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Decreased Autophagic Activity in Triple Negative Breast Cancer Cells upon Hydroxychloroquine and Thymoquinone Combination Treatment

Triple Negatif Meme Kanseri Hücrelerinde Hidroksiklorokin ve Timokinon Kombinasyon Uygulaması Sonrası Otofajik Aktivitenin Azalması

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Decreased Autophagic Activity in Triple Negative Breast Cancer Cells upon Hydroxychloroquine and Thymoquinone Combination Treatment

ABSTRACT

Objective: Autophagy plays a significant role in breast cancer tumorigenesis, including triple-negative breast cancer. Research indicates that hydroxychloroquine and thymoquinone modulate autophagy, potentially suppressing its activity. However, their combined effects on autophagy in triple-negative breast cancer remain unexplored. In this study, we investigated the potential anti-cancer and autophagy-modulating effects of hydroxychloroquine-thymoquinone combination on triple-negative breast cancer cells in vitro.

Material and Method: The viability of MDA-MB-231 cells was evaluated after treatment with hydroxychloroquine (10-210 µM) and thymoquinone (5-45 µM) for 24 and 48 hours using the WST-1 assay. Combination effects were analyzed using the Chou-Talalay method with CompuSyn (v.10). Autophagic vesicles were visualized using an Autophagy Detection Kit and fluorescence microscopy to investigate their role in the decrease in cell viability. Statistical analysis was performed with GraphPad Prism (v.8.3.0).

Results: At both 24- and 48-hour intervals post-treatment, a significant decrease in viability was observed for both hydroxychloroquine and thymoquinone treatments individually *($p<0.0001$)*. The combination of these drugs revealed pronounced synergistic effects at 24 hours, whereas antagonistic effects were noted at 48 hours (combination index>1). At 24 hours, favorable dose reduction effects were evident (dose reduction index >1), while the 48-hour results showed an unfavorable reduction (dose reduction index<1). Consequently, the 24-hour synergistic effects resulted in a reduction in autophagic vesicles *($p<0.0001$)*.

Conclusion: This study revealed, for the first time, a time-dependent decrease in triple-negative breast cancer cell viability via the autophagy mechanism induced by hydroxychloroquine and thymoquinone, highlighting their novel implications for triple-negative breast cancer treatment and autophagy modulation.

Keywords: Autophagy, breast cancer, drug-combination, hydroxychloroquine, thymoquinone.

ÖZET

Amaç: Otofaji, triple negatif meme kanseri alt tipi dahil olmak üzere meme kanserinde tümör oluşumunda etkili bir mekanizmadır. Araştırmalar, hidroksiklorokin ve timokinon'un otofajiyi düzenleyerek aktivitesini potansiyel olarak baskıladığını göstermektedir. Ancak, bu maddelerin kombine uygulanmasının triple negatif meme kanserinde otofaji üzerindeki etkileri henüz aydınlatılmamıştır. Bu çalışma, hidroksiklorokin ve timokinon kombinasyonunun triple negatif meme kanseri hücrelerinde hücrelerinde antikanser ve otofajik etkilerini in vitro olarak incelemeyi hedeflemektedir.

Gereç ve Yöntem: Hidroksiklorokin (10-210 µM) ve timokinon (5-45 µM)'nun 24 ve 48 saat boyunca MDA-MB-231 hücrelerine uygulanması sonucu hücrelerin canlılığı WST-1 testi ile değerlendirildi. Kombinasyonlarının etkileri, CompuSyn (v.10) ile kombinasyon indeksi ve doz azaltma indeksi hesaplanarak Chou-Talalay yöntemiyle analiz edildi. Hücre canlılığının azalmasındaki otofaji etkisini gözlemlemek adına, otofajik veziküllerin tanımlanması ve görüntülenmesi için "Autophagy Detection Kit" ve floresan mikroskobu kullanıldı. İstatistiksel analizler, GraphPad Prism (v.8.3.0) kullanılarak gerçekleştirildi.

Bulgular: Hidroksiklorokin ve timokinon'un 24 ve 48 saatlik uygulamaları sonucu hücre canlılığında anlamlı bir azalma gözlemlendi *($p<0.0001$)*. Kombinasyon uygulamaları sonucu sinerjistik etkileri 24 saatte belirgin olarak görülürken, 48 saat uygulanmaları sonucu ise antagonistik etkiler elde edildi (kombinasyon indeksi>1). Doz azaltmada olumlu etkiler 24 saatte belirgin iken (doz azaltma indeksi >1), 48 saatte tam tersi etkiler elde edildi (doz azaltma indeksi<1). Sonuç olarak, kombinasyonun 24 saatlik uygulamasıyla karşılaşılan sinerjistik etkilerin otofajik veziküllerı azalttığı gösterildi *($p<0.0001$)*.

Sonuç: Bu çalışma, hidroksiklorokin ve timokinon tarafından indüklenen otofaji mekanizması aracılığıyla triple negatif meme kanseri hücrelerinde zamanla bağımlı canlılık azalmasını ilk kez ortaya koymuştur. Bu kombinasyon ileriki çalışmalarla birlikte triple negatif meme kanseri tedavisi ve otofaji modülasyonunda etkin rol oynama potansiyeli taşımaktadır.

