



CLINICAL GROUP



## Research Article

# The effects of thymoquinone and cytozine arabinoside on apoptosis and cell proliferation in acute myeloide leukemia

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## Abstract

**Purpose:** The aim of this study was to investigate the effects of a chemotherapeutic agent Cytosine Arabinoside (Ara-C) and a natural anticancer agent of Thymoquinone (TQ) on apoptosis and cell proliferation of AML cell lines (Kasumi-6) both alone and in combined form.

**Material and method:** Kasumi-6 AML cells were treated with three different doses of Ara-C (0.1, 0.5 and 1 µmol) and TQ (25, 50 and 100 µM) for 48 and 72 hours incubations. After Annexin V and Propidium Iodide (PI) staining, apoptosis, viability, and cell proliferation were evaluated for each group in flow cytometry.

**Results:** As a result, AML cell lines showed a statistically significant difference in a single treatment of the active substances. Their combined treatment showed an increase in apoptosis and a decrease in viability in both groups at 48 and 72 hours incubation times ( $p < 0.001$ ). In each group, it was observed that apoptosis was increased and viability was decreased and consequently cell proliferation was suppressed.

**Conclusion:** Ara-C was used for the first time in this study with TQ in AML. It was determined that the combined use of TQ and Ara-C did not have a synergistic effect on apoptosis.

## Introduction

AML is a group of malignant diseases that occur with the uncontrolled and clonal proliferation of cells with increased proliferation rate and decreased apoptosis compared to normal cells and it occurs when these rapidly proliferating cells invade the bone marrow [1,2].

The main consideration for the treatment of AML is an effective combination of cytosine arabinoside (Ara-C) and chemotherapy. It's known that the aim of chemotherapy is to restore the production of normal blood cells and to achieve complete remission in the patient. However, chemotherapy causes the death of normal healthy cells, and therefore, AML patients experience side effects such as nausea, weakness, and a

high risk of infection [3]. The standard treatment is highly toxic and the development of resistance to the chemotherapeutic drugs used hinders the treatment. Therefore, the development of new therapeutic agents and protocols is needed to improve outcomes in AML patients. Ara-C used in AML is an effective apoptotic drug. Thymoquinone (TQ) is an extract of black cumin, a natural product with anti-carcinogenic effects. Its seeds can be used as a spice and in the treatment of many diseases in traditional medicine. It is reported that it can be used in alternative medicine together with chemotherapy in cancer patients [3,4]. TQ is a herbal anti-carcinogen with no side effects and has an important effect on cancer cells in terms of showing cytotoxic effects against tumor cells [5]. It has been reported to inhibit TNF-induced NF-κappa-B activation in human chronic myeloid leukemia cells through inhibition of TQ phosphorylation and nuclear translocation [6].



Our aim is to examine the effects of TQ and Ara-C on apoptosis and cell proliferation when administered alone or combined in AML cell lines (Kasumi-6).

## Material and methods

### AML cell line and reagents

Kasumi-6 (ATCC No: CRL-2775) myeloblast cells were used for AML-M2 cell culture. Ara-C and TQ 99% (*Nigella sativa*) were used as chemotherapeutic and apoptotic agents. Three different concentrations of Ara-C (0.1, 0.5 and 1  $\mu$ mol) and TQ (25, 50 and 100  $\mu$ M each) were applied to the cells taken into the original medium for 48 and 72 hours. After Annexin V and PI staining, apoptosis, viability, and cell proliferation were evaluated for each group in flow cytometry.

### AML cell line and viability assay

AML-M2 cells were thawed in a 37 °C water bath. After dissolving, it was taken into a 15 ml falcon tube, and 1 ml of RPMI 1640 medium containing bovine serum (FBS) and antibiotics were added. It was centrifuged twice at 1200 rpm for 10 minutes. The supernatant was discarded and 1 milliliter (ml) of the antibiotic-free medium was added to the obtained pellet. Ten microliters ( $\mu$ l) of this suspension was taken and pipetted onto a thoma slide. Viability was determined by adding trypan blue at the same rate. Live and dead cell ratios were measured in the automated cell counter (Bio-Rad). As a result of the measurement, 22% vitality was detected. The number of viable cells of the ready-made AML cell line was measured as  $1.02 \times 10^6$  cells/ml.

