Available online on 15.10.2023 at http://jddtonline.info

Journal of Drug Delivery and Therapeutics

Open Access to Pharmaceutical and Medical Research

Open Access Full Text Article

Research Article

Effect of Thymoquinone on Ovarian Carcinoma Cell Viability (OVCAR-3)

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Article Info:

Article History:

Received 03 August 2023
Reviewed 11 Sep 2023
Accepted 27 Sep 2023
Published 15 Oct 2023

Cite this article as:

Özdemir İ, Ekinci C, Effect of Thymoquinone on Ovarian Carcinoma Cell Viability (OVCAR-3), Journal of Drug Delivery and Therapeutics. 2023; 13(10):76-81

DOI: http://dx.doi.org/10.22270/jddt.v13i10.6263

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Abstract

Aim: Ovarian cancer is the third most common gynecological malignancy worldwide. However, it has the highest mortality rate among cancers due to its asymptomatic course, late diagnosis and recurrence. Doxorubicin (Dox) is one of the most commonly prescribed chemotherapeutics in the treatment of ovarian and breast cancer. The serious side effects of chemotherapeutic drugs and the development of drug resistance restrict the use of these drugs. The use of natural products with anticancer activity may help partially overcome these problems. In this study, the effects of thymoquinone (TQ) and Dox, a powerful chemotherapy agent, on cell growth inhibition and cell viability on OVCAR-3 and human skin keratinocyte cell line (HaCaT) were determined by the MTT method.

Method: Ovarian adenocarcinoma cell lines OVCAR-3 (CCL-2™) and HaCat (RRID: CVCL_0038) were used in the study. To determine the IC50 (inhibitory concentration) doses of Dox and TQ, HeLa and HaCaT cell lines were cultivated with the help of an automatic multipipet. Then, MTT test was applied to analyze cell survival (viability).

Results: OVCAR-3 cell growth was approximately 2.12 nM at the 48th hour in cells treated with Dox, while the IC50 value of TQ at the 48th hour was found to be 62.9 µM.

Conclusion: These results show that TQ potentiates the effect of Dox and the Dox/TQ combination may be a promising alternative to other chemotherapeutic combinations in the treatment of ovarian cancer with lower side effects.

Keywords: Thymoquinone, Cancer, Ovarian adenocarcinoma, MTT

INTRODUCTİON

Cancer is the second leading cause of death worldwide, responsible for an estimated 9.6 million deaths in 2018 [1]. Ovarian cancer is the third most common gynecological malignancy worldwide. However, it has the highest mortality rate among cancers due to its asymptomatic course, late diagnosis and recurrence [2,3]. When diagnosed, it is usually accompanied by omentum involvement, widespread malignant ascites and intraperitoneal metastasis [4,5]. According to available data, ovarian cancer also develops resistance to traditional chemotherapeutics, which contributes to recurrence [6]. The specific etiology for ovarian cancer remains unknown, and since these cancers tend to occur in advanced stages, the early molecular events underlying development remain unknown.

Medicinal plants are important not only as therapeutic agents but also for pharmacological research and drug development. Since many features of the cell come into play in the development of cancer, treatment with a single therapy is rarely effective [7]. For this reason, combination treatments have become preferred. Because of the different pathways targeted and the lower dosage of chemotherapeutic agents used, toxicity is significantly less [8,9]. In addition, significant side effects occur in patients with the use of chemically synthesized drugs. Therefore, the discovery and development of new drugs based on natural products has become the focus of research [10,11].

Thymoquinone (TQ) is one of the major bioactive components of Nigella sativa (black cumin) essential oil. It has proven its effectiveness against various diseases thanks to its many medical and pharmacological activities such as anti-inflammatory, antioxidant, antihistamine, antitumor, analgesic, anti-Alzheimer, hepatoprotector, neuroprotector, histone protein modulator, insecticidal effects, anti-ischemic, leishmanicides, radioprotectors [12, 13]. TQ alone has demonstrated anticancer activity in various in vitro and in vivo studies as well as in adjuvant therapy to prevent carcinogenesis or enhance the effectiveness of conventional therapeutic techniques [14].

In this study, the possible synergistic or antagonistic effect of TQ with Dox alone and in combination was investigated. In this study, the anticancer effects of TQ, one of the agents currently being studied in the current literature, were investigated in order to identify agents that would reduce the effects of chemotherapy as alternative treatments and to reveal their effectiveness.