Anahtar Sözcükler: Hidroksiklorokin, ilaç kombinasyonu, meme kanseri, otofaji, timokinon.

Introduction

Triple-negative breast cancer (TNBC), accounting for 15–20% of newly diagnosed breast cancers (BCs), uniquely lacks targeted treatment compared to other BC subtypes. It is characterized by the absence of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor-2 (HER2) expression, and represents a particularly aggressive form of BC. This highly metastatic and heterogeneous disease demonstrates a notably poorer prognosis and relapse rates. Despite considerable research efforts to address TNBC, therapeutic interventions remain insufficient. Thus, there is a critical necessity for the development of innovative approaches. Advancements in comprehending its biological mechanisms, and drug development have facilitated the integration of targeted therapeutic approaches which potentially revolutionize the therapeutic strategies of TNBC (1,2).

Autophagy is a cellular degradation process that involves the breakdown and recycling of long-lived proteins and organelles through lysosomal activity. Autophagy plays a crucial role in both normal physiology and tumor biology. It serves as a tumor suppressor by preventing the accumulation of genetic defects that could lead to tumor formation. However, autophagy can also promote tumor progression, particularly in aggressive cancers with high metabolic demands. In such cases, autophagy provides energy to cancer cells by recycling cellular components as an alternative metabolic pathway in nutrient-deprived and hypoxic environments. Like in other cancer types, autophagy displays a wide-ranging influence in BC, potentially acting as both a promoter and inhibitor of tumor growth at different stages of tumorigenesis (3,4). It functions as both a promoter and inhibitor of metastasis in the BC progression. The role of autophagy in BC presents a challenge for effective therapy, as it can lead to drug and radiotherapy resistance. It promotes tumor stemness, and inhibits apoptosis, contributing to carcinogenesis (5). Autophagy plays a role in maintaining BC stem cells in TNBC through the Epidermal growth factor receptor (EGFR)/ Signal transducer and activator of transcription 3 (STAT3), Transforming growth factor beta (TGFβ)/Sma and Mad related protein (SMAD) and Interleukin-6 (IL6)/ Signal Transducer and Activator of Transcription 3 (STAT3) signaling pathways (3). Autophagy also interacts with tumor microenvironment components like macrophages and can be modulated by anti-tumor compounds like parthenolide and honokiol. Its consideration in BC therapy is essential due to its multifaceted nature, dual pro-survival and pro-death functions, and its crosstalk with critical molecular pathways such as apoptosis (5,6).

Hydroxychloroquine (HCQ) is a compound that is used in the treatment of malaria, lupus erythematosus and rheumatoid arthritis. Recent research supports using HCQ as an adjuvant therapy to enhance cancer treatment effectiveness and mitigate drug resistance. This is attributed to HCQ's ability to inhibit lysosomal acidification and impede autophagy (7). HCQ functions in the later stages of autophagy by elevating the pH in lysosomes. This elevation prevents the fusion between autophagosomes and lysosomes, consequently hindering the degradation of proteins within lysosomes (8). There are numerous phase I and phase II clinical trials investigating the use of HCQ in treating patients with diverse cancer types, including BC. Aggressive tumors such as TNBC exhibit elevated levels of autophagy, which aids in tolerating cellular stress encountered during the metastatic process (9). Elucidating HCQ's effects as an autophagy inhibitor is crucial for addressing treatment challenges in these cancers.

Thymoquinone (TQ) is the most abundant natural bioactive compound found in Nigella sativa, known for its anti-cancer, anti-inflammatory and antioxidant effects (10,11). It has also gained attention for its potential in regulating autophagy. TQ-induced autophagic cell death can either inhibit or induce autophagy, depending on the specific cellular context. Studies have demonstrated that TQ induces autophagic cell death in renal carcinoma, BC and colon cancer (12). In TNBC cell line MDA-MB-231, TQ treatment significantly inhibited cell proliferation, migration, and autophagic activity by suppressing the expression of microtubule associated protein 1 light chain 3 α (LC3, also referred to as MAP1LC3A) and Beclin-1, suggesting its potential as a candidate for controlling autophagic activity in TNBC (13). Conversely, in cells like glioblastoma multiforme, which utilize autophagy as a survival mechanism,

TQ promotes cell death by inhibiting autophagy. It has been shown to inhibit autophagy in glioblastoma cells by disrupting the lysosomal membrane, and inducing cathepsin translocation, leading to caspase-independent apoptosis (11). While the understanding of the relationship between TQ and autophagy mechanism is still limited, studies suggest its ability to modulate autophagy pathways in cancer.

The effects of HCQ and TQ have been studied individually in various cancers both in vitro and in vivo. However, the combined effects of these two compounds on autophagy in cancer have not been previously reported. Therefore, the current study aims to investigate the combined anticancer and autophagy effects of TQ and HCQ on the aggressive BC subtype, TNBC, in vitro.

Material and Methods

Cell Culture

The MDA-MB-231 TNBC cell line was obtained from the American Type Culture Collection (ATCC) and provided by the Proteomics Laboratory of Kocaeli University. Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, USA) supplemented with high glucose and 10% fetal bovine serum (FBS, Gibco, Thermo Fisher Scientific, USA), as well as penicillin (100 U/mL) and streptomycin (100 µg/mL) (Gibco, Thermo Fisher Scientific, USA), were used to culture MDA-MB-231 cells. The cells were maintained in a humidified incubator at 37°C with 5% CO_2 (Thermo Fisher Scientific, USA). Since a commercially available cell line was used, ethical approval was not required.