### Cell culture

Due to the low number of cells, multiple passages were applied. AML cells were seeded into flasks in 4 ml RPMI 1640 medium and cultured. AML cells were checked under the microscope and their cell densities were examined. All flasks were collected in a single 50 ml falcon tube and washed in accordance with the previously applied wash procedure. Thirty-six ml of medium was added to the cells remaining at the bottom of the tube. Ten  $\mu$ l was taken from it and the measurement was made by an automated cell counter device for the determination of viability. As a result of standard cell culture, a total of  $7 \times 10^6$  cells/ml was obtained.

The cells suspended in a nutrient medium were seeded into 12-well cell culture dishes at  $2 \times 10^5$  cells per milliliter and left in the incubator for 24 hours without adding any substance. TQ (25, 50, 100  $\mu$ M each) and Ara-C (0.1, 0.5, 1  $\mu$ mol each) were added to all samples at the determined rates at the end of the 24<sup>th</sup> hour and DMSO was added to the control groups at the same rates. It was incubated again and at the end of the 48<sup>th</sup> hour, the cultures were taken into 5 ml falcon tubes and prepared for staining for apoptosis and viability determination by flow cytometric analysis. The same procedures were repeated for the 72-hour groups at the end of the 72<sup>nd</sup> hour.

### Apoptosis measurement by flow cytometry

Cells are washed 2 times with cold PBS. It is suspended

in 1  $\times$  Binding Buffer. 100  $\mu$ l of the obtained suspension is transferred to culture tubes. 5  $\mu$ l Annexin V and 5  $\mu$ l PI pipetted and vortexed. And following incubated for 15 minutes in the room. Following 400  $\mu$ l of 1  $\times$  Binding Buffer was added. It was read within 1 hour. With this staining technique, four different phenotypes are determined; viable cells (stained with annexin-V and PI), early apoptotic (stained only with annexin-V), late apoptotic (stained with annexin-V and PI) and dead/damaged cells (stained with PI only).

### Statistical analysis

MINITAB Statistical Software 15.0 Two proportion test was applied.  $p < 0.05$  was accepted as statistically significant.

## Results

### Cell culture and viability

The number of viable cells of the ready cell line was measured as  $1.02 \times 10^6$  cells/ml. A total of  $7 \times 10^6$  cells/ml were obtained as a result of the normal cell culture performed to multiply the cells before the anti-carcinogen application.

