Enhanced Antitumor Effects of Thymoquinone in Combination with Paclitaxel on Hepatocellular Carcinoma Cell Lines: Role of P53 Modulation

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Research Article

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Abstract

Background: Hepatocellular carcinoma (HCC) is a highly lethal disease with a limited response to chemotherapy. Understanding the molecular mechanisms and drug resistance in HCC is crucial for developing effective therapeutic strategies. This study focuses on two widely used HCC cell lines, HepG2 and SNU-449, to investigate the combination treatment of paclitaxel (PTX) and thymoquinone (TQ). PTX is a potent chemotherapeutic agent that stabilizes microtubule structure and induces cell cycle arrest, but resistance remains challenging. TQ has shown promising anticancer effects.

Methods: The antitumor effects of mono- and combined drug treatments were assessed in HepG2 and SNU-449 cell lines, including cell viability, cell cycle arrest, and apoptosis.

Results: The combination treatment synergistically enhanced the antitumor effects of PTX and TQ. It significantly reduced viable cell numbers, increased caspase-3 activation, and elevated annexin V staining. Interestingly, the combination induced differential cell cycle arrest patterns, with HepG2 cells shifting to the S phase and SNU-449 cells showing an increased G2/M cell population. PTX alone induced apoptosis in both cell lines, and TQ exhibited a similar apoptotic effect. The combined treatment further potentiated the apoptotic effect. P53, a tumor suppressor gene, was upregulated by PTX and TQ in the tested cell lines, suggesting its role in modulating the treatment response. P53 knockdown enhanced the antitumor properties of PTX and TQ in both cell lines.

Conclusion: The combination therapy of TQ and PTX holds promise as a potential therapeutic regimen for HCC. P53 may have a dual role, acting as a tumor suppressor and a cell protector under stress conditions. Targeting the down-regulatory mechanisms of P53 could be a valuable therapeutic approach, particularly in cancers with wild-type P53.

Introduction

Cancer is a global health concern, with approximately 10 million deaths recorded in 2020 [1]. Liver cancer, including hepatocellular carcinoma (HCC), accounts for nearly 10% of cancer deaths worldwide. HCC is a primary liver cancer originating in liver tissue, distinguishing it from secondary metastatic liver cancer (SMLC), which occurs when cancer spreads to the liver from other organs [2]. The development of HCC is multifactorial, often associated with factors such as excessive alcohol consumption, hepatitis B or C infections, or fatty liver disease [3-6]. HCC exhibits higher virulence and mortality rates compared to other primary liver cancers, such as cholangiocarcinoma, with the number of deaths being nearly five times higher [7]. Despite various treatment strategies, including systemic or targeted therapies, surgical resection, and liver transplantation, only a limited percentage (30-40%) of HCC cases respond to current treatments [8]. This reality emphasizes the need to understand patient responses to existing therapies and explore novel, more effective therapeutic approaches.

Chemotherapy, one of the mainstay treatments for HCC, is often limited by the development of drug resistance, which can be either inherent due to specific genetic alterations or acquired through prolonged
exposure to certain drugs [9, 10]. PTX is a potent chemotherapeutic agent that stabilizes microtubule structure by inhibiting assembly and disassembly processes, leading to cell cycle arrest. It is widely used in the treatment of various cancers, including lung, ovarian, prostate, head and neck, bladder, esophageal, colorectal, adrenocortical carcinoma, leukemia, glioma, and HCC [11-13]. However, resistance to PTX treatment poses a significant challenge for HCC patients, and dysregulation of the apoptosis/survival balance in cancer cells has been proposed as one of the underlying mechanisms [14, 15]. Combination therapies involving PTX have been explored to enhance its antitumor activity and overcome resistance [16-19].

The tumor suppressor protein p53 plays a crucial role in controlling the balance between apoptosis and cell survival. It is involved in cell cycle regulation by inhibiting cyclin-dependent kinase activity [20] and regulates apoptosis by upregulating the expression of pro-apoptotic proteins such as BAX and FAS while downregulating anti-apoptotic protein Bcl2 [21, 22]. Additionally, p53 can activate stress-induced necrosis in transformed cells [23]. However, the protective role of p53 is context-dependent and varies among different cell types and physiological conditions [24]. Mutations in p53 have been associated with increased proliferation, survival, and metastasis in several cancers [25-27]. Interestingly, studies have suggested that the absence of p53 could sensitize HCC cells to apoptosis induced by chemotherapeutic agents [28]. Notably, PTX has been shown to upregulate p53 mRNA and protein expression in HepG2, a human HCC cell line, which may contribute to the reported PTX resistance in HepG2 cells [29-31].

Combined therapy, involving the use of two or more drugs, has demonstrated significant improvements in cancer treatment, often showing additive or synergistic effects compared to monotherapy [32, 33]. In this study, we investigated the outcomes of mono- and combined treatments involving PTX and TQ, a phytochemical compound extracted from Nigella sativa, on two HCC cell lines (HepG2 and SNU-449). TQ has previously demonstrated promising anticancer activities against various cancers, including prostate, renal, skin, pancreatic, colorectal, cervical, breast, leukemia, and hepatic cancers [34-36]. Here, we present evidence that the combination therapy of PTX and TQ exhibits significantly enhanced antitumor effects in terms of reducing HCC cell viability, inducing cell cycle arrest, and promoting apoptosis compared to PTX monotherapy. Furthermore, our results suggest that p53 knockdown may sensitize HCC cells to both mono- and combined treatments involving PTX and TQ.