Preparation of HCQ and TQ

The stock solutions of HCQ (TRC, Canada) and TQ (Glentham Life Science, United Kingdom) were prepared in water and dimethyl sulfoxide (DMSO) (Sigma-Aldrich, USA) at concentrations of 5000 µM and 600 µM, respectively. Next, dilutions were prepared to achieve the desired concentrations of HCQ (10, 30, 60, 90, 120, 150, 180, 210 µM) and TQ (5, 15, 25, 35, 45 µM).

WST-1 Assay

To assess the effects of HCQ and TQ on the viability of MDA-MB-231 cells, WST-1 assay (Roche Applied Science, Indianapolis, IN, USA) was employed. Initially, $1x10^4$ cells in 100 µl were seeded into the wells of a 96-well plate. After 24 hours of incubation at 37°C, the adherent cells were treated with drugs prepared at the designated concentrations for 24 and 48 hours. Subsequently, 10 µL of WST-1 reagent (Roche Applied Science, Indianapolis, IN, USA) was added to each well, and incubated for 2 hours at 37°C in darkness. Cell viability was then determined at a wavelength of 450 nm using a microplate reader, and viability percentages were calculated. The experiment was carried out with three replicates.

Combination of HCQ and TQ

In drug combination trials, the combination effect was examined for doses demonstrating viability percentages within the range of 60-70%, wherein the doses were found not to exert a significant impact on viability, as assessed by the WST assay. For the 24-hour period, we selected doses of 150, 180, and 210 µM HCQ combined with 15 and 25 µM TQ at 37°C. For the 48-hour period, doses of 10 and 30 µM HCQ combined with 15 and 25 µM TQ were applied to the cells at 37°C. Subsequently, the impact of these combinations on cell viability was determined using the WST-1 assay protocol, as provided above. Then, we employed the Chou-Talalay method to analyze the synergistic effects of HCQ and TQ and calculated the combination index (CI) using CompuSyn v.10 (14). Based on this assessment, a CI value below 1 signified synergism, a CI value of 1 suggested additivity, and a CI value exceeding 1 indicated antagonism. We also calculated dose reduction index (DRI) using the CompuSyn software. DRI values were divided into three categories: DRI<1 indicated an unfavorable reduction in dose, DRI=1 indicated no reduction in dose, and DRI>1 indicated a favorable reduction in dose.

Detection and Monitoring of Autophagy

Autophagy Detection Kit (ab139484, Abcam) was used to detect in vitro development of autophagic vesicle upon HCQ and TQ administration. According to the kit protocol, trypsinized cells were washed with 1x Assay Buffer. A staining solution was then prepared using 1:1000 Green Detection Reagent, 1:1000 Hoechst nuclear stain, and 5% FBS in this buffer, and cells were incubated with this solution for 30 minutes for staining at 37°C. Following fixation with 4% formaldehyde for 20 minutes at room temperature, autophagic vesicles were visualized

using a fluorescence microscope (Olympus, Tokyo, Japan). Finally, vesicles in three randomly selected fields were counted and statistically compared between groups.

Statistical Analysis

Statistical analysis was conducted using GraphPad Prism software (version 8.3.0), with data presented as mean ± standard deviation from three independently replicated experiments. Multiple comparisons were assessed through two-way analysis of variance (ANOVA) followed by Tukey's test. Moreover, CompuSyn (version 1.0) software was utilized to investigate the confidence intervals of the CI of HCQ combined with TQ. One-way ANOVA was employed to compare the percentages of autophagic vesicles among groups.

Results

Effect of HCQ and TQ on MDA-MB-231 Cell Viability

The cytotoxic effects of HCQ and TQ on MDA-MB-231 cells were assessed to determine the appropriate concentrations and durations for subsequent combination studies. The findings demonstrated that both HCQ and TQ reduced cell viability in a dose- and time-dependent manner. At 24- and 48-hours following drug application, a statistically significant decrease in viability compared to the negative control was observed from the first dose onwards *($p<0.0001$)*. Following the 24-hour application period, the viability of MDA-MB-231 cells treated with HCQ was 68.2%, 67.4% and 58.4% at doses of 150 µM, 180 µM and 210 µM, respectively. On the other hand, the viability of cells treated with TQ was 69.6%, 57% and 55.3% at doses of 15 µM, 25 µM and 35 µM, respectively. After 48 hours of treatment, MDA-MB-231 cell viability was 69.2% and 63.4% with HCQ doses of 10 µM and 30 µM, respectively. Additionally, TQ-treated cells exhibited viabilities of 65.3% and 56.2% at 15 µM and 25 µM, respectively (Figure I).

