www.biodicon.com Biological Diversity and Conservation

ISSN 1308-8084 Online ISSN 1308-5301 Print

Research article/Araştırma makalesi
DOI: 10.46309/biodicon.2024.1445711
17/3 (2024) 190-197

Combinational synergistic role of thymoquinone and celastrol in colon carcinoma cell line

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Abstract

Colon carcinoma (HCT-116) cells are highly aggressive cell line and cell proliferation of colon carcinoma cells are rapid and uncontrolled. Treatment of colorectal cancer cells can be achieved through the use of chemotherapeutic agents. However, the treatment with a single type of chemical may require high dosages, which leads to toxicity. To resolve this issue, synergistic combinational treatment of Thymoquinone (TQ) and Celastrol (CLS) can be promising strategy to reduce proliferation and cell viability of the colorectal cancer cells. Evaluation of cell viability and cell growth were determined for the combinational and alone treatments of TQ and CLS using MTT assay. The IC50 values of TQ and CLS were determined as 102 μM and 7 μM, respectively. Four different combinations of these two chemical agents were tested and the results revealed strong synergistic effect against HCT-116 colon cancer cells. Reactive oxygen species (ROS) production was also evaluated by monitoring the production of highly fluorescent DCF from DCFH-DA. Compared to the alone treatments of the both drugs, overproduction of ROS in combinational treatments supported the results obtained from cell viability. Our findings demonstrated that combinational strategy of TQ and CLS has strong synergistic activity against the HCT-116 cancer cells and it can be a promising strategy to increase the effect of the drugs.

Keywords: Colon cancer, thymoquinone, celastrol, synergistic combination, ROS production

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Kolon karsinoma hücre hattında timokinon ve celastrolün kombinasyonel sinerjistik rolü

Özet

Kolon karsinoma (HCT-116) hücreleri oldukça agresif hücre dizisidir ve bu hücrelerinin proliferasyonu hızlı ve kontrolsüzdür. Kolorektal kanser hücrelerinin tedavisi kemoterapötik ajanların kullanılmasıyla sağlanabilir. Ancak tek tip kimyasalla tedavi yüksek dozlar gerektirebilir ve bu da toksisiteye yol açabilmektedir. Bu sorunu çözmek için, Timokinon (TQ) ve Celastrol'ün (CLS) sinerjistik kombinasyon tedavisi, kolorektal kanser hücrelerinin çoğalmasını ve hücre canlılığını azaltmak için umut verici bir strateji olabilir. Hücre canlılığı ve hücre büyümesinin değerlendirilmesi, TQ ve CLS'nin kombine grupları ve tek başına tedavileri için MTT methodu kullanılarak belirlendi. Bu iki ajanın kombinasyon konsantrasyonları ve kombinasyon indeksleri CompuSYN yazılım programı ile belirlendi. TQ ve CLS'nin yarı maksimum inhibitör konsantrasyonları (IC50) sırasıyla 102 μM ve 7 μM olarak belirlendi. Bu iki kimyasal ajanın kombine grupları, HCT-116 kolon kanseri hücrelerine karşı güçlü sinerjistik etkiyi ortaya çıkardı. Reaktif oksijen

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 Received: 1.03.2024; Published: 15.12.2024 BioDiCon. 1145-010324

türlerinin üretimi ayrıca DCFH-DA'dan yüksek derecede floresan DCF üretiminin izlenmesiyle değerlendirildi. Her iki ilacın tek başına tedavileri ile karşılaştırıldığında kombinasyon tedavilerinde ROS'un aşırı üretimi hücre canlılığından elde edilen sonuçları destekledi. Bulgularımız, TQ ve CLS'nin kombinasyon stratejisinin, HCT-116 kanser hücrelerine karşı güçlü sinerjistik aktiviteye sahip olduğunu ve ilaçların etkisini arttırmak için umut verici bir strateji olabileceğini gösterdi.

Anahtar kelimeler: Kolon kanseri, timokinon, celastrol, sinerjistik kombinasyon, ROS üretimi.

1. Introduction

Colon carcinoma (HCT-116) cells are highly aggressive cell line and cell proliferation of colon carcinoma cells are rapid and uncontrolled. Researchers are trying to develop targeted therapies to inhibit proliferation of colon carcinoma cells. Chemotherapeutic agents have been long to be used to treat colorectal cancer, however, the use of these agents limited due to the severe side effects such as toxicity. To avoid potential adverse effects, synergistic combinations of anti-cancer medications with natural substances have been proposed as an alternative technique for treating a variety of cancer cell lines. In addition to toxic chemotherapeutic drugs, effect of natural agents may also improved via combination with another natural agent. Synergistic combination of natural flavonoids curcumin and quercetin have been shown to have better anti-cancer activity compared to single administration of these compounds. Similarly, synergistic avtivity of apigenin and curcumin against lung epithelium cancer [1]. Using natural anticancer agents minimize the side effects by lowering the dosage requirement for the cancer treatments.