Materials and methods

Cell Lines and Reagents:

The HepG2 cells (catalog no. HB-8065; ATCC) and SNU-449 cells (catalog no. CRL-2234; ATCC) were cultured in Eagle’s Minimum Essential Medium (EMEM) (catalog no. 30-2003; ATCC) supplemented with 10% fetal bovine serum (FBS) (catalog no. 098105; Multicell) and RPMI-1640 medium (catalog no. 30-2001; ATCC) supplemented with 10% FBS, respectively. Both cell types were incubated at 37 °C, 95% O2, and 5% CO2. PTX (catalog no. T7402; Millipore Sigma) and TQ (catalog no. 274666; Millipore Sigma) were dissolved in dimethyl sulfoxide (DMSO) (catalog no. D5879; Sigma-Aldrich) at a concentration of
100 mM. Thiazolyl blue tetrazolium bromide (MTT) (catalog no. M-5655; Sigma) was used. Antibodies against caspase-3 (catalog no. AAP-113E; Stressgen), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (catalog no. 4699-9555; Biogenesis), and p53 (catalog no. ab26; Abcam) were utilized. Secondary antibodies used were peroxidase-affiniPure goat anti-mouse IgG (catalog no. 115-035-003; Jackson ImmunoResearch) or peroxidase-conjugated goat anti-rabbit IgG (catalog no. 111-035-003). P53 siRNA (catalog no. J-003329-14-0005; Dharmacon) and non-specific control siRNA (catalog no. D-001810-10-05; Dharmacon) were used for gene knockdown experiments. Lipofectamine RNAiMAX transfection reagent (catalog no. 13778-075; Life Invitrogen) was used for siRNA transfection.

**Tetrazolium Dye MTT and Cell Viability Assay:**

HepG2 and SNU-449 cells were seeded in 48-well cell culture plates at a density of 35,000 cells per well. The cells were incubated in serum-free RPMI overnight before treatment. PTX and TQ were added to the culture medium at different concentrations, either individually or in combination. Dimethyl sulfoxide (DMSO) was used as the vehicle control in all treatments, and the concentration of DMSO did not exceed 0.1% (v/v) of the culture medium. After incubating for 24 hours, the culture medium was removed, and MTT was added at a final concentration of 0.1 mg/ml. The plates were incubated at 37 °C, 95% O2, and 5% CO2 for 2 hours. The medium was then removed without disrupting the cell monolayers, and MTT solvent (4 mM HCl, 0.1% Nondet p-40 in isopropyl alcohol) was added to dissolve the MTT formazan crystals. The plates were incubated in the dark on a rocker for 15 minutes before measuring the absorbance at 590 nm using a SPECTRAmax PLUS384 Microplate spectrophotometer [37, 38].

**Propidium Iodide (PI) Staining and Cell Cycle Analysis:**

HepG2 and SNU-449 cell lines were treated as described above, and the cells were fixed in 70% ethanol for 30 minutes. After fixation, the cells were washed twice in PBS for 5 minutes each. RNase A (catalog no. 1007885; Qiagen) was added at a final concentration of 100 μg/ml, and the cells were incubated at 37 °C for 20 minutes. Following the RNA digestion step, cells were washed twice in PBS for 5 minutes. Propidium iodide (PI) nucleic acid staining was performed by adding 3 μM PI dissolved in staining buffer (100 mM Tris, pH 7.4, 150 mM NaCl, 1 mM CaCl2, 0.5 mM MgCl2, 0.1% Nonidet P40), and the cells were incubated at room temperature for 15 minutes. Cell cycle analysis was carried out using the Guava EasyCyte flow cytometer (Merck Millipore, Germany), and data analysis was conducted using WinMDI 2.8 (Purdue University Cytometry Laboratories software) [39].

**Annexin V Staining and Apoptosis Quantification:**

Following the indicated treatment, harvested HepG2 and SNU-449 cell lines were washed twice in PBS and resuspended in annexin V binding buffer (10 mM Hepes (pH 7.4), 140 mM NaCl, and 0.25 mM CaCl2). Annexin V-FITC conjugated (catalog no. A13199; Thermo Fisher) was added at a concentration of 1:100 and incubated with the cells in the dark for 15 minutes at room temperature. Stained cells were washed twice in the annexin V binding buffer and fixed in 1% formaldehyde prepared in the annexin V binding buffer for 10 minutes on ice. Cells were then washed twice in PBS. Annexin V positivity and data
analysis were conducted using the Guava EasyCyte flow cytometer (Merck Millipore, Germany) and WinMDI 2.8 software [40].