Time-dependent Effects of HCQ and TQ Combination on Cell Viability

The findings obtained from individual drug treatments led to the determination of combination doses for the subsequent combination studies. Specifically, doses of 15 and 25 µM for TQ and 150 µM, 180 µM, and 210 µM for HCQ were selected for the 24-hour treatment period, while doses of 15 and 25 µM for TQ and 10 µM and 30 µM for HCQ were identified for the 48-hour treatment period. After analysis of the combination results for 24 hours, a significant reduction in the viability of MDA-MB-231 cells was observed ($p<0.0001$), with viability decreasing to as low as 30%. Conversely, data obtained for the 48-hour period indicated an increase in viability with combination doses ($p<0.0001$) (Figure II). Typically, viability increase is observed within the range of 80%; however, the combination of 30 µM HCQ + 25 µM TQ reached 94.6%.

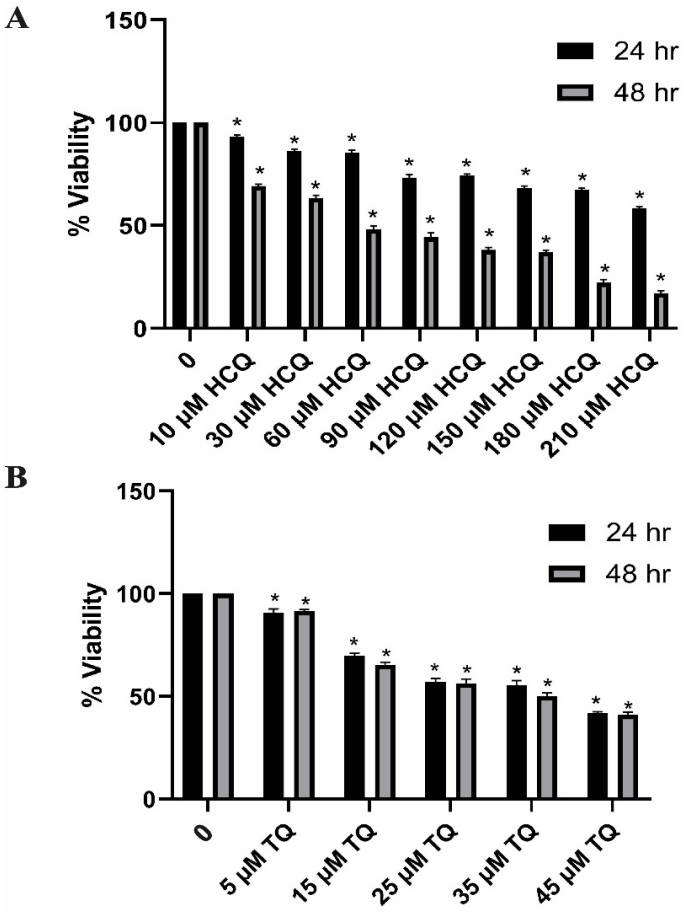

Figure I: Viability of MDA-MB-231 cells following administration of varying doses of HCQ (A) and TQ (B) at 24 and 48 hours. Each data point represents the mean of three independent experiments, and error bars indicate the standard deviation (*: $p<0.0001$).

Analysis of Synergistic Effects of HCQ and TQ

The Chou-Talalay combination index (CI) method, facilitated by Compusyn software, was employed to explore potential synergistic effects of HCQ and TQ. The results obtained for 24 hours indicated that the combination application of HCQ (150, 180, and 210

μM) with 15 μM and 25 μM TQ exhibited a synergistic effect (CI<1), except for the combination dose of 25 μM TQ + 150 μM HCQ (CI=1), which showed an additive effect. However, the results obtained for 48 hours of application showed that all combinations of TQ and HCQ exhibited an antagonistic effect (CI>1). CI data were summarized in Table I.

Table I: CI values of TQ and HCQ combinations applied to MDA-MB-231 cells at 24 and 48 hours

Combinations	Time (hr)			
	24 hr		48 hr	
	15 μM TQ	25 μM TQ	15 μM TQ	25 μM TQ
10 μM HCQ			7.101	7.675
30 μM HCQ			8.879	2.053
150 μM HCQ	0.754	1.047		
180 μM HCQ	0.589	0.822		
210 μM HCQ	0.764	0.826		

HCQ: Hydroxychloroquine, TQ: Thymoquinone

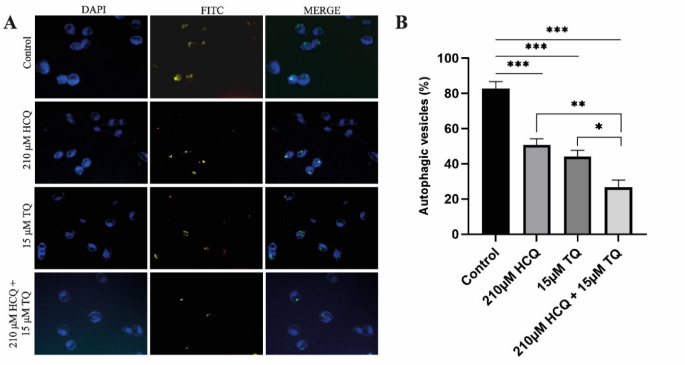

Figure II: The effects of HCQ and TQ combination on MDA-MB-231 cell viability. Each data point represents the mean of three independent experiments, and error bars indicate the standard deviation (*: *p<0.0001*).