Phytochemical agents such as thymoquinone have been reported to have anticancer [2], antioxidant [3], anti-inflammatory [4], and analgesic activities [5]. Chemical structure of thymoquinone is provided in Figure 1. Thymoquinone is a monoterpene and it can upregulates the caspase-3,8,9 and Bax expression. These genes are classified as apoptotic genes and they can induce apoptosis. It is also reported that thymoquinone can terminate JAK-2 and Src initiated phosphorylation of tyrosine kinase receptor. This process inactivates STAT3 genes, which induces apoptosis in colorectal canncer cells [6]. In addition, signalling of MEK-ERK can be distrupted due to TQ mediated alteration in the conformation of PAK1. This process also mediate apoptosis in colorectal cancer cells [7].

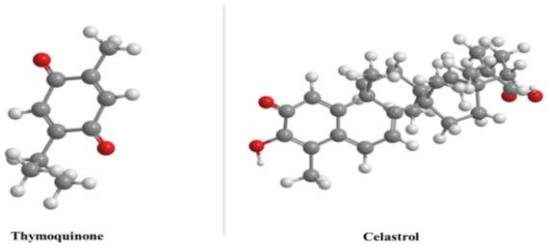

Figure 1. Chemical structures of thymoquinone and celastrol

Celastrol is a triterponoid and reported to have wide range of pharmacological activities. Possible toxicity of celastrol can be interlinked with the intramolecular hydrogen bonds to the carbonyl and hydroxyl groups [8]. Molecular mechanism behind the anticancer activity of celastrol shows variations depending on the type of cancer cell lines. It can reduce the function of NF-κB in colorectal cancer [9], suppress MMP3 and MMP7 via PI3K/AKT signalling [10], stimulates TGF-β1/Smad signalling [11], downregulates miR-21 and PI3K/AKT/GSK-3β pathway [12]. These mechanisms were detected upon celastrol treatments. In this study, synergistic combinations of thymoquinone and celastrol were determined to lower the dosage of administration while improving the anticancer activity against HCT-116 cancer cell lines by minimizing possible adverse effects.

2. Materials and methods

2.1 Materials

MTT and dimethyl sulfoxide (DMSO), 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA), celastrol, PBS and thymoquinone were obtained from Merck. Fetal bovine serum (FBS), penicillin/streptomycin, L-glutamine and trypsin

were supplied from GIBCO Thermo Fisher Scientific (Waltham, Massachusetts, United States. Colorectal cancer cell lines (HCT-116) (ATCC-CCL-247) were obtained from the American Type Culture Collection in Virginia, USA.

2.2 Cell Culture

RPMI-1640 (10% fetal bovine serum (FBS), 1% L-glutamine, and 1% penicillin-streptomycin) was used as growth medium. Cells were detached from the surface of a cell culture flask using 0.25% trypsin/0.02% EDTA solution, and then resuspended in RPMI-1640 cell culture media. Following this process, cell suspension was transferred to samples in 96-well plates. Cells were cultured at 37 °C for 2 hours to allow for cell adherence and plates were incubated for another 48 hours at 37 °C [13].

2.3 Cell Viability Assay

HCT-116 cells ($5x10^3$ per well were seeded on a 96-well plate and cells were treated with various concentrations of thymoquinone (TQ) (10, 20, 60, 80, 100, 150, and 200 µM), celastrol (CLS) (1, 2, 4, 6, and 8 µM), and combinations of TQ and CLS at 37°C. After the incubation, the medium was removed and 10 µl of MTT solution (5 mg/ml in PBS) was added to each well and incubate for 3 hours at 37°C [14]. The produced purple formazan crystals were dissolved in 100 µl DMSO. The absorbance of each well was recorded at 540 nm. TQ and CLS IC50 values against HCT-116 cells were calculated using the Composyn software tool and evaluated with the GraphPad Prism 5 program.

2.4 Combinational Analysis

For the combination study analysis, the Chou-Talalay method was utilized to compute the combination index (CI). The combination index (CI) was determined with CompuSyn software.