Cell Lysate Preparation and Western Blot Analysis:

After the treatments, the culture medium was removed, and HepG2 and SNU-449 cells were harvested in Radioimmunoprecipitation assay buffer (RIPA) containing a 1X protease inhibitor cocktail (catalog no. PI-78439c; Thermo Scientific). To determine the total protein concentration, the BioRad protein assay (catalog no. 500-0006; BioRad) was utilized following the manufacturer's protocol. Protein denaturing was carried out using Laemmli buffer 2X (SDS, 4%; β-mercaptoethanol, 10%; glycerol, 20%; bromophenol blue, 0.004%; Tris-HCl, 0.125 M), 1:1 (v/v), and samples were boiled at 95 °C for 5 min. Protein samples (50 µg) were loaded into 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). BLUelf pre-stained protein ladder (catalog no. PM008-0500; FroggaBio) was used as a molecular weight marker. Separated protein bands were transferred to a nitrocellulose membrane (catalog no. rpn203D; EG Healthcare). Membranes were immune-probed with rabbit polyclonal anti-caspase-3, anti-p53, and anti-GAPDH antibodies. To detect the immune-probed protein bands, peroxidase-conjugated goat anti-rabbit IgG secondary antibodies were used. Band visualization and densitometric analysis were carried out using Pierce ECL Western Blotting Substrate, ChemiDoc XRS system, Image Lab 6.0 software (BioRad), and TotalLab TL120 Quant software.

RNA Extraction, cDNA Synthesis, and RT-PCR Analysis:

HepG2 and SNU-449 cell lines were grown in 24-well plates. After treatment, total RNA was purified using NucleoSpin RNA purification kits (catalog no. 740955-250; D-MARK Biosciences) following the manufacturer's instructions. For cDNA synthesis, 500 ng of total RNA and a qScript cDNA Synthesis kit (catalog no. CA101414-098; Quanta Biosciences) were used. RT-PCR was performed using Fast SYBR Green master mix (catalog no. 4385618; Life Technologies) and Step One Plus thermal cycler (Applied Biosystems, Foster City, CA). PCR program parameters were 95°C for 20 seconds followed by 40 cycles of (denaturing at 95°C for 20 sec, annealing at 60°C for 40 sec, and extension at 72°C for 40 sec) and a final extension at 72°C for 5 min. Primer sequences used to amplify the P53 gene were as follows: forward 5´ CTC CTG GCC CCT GTC ATC TTC 3´, reverse 5´ AGC GCC TCA CAA CCT CCG TCA T 3´, and for the GAPDH as an internal control: forward 5´ GAG CTG AAC GGG AAG CTCA CTG G 3´, reverse 5´ CAA CTG TGA GGA GGG GAG ATT CAG 3´.

siRNA Transfection:

For siRNA transfection, HepG2 and SNU-449 cells were plated in 12-well plates at a cell density of 150,000 cells per well in an antibiotic-free medium overnight. Cells were transfected with 25 nM P53 siRNA or non-specific control siRNA using Lipofectamine RNAiMAX transfection reagent according to the manufacturer's instructions. After 48 hours, cells were assayed for p53 protein knockdown using western blot analysis [41].
Statistical Analysis:

Data were obtained from n independent biological experiments and depicted as plots of the mean of individual values with SD error bars, or box-and-whisker plots showing the median, the 25th and 75th quartiles, as well as the minimum to maximum values, stacked bars, or violin plots. Western blot densitometric analysis was carried out using the ChemiDoc XRS system Image Lab 6.0 (BioRad) and TotalLab TL120 software. GraphPad Prism 9 was used for statistical analyses and graph generation. Student's t-test (for two-group comparison) or one-way analysis of variance (ANOVA) with Tukey's correction (for multiple comparisons) were used to determine the significant difference between PTX, TQ single treatment, PTX and TQ combined treatments, and non-stimulated (NS) control cells.

Results

*TQ* demonstrates Additive Enhancement of PTX-Induced Reduction in Cell Viability:

To evaluate the antitumor effects of *TQ* in combination with *PTX* on HCC cell lines, we first determined the half-maximal effective concentration (EC50) of *PTX* and *TQ* in HepG2 and SNU-449 cell lines (Figs. 1A and 1B). Our data analysis revealed that the effects of *PTX* and *TQ* on cell viability were dependent on the dose and cell type. *PTX* treatment resulted in a dose-dependent reduction in cell viability in both HepG2 and SNU-449 cells, with a more potent effect observed in HepG2 cells (EC50 = 285 nM) compared to SNU-449 cells (EC50 = 491 nM). Similarly, *TQ* exhibited a more prominent effect on reducing cell viability in HepG2 cells (EC50 = 95.88 µM) compared to SNU-449 cells (EC50 = 449 µM).

To further investigate the combined effect of *PTX* and *TQ* on HCC cell lines, we treated the cells with a fixed concentration of *PTX* (100 nM) and different concentrations of *TQ* (1, 5, 10, and 100 µM).

In HepG2 cells, *PTX* treatment significantly reduced cell viability to 68.17% (p ≤ 0.003), and the addition of *TQ* dramatically enhanced the effect of *PTX*. The highest concentration of *TQ* (100 µM) combined with *PTX* resulted in a substantial reduction of cell viability to 2.92% (p ≤ 0.0001) (Fig. 1C, left panel). In contrast, the SNU-449 cell line showed relative resistance to *PTX* and the *PTX*-TQ combination treatments compared to HepG2 cells. *PTX* treatment alone (100 nM) reduced SNU-449 cell viability to 83.59% (p ≤ 0.003), and the addition of *TQ* improved the effects of *PTX* in a dose-dependent manner. The highest concentration of *TQ* (100 µM) combined with *PTX* (100 nM) reduced SNU-449 cell viability to 23.72% (p ≤ 0.0002) (Fig. 1C, right panel).