On the other hand, our DRI results indicated that for all combinations applied for 24 hours, the DRI values of HCQ and TQ were greater than 1. Remarkably, in the combination of 15 μM TQ + 180 μM HCQ, the DRI values for TQ and HCQ were 4.625 and 2.685, respectively. Additionally, in the administration of the 15 μM TQ + 150 μM HCQ combination, DRI values of 3.025 and 2.359 were obtained for TQ and HCQ, respectively (Table II). This suggests that these combinations allow for a favorable reduction in dose compared to when each drug is administered individually. Contrary to these findings, DRI values less than 1 were obtained for nearly all doses applied for 48 hours (except for 25 μM TQ + 30 μM HCQ combination), indicating an unfavorable reduction in dose (Table III). Thus, the efficacy of HCQ and TQ combination decreased with prolonged duration.

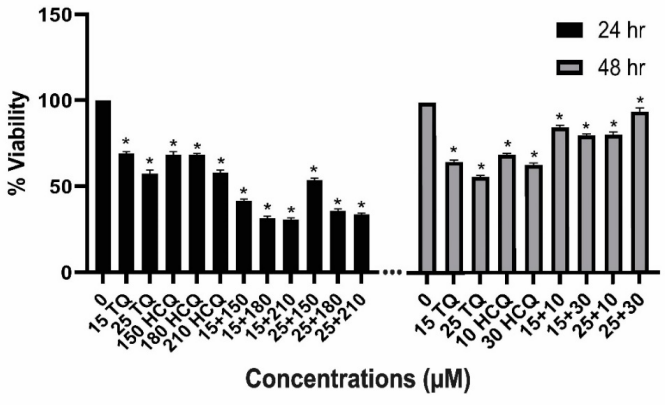

Figure III: Autophagy patterns of MDA-MB-231 cells under fluorescence microscopy (40X) following treatment with HCQ, TQ, and their combination for 24 hours. A. Nuclear morphology was assessed using DAPI staining (blue), while autophagic vacuoles were assessed using Green Detection Reagent (green). B. Comparison of autophagic vesicle percentages among groups (***p<0,0001*, **p<0,0005*, **p<0,005*).

Decrease in Cell Viability Attributed to Autophagy under Synergistic Effect

Autophagy patterns were observed to elucidate whether autophagy underlies the decrease in cell viability observed with the synergistic effect of HCQ and TQ combination treatment in MDA-MB-231 cells after 24 hours of application. Nuclear morphology was examined through DAPI staining, and it was obtained that the nuclei of BC cells remained intact following the application of TQ, HCQ, and their combination. The autophagy assay conducted with the combination of doses that most significantly reduced cell viability (210 μM HCQ + 15 μM TQ) showed a prominent presence of green autophagic vesicles in nearly every cell in the control group. While a decrease in vesicle numbers compared to the control was observed with the individual applications of HCQ and TQ, a notably lower abundance of autophagic vesicles was observed with their combined application (Figure IIIA). For each treated group, a statistically significant reduction in autophagic vesicle percentage was observed compared to the untreated control group (*p<0.0001*). Additionally, a statistically significant decrease was obtained in the HCQ-TQ combination group compared to individual treatment of HCQ (*p<0.05*), and TQ (*p<0.05*) (Figure IIIB). The mean

percentages of autophagic vesicles for the control, 210 µM HCQ, 15 µM TQ, and combination groups were 82.7, 50.7, 44, and 26.7, respectively.

Table II: DRI values of TQ and HCQ combinations applied to MDA-MB-231 cells at 24 hours

	24 hr		
	Combinations	**15 µM TQ**	**25 µM TQ**
150 µM HCQ	DRI TQ	3.025	1.674
	DRI HCQ	2.359	2.224
180 µM HCQ	DRI TQ	4.625	2.430
	DRI HCQ	2.685	2.436
210 µM HCQ	DRI TQ	2.905	2.653
	DRI HCQ	2.380	2.227

HCQ: Hydroxychloroquine, TQ: Thymoquinone

Discussion

Recent research has underscored the roles of TQ and HCQ in oncology, attributed to their capacity to modulate carcinogenesis and autophagy (12,15). While HCQ is an FDA-approved autophagy inhibitor (15), studies investigating the effects of TQ on autophagy are still ongoing. In the clinical implementation, the dosage and timing of administration are essential factors that influence the emergence of side effects (15). Prolonged exposure to cumulative doses of HCQ has been linked with adverse effects such as retinal toxicity, cardiomyopathy and hypoglycemia (16). Identifying the combined effects of herbal products and conventional drugs is crucial, as any enhancements resulting from this synergy can be harnessed for the disease treatment. In complex conditions like cancer, exploring positive synergies between natural compounds and drugs is essential for optimizing outcomes such as enhancement in therapeutic benefits for patients, or minimizing adverse effects (17). Based on this information, in the current study, we investigated the impact of the HCQ and TQ combination on the viability and autophagy patterns of TNBC cells in vitro.

Firstly, we investigated the effects of HCQ and TQ individually on cell viability following 24- and 48-hour treatments, which informed the selection of durations and doses for subsequent combination studies. Our results demonstrated a decrease in cell viability at both time points, which prompts to examine combination effects within these times. Consistent with existing literature, studies have shown that HCQ reduces viability in various cancer cell lines, including MDA-MB-231 cells (18), as well as in leukemia (19), ovarian cancer, gastric cancer, and other BC subtypes (15,20). Similarly, viability studies on TQ align with our findings, indicating a reduction in cell viability for several cancer types (10).