MATERİAL AND METHODS

Culture and passage of cells

In the study, ovarian carcinoma cell line NIH:OVCAR-3 (HTB-161™) and human skin keratinocyte cell line HaCat (RRID:CVCL 0038) were used as the healthy cell line. OVCAR-3 cell line was cultured in RPMI 1640 medium containing 10% FBS, 2 mM L-glutamine and 1% penicillin/streptomycin, and

HaCaT cell line was cultured in DMEM medium containing the same additives, and the cells were cultured in sterile incubators at 37°C and 5% CO2. grown in an incubator containing In all studies, the study started from the 5th passage of the cell lines and ended at the 15th or 20th passage at most.

Determination of IC50 doses of Doxorubicin and Thymoquinone agents in OVCAR-3 and HaCaT cell lines

In the study, stock solutions of Dox and TQ agents were prepared using ultrapure Ethanol (Merck, USA), 5 mM stock solutions were made for Dox and 100 mM stock solutions were made for TQ, and these were portioned and stored at -20ºC. In the applications performed, the final concentration of the vehicle in the 96-well culture dish was reduced to 0.1%.

To determine Dox and TQ IC50 doses, OVCAR-3 and HaCaT cell lines were cultivated in 96-well culture plates (plates) with automatic multipipetting, 3000-5000 cells in each well, respectively. At the end of one night (approximately 16 hours), Dox was applied at 9 different concentrations obtained by serial dilution at dose ranges of 0.5-50 µM and TQ 5-500 µM, and the plates were incubated for 48 hours. In MTT cell viability analysis, the wells on the outside of the 96-well culture dish were excluded to reduce trial error, and each chemotherapy agent and vehicle control groups consisted of 6 wells. MTT test was performed for cell survival (viability) analysis after incubation. For this purpose, the "Yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide)" test solution prepared at a dose of 5 mg/ml was pipetted into all wells at 20 µl/well. Then, the plates were left to incubate for 4 hours, after the incubation, the medium in the wells was completely removed and 200µl ultra puree DMSO (Merk, USA) was added to each well and kept in the incubator under dark conditions for 2-4 hours. At the end of this period, the plates were read spectrophotometrically at 492, 570 and 650 nm wavelengths with a Multiskan GO microplate reader (Thermo Scientific, USA). The value obtained from the control group applied to the vehicle was determined as a comparative viability rate based on 100% viability. IC50 results statistics for each tumor cell line and chemotherapy agents in the control and experimental groups were calculated using probit analysis with the SPSS 20.0 package program.

RESULTS

Within the scope of the study, the % cell viability obtained as a result of the MTT test in the OVCAR-3 cell series after Dox application and the IC50 value calculated using probit analysis and statistical analysis compared to the control are given in Table 1. The data obtained showed that the IC50 value was found to be 2.12 µM as a result of Dox application to the OVCAR-3 cell line for 48 hours. No IC50 value was found in 24-hour Dox application. It was observed that there were significant decreases in cell proliferation as the dose increased. The application started with the cultivation of 100,000 cells, and the number of cells was obtained as 23.31 in Dox application at 50 µM concentration (Table 1). After finding the IC50 value as a result of statistical analysis, it was determined that cell viability decreased significantly after 5 nM Dox application compared to the vehicle group (Figure 1).

Table 1. It was obtained by serial dilution in the OVCAR-3 ovarian carcinoma cell line in the concentration range of 0.5-50 µM. % cell viability, standard deviation and standard error values, 95% confidence interval and minimum maximum values obtained after Doxorubicin application at 9 different concentrations for 48 hours.

OVCAR-3-Dox		N	Cell viabilityı (%)	standard deviation	Std. error	95% confidence interval		Minimum	Maximum
						lower boundary	upper limit		
48 h	Vehicle	6	100,0000	1,49238	0,60926	98,4338	101,5662	98,74	102,89
	0,5 uM	6	64,5056	2,22490	0,90831	62,1707	66,8405	61,87	68,15
	0,75 uM	6	62,1733	2,85494	1,16552	59,1772	65,1693	60,21	67,72
	1 uM	6	55,1718	3,19302	1,30355	51,8209	58,5227	51,10	60,10
	2,5 uM	6	45,9899	2,32729	0,95011	43,5476	48,4322	42,97	49,27
	5 uM	6	43,2242	2,12608	0,86797	40,9930	45,4553	40,21	45,63
	7,5 uM	6	39,1984	0,85978	0,35100	38,2961	40,1007	37,99	40,13
	10 uM	6	34,5069	0,69297	0,28290	33,7797	35,2342	33,75	35,52
	25 uM	6	25,5038	0,71828	0,29324	24,7500	26,2576	24,85	26,81
	50 uM	6	23,3100	2,33771	0,95436	20,8567	25,7632	21,39	27,80