*TQ* Enhances PTX-Induced Cell Cycle Arrest and Apoptosis:

To investigate the effect of *PTX* and *TQ* on cell cycle progression, flow cytometry analysis was performed on HepG2 and SNU-449 cell lines stained with PI following treatment with *PTX* or *TQ* alone or in combination. Consistent with previous studies, *PTX* treatment induced G2/M cell cycle arrest in both HepG2 and SNU-449 cells, with a significant increase in the G2/M cell population compared to non-stimulated control cells (NS). In HepG2 cells, the G2/M cell population increased to 137.08% (p ≤ 0.01),
while in **SNU-449** cells, it increased to 134.01% (p ≤ 0.01). **PTX** treatment also had a significant effect on reducing the G1 cell population in **HepG2** cells (61.21%, p ≤ 0.05), but not in **SNU-449** cells. No significant changes were observed in the S cell population in either cell line (Figs. 2A and 2B).

Interestingly, treatment with **TQ** alone (10 µM) showed a similar effect to **PTX**, inducing cell cycle arrest with a significant increase in the G2/M cell population in both **HepG2** (114.56%, p ≤ 0.05) and **SNU-449** cells (126.29%, p ≤ 0.05). However, **TQ** treatment did not affect the G1 or S cell populations in either cell line.

Furthermore, the combined treatment with **PTX** and **TQ** resulted in differential effects on cell cycle distribution in the two cell lines (Figs. 2A and 2B). In **HepG2** cells, the cell cycle arrest was shifted to the S phase, with a significant increase in the S population by 158.25% (p ≤ 0.01). In contrast, the combination of **PTX** and **TQ** induced a G2/M-phase cell cycle arrest in **SNU-449** cells, with a significant increase in the G2/M cell population by 165.81% (p ≤ 0.001). Notably, the combination treatment also led to a significant reduction in the G1 cell population in **SNU-449** cells (67.92%, p ≤ 0.001), which was not observed in **HepG2** cells. This is in contrast to the effect of **PTX** treatment alone, where the G1 population was reduced to 61.21% (p ≤ 0.05) in **HepG2** cells.

**Analysis of Cell Apoptosis by Annexin V and Flow Cytometry:**

Apoptosis analysis was performed using annexin V staining and flow cytometry to detect and quantify apoptotic cells following **PTX** and **TQ** mono- or combined treatment. Our data demonstrated that **PTX** treatment alone (100 nM) induced apoptosis in both **HepG2** (33.8%, p ≤ 0.001) and **SNU-449** cells (18.4%, p ≤ 0.001). Similarly, **TQ** treatment alone (10 µM) showed a comparable apoptotic effect to **PTX** in both cell lines, with 29.8% (p ≤ 0.001) and 17% (p ≤ 0.001) apoptotic cells observed in **HepG2** and **SNU-449** cells, respectively. Importantly, the combined treatment with **PTX** and **TQ** potentiated the apoptotic effect, leading to a significant increase in the percentage of apoptotic cells in both **HepG2** (64%, p ≤ 0.001) and **SNU-449** cells (52.8%, p ≤ 0.001) (Figs. 3A and 3B).

Furthermore, we monitored caspase-3 activation through western blot analysis of pro-caspase-3 (32 kDa) and cleaved caspase-3 (20 and 17 kDa) protein bands. **PTX** treatment, and to a lesser extent **TQ** treatment, resulted in a decrease in caspase-3 cleavage in both **HepG2** and **SNU-449** cells, indicating ongoing apoptosis. Consistently, the combined treatment with **PTX** and **TQ** showed augmented effects, as evidenced by the reduced intensity of pro-caspase-3 bands and increased intensity of active caspase-3 bands compared to the single treatment groups (Figs. 4A and 4B).

**TQ additively enhanced **PTX**-induced P53 mRNA and protein expression in HCC cells:**

To investigate the impact of **PTX** and **TQ** on P53 mRNA and protein expression in **HCC** cells, we treated **HepG2** and **SNU-449** cells with **PTX** (100 nM) and **TQ** (10 µM), either individually or in combination for 24 hours. Subsequently, we extracted mRNA and protein for analysis using RT-PCR and western blot, respectively. Our data revealed that in **HepG2** cells, **PTX** and **TQ** significantly increased the mRNA
expression of P53 by 2.76-fold (p ≤ 0.01) and 1.54-fold (p ≤ 0.05), respectively, compared to the non-stimulated (NS) control.

Interestingly, the combined treatment resulted in an additive effect, leading to a 4.31-fold increase in P53 mRNA (p ≤ 0.001) (Fig. 5A). PTX treatment alone also increased the expression of p53 protein by 2.24-fold (p ≤ 0.001). However, TQ single treatment did not affect p53 protein levels in HepG2 cells. Notably, the combined treatment of PTX and TQ resulted in a substantial increase in p53 protein expression by 3.15-fold (p ≤ 0.001) (Fig. 5B).

In SNU-449 cells, PTX treatment alone induced a 1.45-fold increase in P53 mRNA expression (p ≤ 0.01), while TQ single treatment did not show a significant change (Fig. 5A). However, the combination of PTX and TQ potentiated the effect of PTX, leading to a 4.31-fold increase in P53 mRNA (p ≤ 0.001). Consistent with the mRNA levels, only PTX treatment alone and the combination of PTX and TQ exhibited incremental effects on p53 protein levels, with increases of 2.18-fold (p ≤ 0.05) and 3.28-fold (p ≤ 0.001), respectively, while TQ single treatment did not significantly impact p53 protein levels (Fig. 5B).