2.5 Determination of ROS Assay

The determination of ROS levels is critical for understanding their relationship with apoptosis, aging, stress, and tumor growth. Reactive oxygen species oxidize the 2'7'-dichlorodihydrofluorescein diacetate (DCFH-DA) dye molecules to DCF. DCF molecules are fluorescent molecules and may be identified. HCT-116 cells are sown onto 96-well plates and incubated for 24 h. For 48 h, cells were treated with TQ, CLS, and their mixtures at their IC50 concentration. After incubation, DCFH-DA dye solution (20 µM) was applied to each well at 37 °C for 45 min. The fluorescence intensity of DCF was measured at 488 nm and 525 nm with a multiplate reader [13].

2.6 Statistical Analysis

To establish significant differences between treatments and controls, one-way ANOVA and Dunnett's multiple comparison test were utilized.

3.Results

3.1 Cell viability

The effect of different concentrations of TQ and CLS were tested on HCT-116 cell viability and results were presented in Figure 2a and 2b. After treatment with of TQ (10, 20, 60, 80, 100, 150 and 200 µM), CLS (1, 2, 4, 6 and 8 µM) alone, there was a significant reduction in the cell viability percentages for 48h. IC50 values of TQ and CLS were determined using Compusyn Software program. After 48 h treatment, IC50 values of TQ and CLS were found to be 102 µM and 7 µM, respectively.

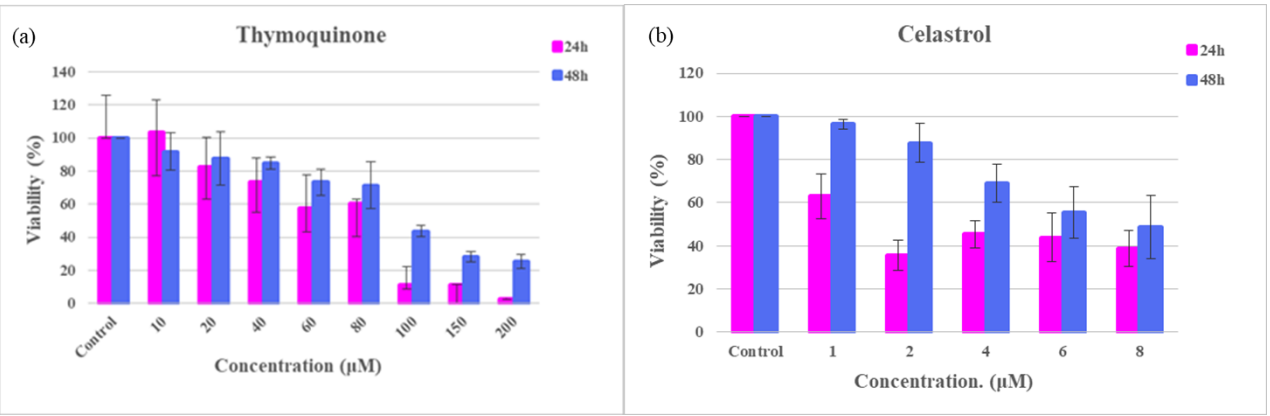

Figure 2. Cell viability % of various concentrations of **(a)** TQ, and **(b)** CLS toward the HCT-116 cancer cell line for 24 and 48 h

3.2 Combined Effect of TQ and CLS on HCT-116 Cancer Cells

Combinations of TQ and CLS enhanced their anticancer effects against HCT-116 cells. The dose-effect curves of TQ and CLS and their combinations are presented in Figure 3.

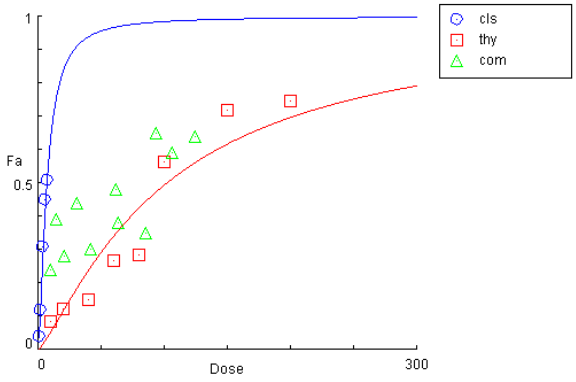

Figure 3. The dose-effect curves of thymoquinone, celastrol and their combinations

Combination index (CI) values were reported for the combination of TQ and CLS in Table 1. CI values are used to evaluate the synergistic activity between TQ and CLS. CI values expected to be smaller than 1 for synergistic activity. The strongest synergistic activity can be observed with smaller CI values. Based on CI values, 14 µM TQ with 1 µM CLS (CI: 0.38316, effect value: 0.39) and 10 µM TQ with 0.7 µM CLS (CI: 0.45194, effect value: 0.24) were determined as the best combination found due to smaller CI values compared to other combinations. It was observed that when TQ and CLS were used together, the same 50 % inhibitory concentration on HCT-116 cancer cells was achieved with a concentration approximately 3.5 times lower than when used alone. It has been observed that combination strategy of these two drugs significantly reduced the required dosage of each drug alone as shown in Figure 2 and this can minimize the possible toxic side effect of these chemicals.