Table III: DRI values of TQ and HCQ combinations applied to MDA-MB-231 cells at 48 hours

	48 hr		
	Combinations	**15 µM TQ**	**25 µM TQ**
10 µM HCQ	DRI TQ	0.221	0.178
	DRI HCQ	0.387	0.483
30 µM HCQ	DRI TQ	0.325	1.385
	DRI HCQ	0.172	0.751

HCQ: Hydroxychloroquine, TQ: Thymoquinone

During the determination of combination doses, we selected concentrations potent enough to significantly affect cell viability while still allowing some cells to survive. Significant decreases in viability were observed compared to the control in 24 hours co-administration of these doses, with a subsequent significant increase in viability observed after 48 hours. The time-dependent effects of the combination imply potential synergistic and antagonistic actions of HCQ and TQ. These effects were verified using Chou-Talalay CI analysis. Overall, when HCQ and TQ were co-administered, they decreased viability with a synergistic effect in 24 hours (except only for 25 µM TQ + 150 µM HCQ), while they increased viability by working antagonistically in 48 hours. Changes in drug toxicity over time are a well-documented phenomenon in the literature, demonstrated in various subtypes of BC, including TNBC, as well as in other cancers (21–24). For instance, the significance of the time factor has been highlighted in the synergistic effects observed when tyrosine kinase inhibitors targeting the human epidermal growth factor receptors were combined with Doxorubicin in MCF-7 and MDA-MB-231 cells (24). The time-dependent differential effects of the combined drugs, HCQ and TQ, on cell viability observed in our study may be attributed to pharmacological mechanisms. One possible explanation is the dynamic interplay between the pharmacokinetics and pharmacodynamics of HCQ and TQ over time (25,26). At 24 hours post-administration,

the drugs may reach peak concentrations in the cellular microenvironment, leading to a maximal inhibitory effect on key cellular processes such as autophagy and proliferation. This synergistic action may result from the combined targeting of multiple pathways involved in cell survival and proliferation, thereby exerting a greater inhibitory effect on cell viability. However, as the duration of exposure increases to 48 hours, the pharmacokinetic profiles of HCQ and TQ may change, leading to altered drug concentrations and distribution within the cells. This temporal shift in drug exposure may result in the activation of compensatory cellular mechanisms, or the development of drug resistance, ultimately attenuating the inhibitory effects of the combination therapy (26,27). Individually, previous studies have supported the duration-dependent effects of both drugs, demonstrating the time-dependent cytotoxic effects of TQ on BC cells including MDA-MB-231 cell line, (28) and the time-varying inhibition of cholangiocarcinoma cells by HCQ (29). Overall, the time-dependent effects of combined HCQ and TQ therapy likely stem from a complex interplay of pharmacokinetic and pharmacodynamic factors, emphasizing the importance of considering temporal dynamics in drug response for the development of effective cancer treatment strategies.

Following the analysis of HCQ-TQ interaction effects, DRI values were calculated. The DRI analysis quantifies how much the combined dose of drugs in a synergistic combination can be reduced compared to the individual doses of each drug alone, while still achieving the same therapeutic effect. It helps optimize combination therapies by minimizing individual drug doses while maintaining efficacy (30). Specifically, the DRI values greater than 1 for all combinations applied for 24 hours indicate a favorable reduction in dose, suggesting a synergistic effect of the drugs in inhibiting cell viability within this time interval. This observation aligns with the notion of maximal inhibitory effects occurring at 24 hours, as discussed earlier. In the literature focusing on cancer research, DRI values have not been reported for HCQ, whereas some data exist for TQ. For instance, DRI values obtained with the combination of paclitaxel and TQ are similar to those in our study, primarily concentrating around 2. They have shown that TQ and paclitaxel which is an FDA-approved chemotherapy drug, significantly reduce each other's effective doses in MDA-MB-231 cells. The underlying mechanism for this phenomenon is attributed to the increased expression levels of Beclin-1, ATG-5, and ATG-7, which trigger autophagy (31). In other studies, these combinations have been shown to suppress BC cells by upregulating apoptosis through Caspases 12, 7, and 3, and also tumor suppressors such as BRCA1, p53 and p21 (32,33). In a study on colorectal cancer, different DRI values were observed as compared to our investigation regarding the synergistic co-administration of TQ and Imatinib. It has been demonstrated that TQ enhances the effectiveness of Imatinib through the regulation of uptake/efflux genes (34). Conversely, the DRI values which was obtained as less than 1 for nearly all doses applied for 48 hours. This indicates an unfavorable reduction in dose, reflecting a diminished efficacy of the combination therapy with prolonged duration, which aligns with the previously observed antagonistic effect. These findings confirm once more the significance of duration factor in the combination HCQ and TQ.