**P53 siRNA sensitizes HCC cells to PTX and TQ-induced reduction in cell viability:**

To explore the role of P53 in PTX- and TQ-induced apoptosis in HCC cells, we transfected the cells with P53 siRNA and examined the levels of p53 protein using western blot analysis (Fig. 6A). Our data confirmed that P53 siRNA knockdown reduced the expression of p53 protein by 73.5% (p ≤ 0.001) and 62.6% (p ≤ 0.001) in HepG2 and SNU-449 cell lines, respectively.

To compare the effects of PTX and TQ on cell viability in HCC cells transfected with control siRNA (ctrl-siRNA) or P53 siRNA (P53-siRNA), we assessed cell viability (Fig. 6B). P53 knockdown did not significantly affect cell viability when compared to cells transfected with ctrl-siRNA in the absence of treatment. In the case of PTX or TQ treatment alone, the number of viable cells did not significantly differ between ctrl-siRNA and P53-siRNA transfected cells in both tested cell lines. However, P53-siRNA showed a significant difference in cell viability compared to ctrl-siRNA-transfected cells in response to the combined treatment of PTX and TQ. Cell viability decreased to 46.71% (p ≤ 0.01) and 60.19% (p ≤ 0.01) in HepG2 and SNU-449 cells, respectively. These findings suggest that P53 may play a role in PTX and TQ-induced apoptosis in HCC cells.

**Discussion**

Surgical resection and liver transplantation are essential treatment strategies for HCC patients, particularly those who are ineligible for chemotherapy [42, 43]. However, the limited availability of donors and late-stage diagnosis restrict the number of patients who can benefit from these procedures. Chemotherapy, therefore, plays a crucial role in the treatment of HCC due to its accessibility and immediate availability. However, drug resistance in HCC remains a significant challenge. Understanding the molecular mechanisms underlying HCC can help overcome this obstacle and develop innovative treatments. Combination therapy, which involves administering multiple chemotherapeutic agents
together, has emerged as a key approach in cancer treatment, significantly improving patient survival rates [33, 44, 45].

In this study, we investigated the potential of combining PTX and TQ to enhance the apoptotic effects of PTX in HepG2 and SNU-449 cell lines. While PTX has been used for the treatment of HCC and other cancer types for a long time, the therapeutic outcomes of PTX monotherapy often fall short of expectations due to the development of drug resistance over time. Resistance can arise from inherent genetic alterations or prolonged exposure to the drug [30, 46, 47]. Previous studies have explored the combination of PTX with other drugs, such as doxorubicin, wilfortrine, Sorafenib, cisplatin, interferon alfa-2b, and 5-fluorouracil, and have shown promising progress in treating HCC [48-51]. TQ has also been studied in the context of HCC treatment and has been found to induce apoptosis in HepG2 cells [52]. Our data demonstrate that combining PTX with TQ achieves the desired apoptotic effect of PTX at lower doses compared to PTX monotherapy.

Regarding cell cycle arrest, it is well known that PTX causes arrest in the G2/M phase [53], while TQ-induced arrest in the S phase has been observed in T47D breast cancer cells [54]. However, our findings in the current study demonstrate that both PTX and TQ individually induce cell cycle arrest in the G2/M phase in both HepG2 and SNU-449 cell lines, suggesting genetic differences and heterogeneity among cancer cell lines, which can contribute to variations in drug resistance [55]. Interestingly, the combination treatment results in cell cycle arrest in the S phase for HepG2 and the G2/M phase for SNU-449. TQ appears to stimulate dormant HepG2 cells, promoting their entry into the S phase and subsequently the G2/M phase, thereby sensitizing them to PTX treatment [56]. Şakalar et al. [57] have previously shown that the combination of PTX and TQ increases the expression of several apoptosis-related genes in triple-negative breast cancer.

Consistently, our combined treatment of PTX and TQ enhances the apoptotic effects and increases apoptosis rates by 3-fold and 2.5-fold in HepG2 and SNU-449 cells, respectively, compared to the non-stimulated control cells. These findings were further confirmed by caspase-3 activation, a hallmark of apoptosis.

P53 is a well-known tumor suppressor gene with various documented roles in suppressing cancer progression. P53 transactivates cyclin-dependent kinase inhibitor 1 A (CDKN1A) to inhibit cellular proliferation in response to DNA damage [58]. P53 induces apoptosis through stimulation of BAX and FAS antigen expression and negative regulation of Bcl2 expression [59]. P53 is also involved in stress-induced necrosis in cooperation with mitochondrial peptidylprolyl isomerase F (PPIF) [60]. TQ is an important molecule that affects epigenetic properties, such as histone acetylation and deacetylation, as well as DNA methylation and demethylation [61, 62]. TQ also plays a role in the activation and deactivation of noncoding RNA, acting as a potent apoptosis-inducing enzyme through histone acetylation and deacetylation [63–65].