3.3 Effect on Reactive Oxygen Species of TQ, CLS and Their combinations

Intracellular ROS production percentages for TQ, CLS and their combinations were presented in Figure 4. Based on experimental results, it was determined that TQ and CLS reduced intracellular ROS levels at their IC50 concentrations and alone treatments. Accumulation of ROS levels in cancer cells suppressed via the treatment of TQ and CLS. Combination-1, combination-2 and combination-3 treatments reduced the ROS production significantly at lower concentrations. Intracellular ROS production was effectively inhibited by combination-1 group among other combination groups.

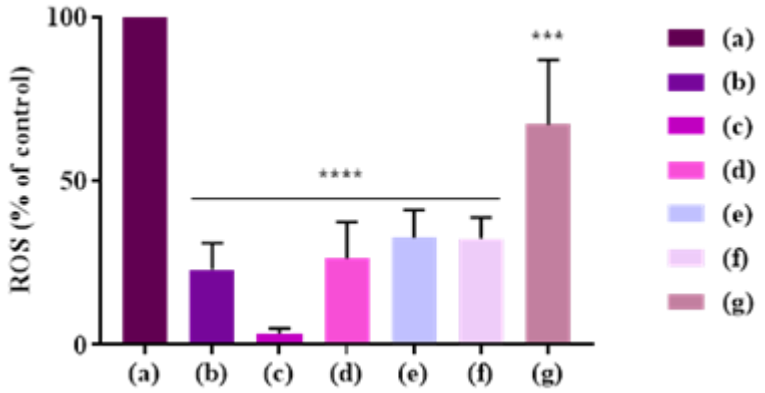

Figure 4. ROS production percentages of (a) control, (b) TQ, (c) CLS, (d) combination 1, (e) combination 2, (f) combination 3, and (g) combination 4. ROS production percentages were provided from three replicated measurements (n= 3). One Way ANOVA, Dunnett's multiple comparison assay used to evaluate the data by comparing with control as P<0.001, **** and p=0.0003, ***.

4. Conclusions and discussion

Synergisic activity is important as it often leads to enhanced therapeutic or beneficial effects compared to individual compounds. Synthetic compounds work together synergistically with natural substances, amplifying their overall impact on health. This synergism may improve absorption, increase antioxidant capacity, or target multiple pathways, making the combined effect more potent and valuable for various health applications.

In this study conducted in a similar manner, TQ alone inhibited HCT-116 cell growth at 48 and 72 hours, with a higher effect than 72 hours. Cell viability dropped to 27.8% at the highest TQ concentration (100 µg/mL), with an IC50 of 4.7 ± 0.21 µg/mL [15].

In different studies, HCT-116 cells treated with 50 µM TQ exhibited significant ($p<0.001$) reduction in cell proliferation. The IC50 of TQ against HCT-116 was calculated to be 64.15 ± 2.80 µM [16]. Fröhlich et al stated that the IC50 of TQ for HCT-116 cell line was 50.1 ± 6.1 µM [28].

In previous study, Kundu et al reported that apoptosis can be induce in HCT-116 cells upon STAT3 inactivation by inhibiting JAK2- and Src-initiated phosphorylation of tyrosine kinase [17]. Induction of apoptosis can also be mediated upon TQ treatment due to upregulation of apoptotic genes such as caspase-3, 8,9 and Bax [18]. Alterations in the conformation of PAK1 can block the signalling of MEK-ERK, which then eventually leads to the decrease in the proliferation of colorectal cancer cells [19]. Overall, oxidative stress mediated by TQ has been reported to downregulates JAK2/STAT3 pathway and this causes initiation of apoptosis in the cancer cells [20].