Based on the individual significant roles of HCQ and TQ in autophagy (7–9,11–13), one of the most plausible mechanisms by which co-administration of these exert their effects on cell viability is through autophagy. In line with this rationale, we evaluated whether the synergistic decline in cell viability observed within a 24 hours administration is mediated through the autophagy mechanism. As a result, we demonstrated that the combinative effect led to an increase in autophagy in TNBC cells. When given simultaneously, HCQ and TQ induced the autophagy mechanism to a greater extent compared to when administered separately. Beyond the cancer perspective, autophagy operates at basal levels in all cells and plays a vital role in maintaining cellular health by eliminating misfolded proteins, clearing damaged organelles like endoplasmic reticulum, peroxisomes and mitochondria, and eliminating intracellular pathogens. Additionally, under stress conditions, such as nutrient deprivation, autophagy facilitates the recycling of cellular resources to sustain cell survival. Moreover, autophagy is intricately involved in cellular differentiation and development

processes. Given its pivotal role in organismal homeostasis, dysregulation of autophagy contributes to the pathogenesis of various human diseases (35). Therefore, illustration of the combinative modulatory effects of HCQ and TQ on autophagy not only holds promise for TNBC treatment strategies but also warrants exploration in other diseases, underscoring its broader therapeutic potential.

This study provides valuable insights into the synergistic effects of HCQ and TQ on TNBC cells, but there are several limitations. The in vitro nature of the study restricts the findings to cellular models, and further validation in animal models or clinical trials is needed to confirm the therapeutic potential. Additionally, the long-term effects and safety of HCQ and TQ combinations have not been fully explored, particularly regarding potential adverse effects and optimal dosing strategies. The observed time-dependent effects underscore the need for further investigation into the pharmacokinetics and pharmacodynamics of these compounds. Future studies should focus on evaluating the combination therapy in vivo, exploring its effects in different cancer models, and investigating the detailed mechanisms underlying autophagy modulation by HCQ and TQ. Additionally, assessing the impact of this combination on other cancer cancers and patient populations could provide broader insights into its potential clinical applications.

Conclusion

Our study is the first to reveal the time-dependent synergistic and antagonistic effects of the HCQ and TQ combination on TNBC cells. This study also demonstrated, for the first time, the time-dependent reduction in cell viability in TNBC through the autophagy mechanism induced by these two drugs, highlighting their novel potential for modulating autophagy and providing new insights into TNBC treatment strategies. Our findings emphasize the critical importance of timing in the co-administration of HCQ and TQ, suggesting that careful consideration of treatment schedules is essential for maximizing therapeutic efficacy. Future research should focus on elucidating the specific mechanisms by which HCQ and TQ influence autophagy, both in vitro and in vivo. Additionally, understanding the transition point from synergistic to antagonistic effects and the underlying pharmacodynamic mechanisms will enhance our perspective of the time-dependent dynamics of this combination therapy. Given the aggressive nature and poor prognosis of TNBC, identifying the key targets and interactions of HCQ and TQ could significantly advance therapeutic strategies for this challenging cancer subtype.