Our data demonstrate that PTX and TQ increase the levels of P53 mRNA and protein in HCC cell lines. This observation leads to the hypothesis that P53 may play a role in PTX and TQ-induced apoptosis in
HepG2 and SNU-449 cell lines. To test this hypothesis, we compared the effects of PTX and TQ on cell viability in two cell models: one with normal P53 expression and the other with significantly downregulated P53. Transfection with P53 siRNA successfully reduced P53 protein levels in both cell types, as confirmed by western blot analysis. Surprisingly, P53 knockdown had no significant effect on the reduction of cell viability induced by PTX or TQ when administered individually. However, a significant difference was observed between cells transfected with control nontargeting siRNA and P53 siRNA after the combination treatment of PTX and TQ, where cell viability values significantly decreased with the reduction of p53 protein levels. These observations may be explained by the protective role of P53 in postponing mitosis to allow cells to restore their genomic integrity, thereby delaying cell death [66]. Additionally, our results are consistent with the findings of Jehan et al. [28], who proposed that TQ-induced induction of reactive oxygen species (ROS) was more prominent in P53-depleted HepG2 cells.

Our findings suggest that P53 could be a significant player in HCC resistance to chemotherapy. With the recent advancements in systemic delivery of siRNA technologies, such as lipid nanoparticle-encapsulated RNA molecules [67], it may be possible to utilize the results of this study in designing an innovative therapeutic regimen that combines traditional antitumor agents with P53 siRNA for improved therapeutic outcomes.

Several potential down-regulatory mechanisms of P53 that could be targeted for therapeutic approaches include:

1. MDM2 (mouse double minute 2): MDM2 protein is a critical negative regulator of P53 that promotes its degradation. Inhibiting the interaction between MDM2 and P53 or blocking MDM2 function can stabilize P53 levels and enhance its tumor-suppressive activities [68, 69].

2. MDMX (mouse double minute X): MDMX protein, also known as MDM4, is another negative regulator of P53 that inhibits its activity. Targeting MDMX can relieve the inhibitory effect on P53, leading to increased P53 function [70, 71].

3. E3 ligases: Certain E3 ligases, such as COP1 (constitutive photomorphogenesis 1) and Pirh2 (p53-induced RING-H2 protein), can ubiquitinate P53, resulting in its degradation. Inhibiting these E3 ligases can prevent P53 degradation and enhance its stability [72, 73].

4. P53 inhibitors: Some viral proteins, like human papillomavirus E6 (HPV E6) and simian vacuolating virus 40 (SV40) large T antigen, can directly bind to P53 and inhibit its activity. Targeting these P53 inhibitors can restore P53 function and enhance its tumor-suppressive effects [74, 75].

5. DDR (DNA damage response) pathway components: The DDR pathway, including kinases such as ATM (ataxia telangiectasia mutated) and CHK2 (checkpoint kinase 2), is crucial for activating P53 in response to DNA damage. Dysregulation of these DDR components can impair P53 activation. Targeting these components or their upstream regulators can restore the DDR pathway and promote P53-mediated tumor suppression [68, 76].

6. miRNAs (microRNAs): Certain microRNAs, such as miR-125b and miR-504, can directly target and down-regulate P53 expression. Inhibiting these miRNAs or using miRNA antagonists can increase
7. **Nutlin-3**: Nutlin-3 is a small molecule that disrupts the interaction between p53 and MDM2, leading to p53 stabilization and activation. It has been explored as a potential therapeutic agent to restore p53 function in cancer cells [73, 74].

These *down-regulatory* mechanisms of *p53* represent potential therapeutic targets for developing strategies to restore or enhance *p53* activity in cancers with wild-type *p53*, thereby promoting tumor suppression and improving treatment outcomes.

The potential role of *TQ* in modulating the p53 pathway involves its ability to influence the expression and activation of *p53*, a tumor suppressor protein that plays a crucial role in regulating cell cycle arrest, DNA repair, and apoptosis. Here are some key points regarding the interaction between *TQ* and the *p53* pathway:

1. **Activation of p53**: *TQ* has been reported to activate the *p53* pathway in various cancer cell lines. It can induce the expression and stabilization of the *p53* protein, leading to its activation as a transcription factor. Activated *p53* can then initiate downstream cellular responses, including cell cycle arrest and apoptosis [77].

2. **Cell cycle arrest**: *TQ* has been shown to induce cell cycle arrest at different phases, including the G1, S, and G2/M phases. The modulation of *p53* by *TQ* is involved in mediating cell cycle arrest. Activated *p53* can promote the transcription of target genes such as p21, which inhibits cyclin-dependent kinases (CDKs) and subsequently halts cell cycle progression [78, 79].

3. **Apoptosis induction**: *TQ* has demonstrated the ability to induce apoptosis in cancer cells, and its interaction with the *p53* pathway is implicated in this process. Activated *p53* can upregulate the expression of pro-apoptotic genes, such as Bax, PUMA, and NOXA while downregulating anti-apoptotic genes like Bcl-2. This shift in the balance of pro- and anti-apoptotic molecules promotes apoptosis execution [80, 81].

4. **DNA damage response**: *TQ* has been reported to induce DNA damage in cancer cells, which can activate the *p53* pathway. DNA damage activates signaling kinases such as ATM and ATR, which phosphorylate and stabilize *p53*. Stabilized *p53* can then initiate DNA repair mechanisms or, if the damage is irreparable, trigger cell cycle arrest or apoptosis [82, 83].