The % cell viability obtained as a result of the MTT test in the OVCAR-3 cell series after TQ application and the IC50 value calculated using probit analysis and statistical analysis compared to the control are given in Table 2. As a result of 48 hours of TQ application, the IC50 value was found to be 62.9 µM. Then, the statistical significance between the vehicle group and different concentrations of TQ was calculated using one-way ANOVA and Tukey HSD test according to $P \leq 0.05$. As a result, it was observed that cell viability decreased significantly after 48 hours of TQ application and 50 µM concentration (Table 2).

Table 2. OVCAR-3 was obtained by serial dilution in the 5-500 µM concentration range in the ovarian carcinoma cell line. % cell viability, standard deviation and standard error values, 95% confidence interval and minimum and maximum values obtained after TQ application for 48 hours at 9 different concentrations.

OVCAR-3- TQ		N	Cell viabilityı (%)	standard deviation	Std. error	95% confidence interval		Minimum	Maximum
						lower boundary	upper limit		
48 s	Vehicle	6	100,0000	2,72142	1,11102	97,1440	102,8560	95,60	103,83
	5 uM	6	100,5343	4,87051	1,98838	95,4230	105,6456	95,48	107,91
	7.5 uM	6	97,9384	3,01008	1,22886	94,7795	101,0973	93,94	102,98
	10 uM	6	100,5192	3,54340	1,44659	96,8006	104,2378	96,45	104,37
	25 uM	6	96,3153	2,04377	0,83436	94,1705	98,4601	93,51	98,93
	50 uM	6	73,6075	4,71916	1,92659	68,6551	78,5600	66,41	79,06
	75 uM	6	28,5297	8,61601	3,51747	19,4877	37,5716	21,05	41,01
	100 uM	6	9,0882	0,62940	0,25695	8,4276	9,7487	8,01	9,77
	250 uM	6	8,7303	0,21527	0,08789	8,5044	8,9562	8,47	9,10
	500 uM	6	9,1638	0,28371	0,11582	8,8660	9,4615	8,71	9,56

The effect of Dox and TQ applications on healthy cell lines was also analyzed and the % cell viability obtained from the MTT test on the HaCaT cell series after the applications of both agents and IC50 values calculated using probit analysis and statistical analysis compared to the control are given in Tables 3 and 4. .

As a result of Dox application to the HaCaT cell line for 48 hours, the IC50 value was found to be 5.32 µM. It was observed that there were significant decreases in cell proliferation as the dose increased. The application started with the cultivation of 100,000 cells, and the number of cells was obtained as 11.1679 in Dox application at 50 µM concentration (Table 3). After finding the IC50 value as a result of statistical analysis, it was determined that cell viability decreased significantly after 7.5 µM Dox application compared to the vehicle group (Figure 3).

Table 3. HaCaT was obtained by serial dilution in the concentration range of 0.5-50 µM in human healthy dermal keratinocyte cell lines. % cell viability, standard deviation and standard error values, 95% confidence interval and minimum maximum values obtained after Doxorubicin application at 9 different concentrations for 48 hours.

HaCaT-Dox		N	Cell viability (%)	standard deviation	Std. error	95% confidence interval		Minimum	Maximum
						lower boundary	upper limit		
48 s	Vehicle	6	100,0000	4,09262	1,67080	95,7051	104,2949	95,55	106,33
	0.5 uM	6	86,3335	3,26373	1,33241	82,9085	89,7586	81,16	90,20
	0.75 uM	6	88,3788	2,86289	1,16877	85,3744	91,3833	83,49	90,58
	1 uM	6	79,7133	4,48618	1,83147	75,0054	84,4213	74,38	85,37
	2.5 uM	6	74,0422	4,80999	1,96367	68,9944	79,0900	67,75	81,53
	5 uM	6	69,4906	7,50001	3,06186	61,6198	77,3614	62,31	82,95
	7.5 uM	6	35,7118	3,46635	1,41513	32,0741	39,3495	32,42	41,23
	10 uM	6	19,3570	1,67547	0,68401	17,5987	21,1153	17,34	21,08
	25 uM	6	22,3242	2,09793	0,85648	20,1226	24,5259	19,57	24,99
	50 uM	6	11,1679	0,91112	0,37196	10,2118	12,1241	9,90	12,23