References

1. Leon-Ferre RA, Goetz MP. Advances in systemic therapies for triple negative breast cancer. BMJ 2023;381:e071674.
2. Dass SA, Tan KL, Rajan RS, et al. Triple Negative Breast Cancer: A Review of Present and Future Diagnostic Modalities. Medicina 2021;57:62.
3. Kim HM, Koo JS. The Role of Autophagy in Breast Cancer Metastasis. Biomedicines 2023;11:618.
4. Barsoum RS, Bassily S, Baligh OK, et al. Renal disease in hepatosplenic schistosomiasis: a clinicopathological study. Trans R Soc Trop Med Hyg 1977;71:387–391.
5. Hashemi M, Paskeh MDA, Orouei S, et al. Towards dual function of autophagy in breast cancer: A potent regulator of tumor progression and therapy response. Biomed Pharmacother 2023;161:114546.
6. Wu Q, Sharma D. Autophagy and Breast Cancer: Connected in Growth, Progression, and Therapy. Cells 2023;12:1156.
7. Liu LQ, Wang SB, Shao YF, et al. Hydroxychloroquine potentiates the anti-cancer effect of bevacizumab on glioblastoma via the inhibition of autophagy. Biomed Pharmacother 2019;118:109339.
8. Abdel-Aziz AK, Saadeldin MK, Salem AH, et al. A Critical Review of Chloroquine and Hydroxychloroquine as Potential Adjuvant Agents for Treating People with Cancer. Futur Pharmacol 2022;2:431–443.
9. Cocco S, Leone A, Roca MS, et al. Inhibition of autophagy by chloroquine prevents resistance to PI3K/AKT inhibitors and potentiates their antitumor effect in combination with paclitaxel in triple negative breast cancer models. J Transl Med 2022;20:290.
10. Korak T, Ergül E, Sazci A. Nigella sativa and Cancer: A Review Focusing on Breast Cancer, Inhibition of Metastasis and Enhancement of Natural Killer Cell Cytotoxicity. Curr Pharm Biotechnol 2020;21:1176–1185.
11. Racoma IO, Meisen WH, Wang QE, Kaur B, Wani AA. Thymoquinone Inhibits Autophagy and Induces Cathepsin-Mediated, Caspase-Independent Cell Death in Glioblastoma Cells. PLoS ONE 2013;8:e72882.
12. Gomathinayagam R, Ha JH, Jayaraman M, Song YS, Isidoro C, Dhanasekaran DN. Chemopreventive and Anticancer Effects of Thymoquinone: Cellular and Molecular Targets. J Cancer Prev 2020;25:136–151.
13. Ünal TD, Hamurcu Z, Delibaşı N, et al. Thymoquinone Inhibits Proliferation and Migration of MDA-MB-231 Triple Negative Breast Cancer Cells by Suppressing Autophagy, Beclin-1 and LC3. Anti-Cancer Agents Med Chem 2020;21:355–364.
14. Chou T, Talalay P. Generalized Equations for the Analysis of Inhibitions of Michaelis-Menten and Higher-Order Kinetic Systems with Two or More Mutually Exclusive and Nonexclusive Inhibitors. Eur J Biochem 1981;115:207–216.
15. Zhou N, Liu Q, Wang X, et al. The combination of hydroxychloroquine and 2-deoxyglucose enhances apoptosis in breast cancer cells by blocking protective autophagy and sustaining endoplasmic reticulum stress. Cell Death Discov 2022;8:286.
16. Verbaanderd C, Maes H, Schaaf MB, et al. Repurposing Drugs in Oncology (ReDO)—chloroquine and hydroxychloroquine as anti-cancer agents. ecancermedicalscience 2017;11:781.
17. Pezzani R, Salehi B, Vitalini S, et al. Synergistic Effects of Plant Derivatives and Conventional Chemotherapeutic Agents: An Update on the Cancer Perspective. Medicina 2019;55:110.
18. Rahim R, Strobl JS. Hydroxychloroquine, chloroquine, and all-trans retinoic acid regulate growth, survival, and histone acetylation in breast cancer cells. Anti-Cancer Drugs 2009;20:736–745.
19. Wang F, Zhang Z, Leung WT, et al. Hydroxychloroquine reverses the drug resistance of leukemic K562/ADM cells by inhibiting autophagy. Mol Med Rep 2019;20:3883–3892.
20. Peng X, Zhang S, Jiao W, et al. Hydroxychloroquine Synergizes With The PI3K Inhibitor BKM120 to Exhibit Antitumor Efficacy Independent of Autophagy. 2021;40:374.
21. Schlotter CM, Vogt U, Allgayer H, Brandt B. Molecular targeted therapies for breast cancer treatment. Breast Cancer Res 2008;10:211.
22. Peleg L, Ashkenazi IE, Carlebach R, Chaitchik S. Time-dependent toxicity of drugs used in cancer chemotherapy: Separate and combined administration. Int J Cancer 1989;44:273–275.
23. Alagkiozidis I, Facciabene A, Tsiatas M, et al. Time-dependent cytotoxic drugs selectively cooperate with IL-18 for cancer chemo-immunotherapy. J Transl Med 2011;9:77.
24. Abrahams B, Gerber A, Hiss DC. Combination Treatment with EGFR Inhibitor and Doxorubicin Synergistically Inhibits Proliferation of MCF-7 Cells and MDA-MB-231 Triple-Negative Breast Cancer Cells In Vitro. Int J Mol Sci 2024;25:3066.
25. Sun L, Mi K, Hou Y, et al. Pharmacokinetic and Pharmacodynamic Drug–Drug Interactions: Research Methods and Applications. Metabolites 2023;13:897.
26. Sandritter TL, McLaughlin M, Artman M, Lowry J. The Interplay between Pharmacokinetics and Pharmacodynamics. Pediatr Rev 2017;38:195–206.
27. Wright DFB, Winter HR, Duffull SB. Understanding the time course of pharmacological effect: a PKPD approach. Br J Clin Pharmacol 2011;71:815–823.

28. Adinew GM, Messeha SS, Taka E, Badisa RB, Antonie LM, Soliman KFA. Thymoquinone Alterations of the Apoptotic Gene Expressions and Cell Cycle Arrest in Genetically Distinct Triple-Negative Breast Cancer Cells. Nutrients 2022;14:2120.
29. Chen J, Pan Q, Bai Y, Chen X, Zhou Y. Hydroxychloroquine Induces Apoptosis in Cholangiocarcinoma via Reactive Oxygen Species Accumulation Induced by Autophagy Inhibition. Front Mol Biosci 2021;8:720370.
30. Chou TC. The mass-action law-based algorithms for quantitative econo-green bio-research. Integr Biol 2011;3:548–559.
31. Khazaei MR, Bozorgi M, Khazaei M, Moradi A, Bozorgi A. Computational and in vitro analyses on synergistic effects of paclitaxel and thymoquinone in suppressing invasive breast cancer cells. Mol Biol Rep 2024;51:388.
32. Soni P, Kaur J, Tikoo K. Dual drug-loaded paclitaxel–thymoquinone nanoparticles for effective breast cancer therapy. J Nanoparticle Res 2015;17:18.
33. Şakalar Ç, İzgi K, İskender B, et al. The combination of thymoquinone and paclitaxel shows anti-tumor activity through the interplay with apoptosis network in triple-negative breast cancer. Tumor Biol 2016;37:4467–4677.
34. Thabet NA, El-Khouly D, Sayed-Ahmed MM, Omran MM. Thymoquinone chemosensitizes human colorectal cancer cells to imatinib via uptake/efflux genes modulation. Clin Exp Pharmacol Physiol 2021;48:911–920.
35. Gómez-Virgilio L, Silva-Lucero M del C, Flores-Morelos DS, et al. Autophagy: A Key Regulator of Homeostasis and Disease: An Overview of Molecular Mechanisms and Modulators. Cells 2022;11:2262.