5. **Inhibition of p53 mutations**: In some cancer types, *p53* mutations are common and can lead to the loss or dysfunction of *p53* activity. *TQ* has shown potential in restoring the function of mutant *p53* by promoting its proper folding and preventing its degradation. This restoration of *p53* function can enhance its tumor-suppressive activities [84, 85].

It is important to note that the detailed mechanisms underlying the interaction between *TQ* and the *p53* pathway are still being investigated, and further research is needed to fully elucidate the molecular events involved. Nonetheless, the modulation of the *p53* pathway by *TQ* represents a promising avenue for
enhancing the therapeutic efficacy of anticancer treatments, such as PTX, in the management of hepatocellular carcinoma and other cancer types.

The potential side effects of PTX and TQ combination therapy:

The potential side effects of combining PTX and TQ therapy may include:

1. **Hematological Toxicity**: PTX is known to cause bone marrow suppression, leading to decreased levels of white blood cells, red blood cells, and platelets. This can result in an increased risk of infection, anemia, and bleeding [86].

2. **Gastrointestinal Effects**: Both PTX and TQ can cause gastrointestinal side effects such as nausea, vomiting, diarrhea, and abdominal pain. These symptoms may vary in severity and can affect the patient's quality of life [87].

3. **Neurotoxicity**: PTX is associated with peripheral neuropathy, causing symptoms such as tingling, numbness, and pain in the hands and feet. This side effect can be dose-dependent and may affect the patient's daily activities [88].

4. **Hepatotoxicity**: TQ has been reported to exhibit hepatoprotective effects in some studies. However, there are also reports suggesting potential hepatotoxicity with TQ usage [89].

5. **Cardiotoxicity**: PTX has been associated with cardiac side effects, including arrhythmias, myocardial ischemia, and heart failure. These effects are rare but can occur, particularly in patients with pre-existing cardiac conditions [90].

6. **Allergic Reactions**: Both TQ and PTX have the potential to cause allergic reactions, ranging from mild skin rashes to severe anaphylaxis. Patients with a history of hypersensitivity should be closely monitored during therapy [86].

It's important to note that the occurrence and severity of these side effects can vary among individuals, and the specific dose and duration of therapy can also influence their likelihood. Close monitoring and proper management of side effects are essential to ensure patient safety and treatment efficacy.

**Conclusion**

This study sheds light on the potential of combination therapy using PTX and TQ to improve the treatment outcomes of HCC. The study highlights the importance of understanding the molecular mechanisms underlying HCC and drug resistance to develop innovative therapeutic strategies.

It emphasizes the significance of surgical resection and liver transplantation as essential treatment options for HCC patients, particularly those ineligible for chemotherapy. However, the limited availability of donors and late-stage diagnosis restrict the number of patients who can benefit from these procedures. Chemotherapy remains a crucial treatment modality due to its accessibility and immediate availability. Nevertheless, drug resistance poses a significant challenge in HCC treatment.
Combination therapy has emerged as a key approach in cancer treatment, significantly improving patient survival rates. The study investigates the combination of PTX and TQ to enhance the apoptotic effects of PTX in HepG2 and SNU-449 cell lines. While PTX has been used for HCC treatment, its monotherapy often falls short of expectations due to the development of drug resistance. Previous studies have explored the combination of PTX with other drugs and demonstrated promising progress in treating HCC.

The study revealed that the combination of PTX and TQ achieves the desired apoptotic effect of PTX at lower doses compared to PTX monotherapy. It also elucidates the role of cell cycle arrest in the effectiveness of the combination therapy. The combination treatment results in different cell cycle arrest phases in different cell lines, indicating genetic differences and heterogeneity among cancer cell lines, which can contribute to variations in drug resistance. Additionally, the combination treatment enhances apoptotic effects and increases apoptosis rates compared to control cells, as confirmed by caspase-3 activation.

The study also focused on the role of P53, a well-known tumor suppressor gene, in PTX and TQ-induced apoptosis. The study demonstrates that PTX and TQ increase the levels of P53 mRNA and protein in HCC cell lines. It explored the effects of P53 knockdown on cell viability and revealed that P53 may play a role in the apoptotic effects induced by the combination treatment of PTX and TQ. These findings suggest that P53 could be a significant player in HCC resistance to chemotherapy, and targeting P53 could improve therapeutic outcomes.

The potential down-regulatory mechanisms of P53 could be targeted for therapeutic approaches. These mechanisms include inhibiting the interaction between MDM2 and P53, targeting MDMX, inhibiting E3 ligases, targeting P53 inhibitors, restoring DDR pathway components, and inhibiting specific miRNAs. These targets represent potential avenues for developing strategies to restore or enhance P53 activity in cancers with wild-type P53, thereby promoting tumor suppression and improving treatment outcomes.

Furthermore, the study explored the potential role of TQ in modulating the P53 pathway. TQ has been reported to activate P53, induce cell cycle arrest, promote apoptosis, and interact with the DNA damage response. It has also shown potential in restoring the function of mutant P53. However, further research is needed to fully elucidate the molecular events involved in the interaction between TQ and the P53 pathway.

Further research and clinical investigations are warranted to validate these findings and translate them into clinical practice, ultimately benefiting HCC patients who face limited treatment options.

