V-FITC and PI are either in the end stage of apoptosis, are undergoing necrosis, or are already dead.

Western blot

Cells were plated and cultured to reach confluence, then treated with TQ (40 µM), TP (0.6 µM), or a combination for 24 and 48 h. The cells were scraped and centrifuged at 1500 rpm at 4 °C. Protein was extracted using the Qproteome Mammalian Protein kit. Proteins were quantified using a Bradford assay. Western blot analysis was performed to measure the protein expression of p53, p21, Bax, Bcl2, caspase-3, caspase-9, and caspase-8.

Using SDS-PAGE, proteins were separated on 10% gels and transferred to polyvinylidene fluoride membranes at 0.25 mA for 90 min. The membranes were then blocked with 5% dry skim milk in PBS containing 0.05% Tween-20 for 1 h at room temperature with shaking, or overnight at 4 °C. The membranes were then incubated with primary antibodies using mouse monoclonal IgG anti-β-actin, anti-Bax, anti-p21, anti-Bcl2, anti-caspase-3, anti-caspase-8, and anti-caspase-9 antibodies (Santa Cruz Biotechnology) at a concentration of 1 : 250 (Bax, Bcl2, p21, caspase-3, caspase-9, caspase-8) or 1 : 2000 (p53) for 1 h at room temperature with gentle shaking. After washing the membranes for 1 h using 1X PBS with 0.5% Tween-20, they were incubated with the secondary antibody (anti-mouse IgG HRP-conjugated secondary antibody obtained from Promega) at a concentration of 1 : 5000 for 1 h at room temperature. The membranes were then washed and developed using Western blotting-enhanced chemiluminescent reagent (ECL; GE Healthcare), and pictures were taken using a ChemiDoc XRS+ machine (BIO-RAD).

Statistical analysis

All the experiments were carried out in triplicate, and each experiment was repeated three times. The results are reported as the mean ± standard deviation. The data were analyzed using two-way ANOVA. The level of significance upon comparing control versus treatment was set at $p < 0.05$.

Acknowledgements

This work was supported and funded by the School Research and Development Council at the Lebanese American University (SRDC-t2013–47).

Conflict of Interest

The authors report no conflicts of interest related to this work.

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