The effect of TQ applications on healthy cell lines was also analyzed and the % cell viability obtained as a result of the MTT test in the HaCaT cell series and IC50 values calculated using probit analysis and statistical analysis compared to the control are given in Table 4.

No IC50 value was found as a result of 24-hour GA application to the HaCaT cell line. With 48 hours of TQ application, the IC50 affecting the viability of the HaCaT cell line was found to be 346.4 µM. It was observed that there were significant decreases in cell proliferation as the dose increased. The application started with the cultivation of 100,000 cells, and the number of cells was obtained as 39.3925 in TQ application at 500 µM concentration (Table 4). After finding the IC50 value as a result of statistical analysis, it was determined that

cell viability decreased significantly after 100 µM TQ application compared to the vehicle group (Table 4).

The findings show that Thymoquinone can increase the therapeutic effect and reduce the toxicity on healthy cells when combined with chemotherapy drugs used in the treatment of ovarian cancer. It has been shown that TQ can exhibit a synergistic effect with Dox and also play a preventive role against doxorubicin toxicity.

Table 4. HaCaT was obtained by serial dilution in the concentration range of 5-500 µM in human healthy dermal keratinocyte cell lines. % cell viability, standard deviation and standard error values, 95% confidence interval and minimum maximum values obtained after TQt application at 9 different concentrations for 48 hours.

HaCaT- TQ		N	Cell viability (%)	standard deviation	Std. error	95% confidence interval		Minimum	Maximum
						lower boundary	upper limit		
48 s	Vehicle	6	100,0000	9,07418	3,70452	90,4772	109,5228	91,25	116,42
	5 uM	6	111,1961	4,55704	1,86040	106,4137	115,9784	107,00	119,38
	7.5 uM	6	109,6590	6,36265	2,59754	102,9818	116,3362	102,27	120,45
	10 uM	6	107,9804	5,91253	2,41378	101,7756	114,1852	100,35	116,13
	25 uM	6	111,6531	7,66897	3,13084	103,6050	119,7012	100,45	121,83
	50 uM	6	107,9036	7,17865	2,93067	100,3700	115,4371	97,68	118,92
	75 uM	6	96,8127	8,00446	3,26781	88,4125	105,2129	83,92	105,93
	100 uM	6	79,3431	8,83223	3,60574	70,0743	88,6120	72,54	95,69
	250 uM	6	60,3365	14,66824	5,98829	44,9432	75,7299	34,10	79,04
	500 uM	6	39,3925	20,53110	8,38178	17,8464	60,9385	8,15	69,36

DİSCUSSİON

Although chemotherapeutic drugs are very effective, their use is restricted due to their serious side effects and the development of drug resistance. Natural products with anticancer activity can provide solutions at this point. In this study, we found that TQ could increase the anti-cancer activity with the MTT method in combination with Dox in the OVCAR-3 cell line. Our results showed that the most promising treatment was not the use of Dox and TQ alone, but their combination, as it showed the highest cytotoxic effect among the groups by MTT analysis.

Ovarian cancer is the leading cause of gynecological cancer deaths in developed countries and often occurs at an advanced stage. [15]. The current standard treatment for advanced ovarian cancer is cytoreductive surgery and platinum/taxane-based chemotherapy [16-18]. The response rate to treatment is approximately 80-90%, but many often relapse and develop resistance to chemotherapy [14]. Therefore, alternative approaches to the diagnosis and treatment of ovarian cancer are needed. Over the past three decades, a number of preclinical and clinical studies have been conducted to identify potential drug candidates (as mono or combined treatments) or to improve the therapeutic efficacy of existing chemotherapy regimens against ovarian cancer [19].