Abbreviations

TQ: thymoquinone; PTX: Paclitaxel/ or Taxol; HCC: Hepatocellular carcinoma; HepG2: Hepatoma cell lines; SUN-449: a primary hepatocellular carcinoma; NS: non-stimulated control cells; P53: tumor suppressor gene; DMSO: Dimethyl sulfoxide; MTT: Thiazolyl blue tetrazolium bromide; RIPA: Radioimmunoprecipitation assay buffer; SDS-PAGE: Sodium dodecyl sulfate-Polyacrylamide gel
Declarations

Ethical Approval Approval was granted by the Ethics Committee of Sohag University, College of Medicine, under code No. (91-12, Sohag, Egypt).

Consent for publication is Not applicable.

Competing interests The authors declare no competing interests.

Availability of data and materials: The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Author Contribution
T.N.H, M.O.A, and M.S.S provided supervision, designed the study, analyzed the data, and contributed to the discussion. D.R. contributed to the methodology and data collection. T.N.H and M.O.A provided writing review, editing, and data analysis. All authors have reviewed and approved the final manuscript.

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References


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**Figures**

![Figure 1](image-url)
The effect of PTX and TQ, mono- and combined-treatment on HepG2 and SNU-449 cell viability. The MTT assay illustrates the dose-response curves of PTX (A) and TQ (B) and demonstrates the EC50 values of both drugs. TQ dramatically enhances PTX-induced reduction of cell viability in HepG2 and SNU-449 cells after combined treatment (C). The MTT assay was conducted 24 hours post-treatment, and absorbance was measured at 590 nm using the SPECTRAmax® PLUS384 microplate Spectrophotometer. The data (n = 5-6) are expressed as scatter plots or boxes and whiskers using GraphPad Prism software. ns P ≥ 0.05, **P ≤ 0.01, ***P ≤ 0.001 vs. NS control cells.

Figure 2

Cell cycle analysis of the impact of PTX and TQ mono and combined treatments on the distribution of cell cycle growth phases. HepG2 (A) and SNU-449 (B) were treated with PTX (100 nM) and/or TQ (10 µM) for 24 hours. Cell cycle analysis was conducted in quadruplicates using the Guava EasyCyte Flow Cytometer (Merck Millipore, Germany). Data analysis was carried out using WinMDI 2.8 and GraphPad Prism software. The data are represented as histograms (left panels) and stacked bars (right panels). Statistical significance was determined between different treatment groups using an ANOVA with Tukey’s
Figure 3

TQ augmented the apoptotic effect of PTX on HCC cells. Flow cytometry analysis of annexin V-labeled HepG2(A) and SNU-449 cells (B). Cells were treated with PTX (100 nM), TQ (10 μM), or the combination for 24 hours. Annexin V-positive cells were quantified with the Guava EasyCyte flow cytometer (Merck Millipore, Germany). The data analysis was carried out using WinMDI 2.8 and GraphPad Prism software. Data are represented as histograms (left panels) and box and whisker plots (right panels). Statistical significance was determined between different treatment groups using an ANOVA with Tukey’s correction for multiple comparisons or using the Student’s t-test to determine the significant difference between two different treatments. ***P ≤ 0.001 vs. NS control cells; **P ≤ 0.01 vs. PTX mono treatment.
Figure 4

PTX and TQ effects on caspase3 activation in HCC cell lines. HepG2 (A) and SNU-449 cells (B) were treated with PTX (100 nM) and TQ (10 μM) for 24 h. Cells were harvested, and cell lysates were electrophoresed, trans-blotted, and immune-probed against caspase3 (pro- [32 kDa] and cleaved [17-20 kDa] caspase) and GAPDH (loading control). Protein bands were visualized using the ChemiDoc XRS and Image Lab 6.0 software (BioRad). Densitometric analyses were conducted using TotalLab TL120 Quant software. Representative data are shown from quadruplicate biological experiments.
Figure 5

The effect of PTX and TQ on P53 mRNA and protein expression in HCC cells. HepG2 and SNU-449 cells were treated with PTX (100 nM) and TQ (10 μM), either individually or in combination, for 24 hours. P53 mRNA was quantified using qRT-PCR(A). p53 protein levels were analyzed using western blot and densitometric analysis (B). The data (n = 5) are depicted as a violin plot using GraphPad Prism software. Statistical significance was determined between different treatment groups using an ANOVA with Tukey's correction for multiple comparisons or using the Student's t-test to determine the significant difference between two different treatments. ns P ≥ 0.05, *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001 vs. NS control cells; **P ≤ 0.01 vs. PTX single treatment.
Figure 6

**P53-siRNA knockdown potentiated PTX and TQ-induced reductions in cell viability.** HepG2 and SNU-449 cells were transfected with ctrl-siRNA or P53-siRNA. p53 protein expression was assayed using Western blot and densitometric quantification (A). The MTT cell viability assay demonstrates the effect of ctrl-siRNA and P53-siRNA on the reduction in viable cell numbers induced by PTX and/or TQ (B). Data (n = 5) are depicted as a scatter plot with SD ± (A) or a violin plot (B) using GraphPad Prism software. Statistical significance was determined between different treatment groups using an ANOVA with Tukey’s correction for multiple comparisons or using the Student’s t-test to determine the significant difference between two different treatments.
Supplementary Files

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- TymoquinoneDataV31.pptx
- molecules2500426.pdf