Several studies have demonstrated that CLS can effectively decrease tumor proliferation, migration, and angiogenesis in numerous tumor models, both in vitro and in vivo [21, 22]. In the present study, HCT-116 colon cancer cells were inhibited by 50 % at 7 µM of celastrol for 48 hours. Another study showed inhibitory effect to be more than 80 % after 48 h of treatment with 40 µM celastrol against HCT-116 colon cancer cells [23]. In different study, it was observed that CLS treatment caused a significant decrease in the proliferation of HCT-116 colon cancer cells in a dose-dependent manner [24]. All these molecular mechanisms inhibit the NF-κβ activity and blocks the nuclear translocation of NF-κβ [25]. These factors caused a reduction in the number of cancer cells.

Table 1. Dosages, and synergistic combination index values for combined groups of in thymoquinone (TQ) and celastrol (CLS) in HCT-116 cell line

Combination	Thymoquinone (µM)	Celastrol (µM)	CI	Effect Value	Function of CI Values
1	14.0	1.00	**0.383**	0.390	**Strong Synergism**
2	29.0	2.00	**0.672**	0.440	**Synergism**
3	10.0	0.700	**0.451**	0.240	**Strong Synergism**
4	20.0	1.40	**0.776**	0.280	**Synergism**

As previously determined by Moreira et al., CLS induces significant decrease in intracellular ROS production. This considerable reduction in intracellular ROS production can be observed with CLS at concentrations higher than 5 µM. At lower concentrations of CLS (0.1-1 µM), no significant difference or reduction can be observed as shown in our study [26]. It was determined that CLS decreased ROS production even better than TQ in parallel with cell viability results. Intracellular ROS assay further supported the synergistic effect of these combinational strategies against HCT-116 cells.

Recent studies on the combinational strategies of either TQ or CLS with various anticancer drugs are presented in Table 2. Strong synergistic activity of CLS (0.2 µmol/L) combination with 0.5 µg/mL vincristine on HCT-8 cell line determined with 0.18 CI value [27]. Combinations of TQ with 5-fluorouracil (5-FU) were reported to have low combination index values (CI< 0.5), which represent strong synergistic activity on glioblastoma cells. Reduction in the required concentrations of both drugs achieved by combinational treatments compared to alone treatments. This minimizes the possible side effects of the administered drugs [28]. In different study, synergistic activity of 60 µM TQ + 33 µg/mL Cisplatin was determined on MCF-7 cells with a CI value of 0.62 [29]. In this study, the concentration of TQ was further declined down to 14 µM upon combination with 1 µM CLS. This combination enabled the strong synergistic activity with 0.383 CI value.

In two separate studies, combination of TQ with silymarin and piperine showed synergistic effects on EMT6/P cells with CI values of 0.632 and 0.788, respectively [30, 31]. In another study, enhanced autophacy in MCF-7 cell line resulted upon 0.3 µM CLS + 10 µM tamoxifen combinational treatment [32].

Table 2. Recent studies on combinational strategies related to either TQ or CLS with function of CI values

Combinational Strategies	Cancer Cell Line	CI Value	Function of CI Values	References
0.2 µmol/L CLS + 0.5 µg/mL vincristine	HCT-8 cells	0.18	Strong Synergism	[27]
33 µM TQ + 10 µM 5-Fluorouracil	U-251MG glioblastoma	0.31	Strong Synergism	[28]
14 µM TQ + 1 µM CLS	HCT-116 cells	0.383	Strong Synergism	This study
60 µM TQ + 33 µg/mL Cisplatin	MCF-7 cells	0.62	Synergism	[29]
29.95 µM TQ + 25.23 µM Silymarin	EMT6/P Cells	0.632	Synergism	[30]
425 µM piperine + 80 µM TQ	EMT6/P cells	0.788	Synergism	[31]
0.3 µM CLS + 10 µM Tamoxifen	MCF-7 cells	Not available	Not available	[32]

Overall, in this present study it was first time shown that the combination of TQ and CLS decreased the dose requirement compared to the alone administration of these drugs. Reducing the concentration of chemical agents required to treat colon cancer decrease the risk of any toxic, adverse effect of these drugs. Based on CompuSYN software, the combination of these two drugs showed strong synergistic activity against HCT-116 colon cancer cells. Cancer cells shows elavated levels of ROS compared to normal cells. This high level of ROS production can be reduced in dose dependent manner with TQ and CLS. Reduced ROS production in the combinational approaches decreased the HCT-116 cell growth and viability at low concentrations. This study showed that combination of two natural agents can be used to treat colon cancer cells by decreasing the required dose. Further studies required to be performed prior to clinical trails. These studies can be conducted to reveal the molecular basis of the action of combinational treatments on the specific cancer cell lines and also effect of combinational approaches required to be tested with in vivo models.

Acknowledgements

The authors would like to thank Eastern Mediterranean University for financial assistance.

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