New compounds derived from plants have recently gained importance as an alternative to conventional therapies due to their potent activity against carcinogenic cells with limited or negligible side effects. They are particularly focused on producing some promising cytotoxic drugs originating from natural compounds such as rosmarinic acid, alpha lipoic acid, ascorbic acid, curcumin, etc [20-22]. It is mainly aimed to reduce or even eliminate the side effects of existing chemotherapy. Antioxidant therapy can be defined as treatment that prevents or reduces the side effects of free radicals. The effectiveness of exogenous antioxidants in protecting tissues from oxidative stress in vivo is variable and depends on the type of antioxidant, its biopharmaceutical properties, its concentration at the site of action, and the nature of the oxidative stress [22].

Cancer has become a deadly disease in today's world that kills hundreds of thousands of people every year. Standard treatments such as chemotherapy, radiation therapy, or immunotherapy are successful in only a fraction of the patient population due to the heterogeneity of the disease. By targeting a single gene, gene product, or signaling pathway, only a specific group of cells in the tumor can be eliminated, while other genetically distinct variations can easily escape treatment and form tumors in the surrounding area or spread to distant locations. Therefore, in cancers, such features may ultimately lead to drug resistance [23-25]. These poor outcomes are caused by genomic instability and abnormal activation of DNA maintenance genes involved in DNA damage detection and repair. To ameliorate the failure of cancer treatments such as platinum-based chemotherapies, drug redirection (new uses of old drugs) is being implemented as sensitizers of genotoxic therapy by inhibiting the repair of DNA damage and also by causing cell death [26,27]. Based on this information, it was investigated whether the Dox-TQ combination could be a viable new cancer treatment drug. In the MTT test, the cytotoxic effect of Dox (0.5-50 µM), TQ (5-500 µM) alone or in combination on OVCAR-3 and HaCaT cells was evaluated for 24 and 48 hours. According to the results of the effects of Dox and TQ on cell growth rate, it was observed that the cell growth rate of OVCAR-3 cells decreased depending on the 48-hour period and dose. The results obtained were found to be close to the determined IC50 of TQ: 55.3 µM and 50 µM doses in pancreatic cancer lines [28,29]. After 48 hours, the most cytotoxic effect of thymoquinone alone on OVCAR-3 cell viability was found with an IC value of 62.9 µM. In another study, Samerghandien and colleagues showed that thymoquinone induces apoptosis in A549 cells by increasing the Bax/Bcl-2 ratio and positively regulates p53 expression [30]. After thymoquinone treatment alone in A549 cells, a slight

decrease in caspase 3/7 activity was detected in late apoptosis. Spironolactone and thymoquinone compounds, each in combination with other drugs, have been shown in other studies to cause an increase in caspase 3/7 activation in cancer lines [31,32].

The difference between the IC50 values of TQ in the OVCAR-3 cell seen in our study can be explained by the fact that the MTT test has been used in cancer research for 30 years. Since some cell lines can respond resistantly in the analysis based on absorbance measurement, different results can be obtained even in the same measurements [33-35]. A consistent IC50 value is rarely obtained for a given chemical compound against a given cancer cell line. He et al attributed this problem to differences between manufacturers and the formulas used by different laboratories. Even within the same laboratory, MTT test results reported that variable IC50 values could be obtained between different researchers and between different experiment repetitions performed by the same researcher. This discrepancy may be explained by variability in the control wells used as the basis for calculating IC50 values. These variations depend on the initial cell density and proliferation potential of the cell line [36].

In our study, TQ-induced apoptosis was induced as shown by the results of the MTT assay. To understand the role of thimoquinone in inducing apoptosis, it is necessary to examine the expression of apoptotic markers such as P53, Bcl-2 and Caspase-3.

CONCLUSİON

The current study suggests that the combination of Dox and TQ may be a promising protocol for ovarian cancer treatment and an alternative to the widely used current regimen, Paclitaxel/Carboplatin. The Dox/TQ combination offers a number of advantages over existing protocols, such as higher efficacy, lower dose and fewer side effects. More detailed mechanistic and efficacy studies in cell culture, animal models and ultimately clinical trials are recommended to evaluate Dox/TQ combination therapy.

ACKNOWLEDGEMENT

This study was a part of doctorate thesis of İlhan ÖZDEMİR"

Ethic

Ethical approval is not required because commercially available cell lines are used as an in vitro study.

Conflict of Interest: The authors declare that they have no conflict of interest.

Financial Support:

This study was funded by Dicle University Scientific Research Platform (DÜBAP) with project number SBE.22.008.

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