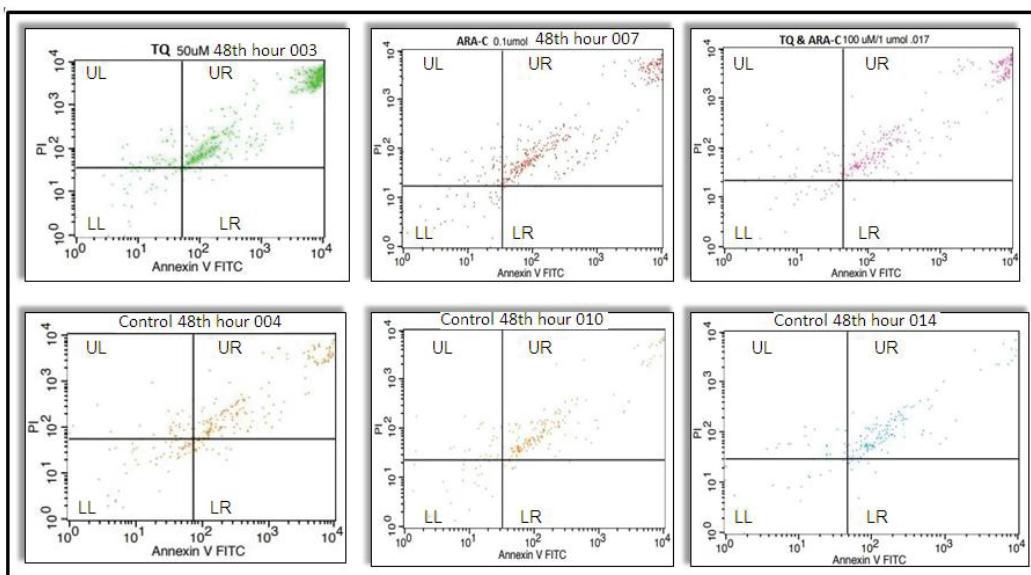
### Flow cytometry results

It was observed that the number of apoptotic cells at three different TQ concentrations (25, 50 and 100  $\mu$ M each) increased significantly ( $p < 0.001$ ) at the 48<sup>th</sup> hour, when the cells in the TQ-applied experimental group underwent the first mitosis ( $p < 0.001$ ), and the viability decreased in the experimental groups compared to the controls ( $p < 0.001$ ), (Figure 1, Table 1). In the 72<sup>nd</sup> hour incubation of this group, it was observed that the number of apoptosis increased ( $p < 0.001$ ) and viability decreased ( $p < 0.001$ ) when the experimental groups were compared with the controls (Figure 2, Table 1).

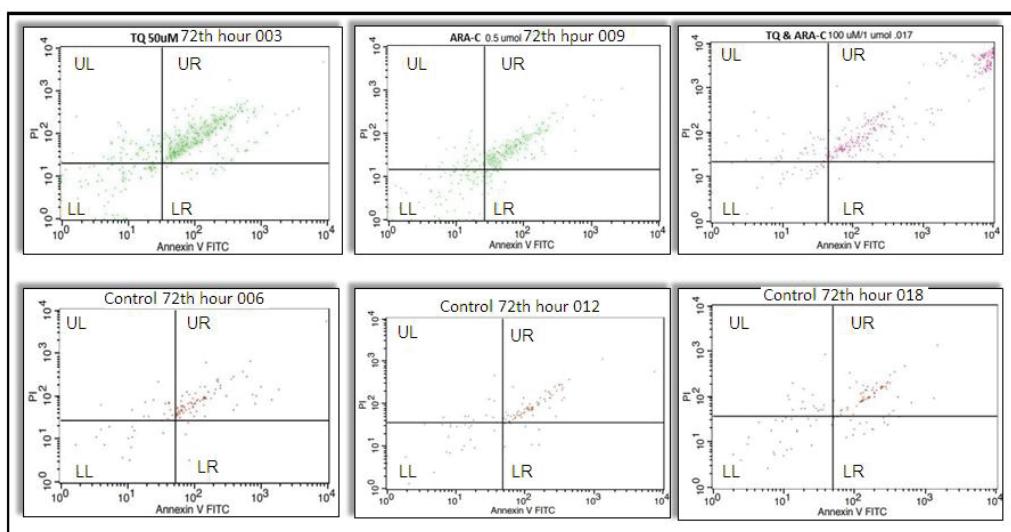
Apoptotic cell numbers increased significantly at three different Ara-C concentrations (0.1, 0.5, 1  $\mu$ mol each) in the 48<sup>th</sup> hour, when the cells in the Ara-C applied experimental group underwent the first mitosis, compared to the controls ( $p < 0.001$ ). It was observed that viability decreased in the experimental groups compared to the controls ( $p < 0.001$ ), (Figure 1, Table 1). In the 72<sup>nd</sup> hour incubation of this group, it was observed that the number of apoptosis increased ( $p < 0.001$ ) and viability decreased ( $p < 0.001$ ) when the experimental groups were compared with the controls (Table 1).

It was observed that the number of apoptotic cells in the experimental group with three different concentrations, in which TQ and Ara-C were applied together, were significantly increased ( $p < 0.001$ ) compared to the controls, at the 48th hour when they underwent the first mitosis, and the viability decreased in the study groups compared to the control groups ( $p < 0.001$ ) (Table 1). It was observed that the number of apoptosis increased ( $p < 0.001$ ) and the viability decreased when compared to the controls in the 72<sup>nd</sup>-hour incubation of this group (Table 1).

The increase in apoptosis and the decrease in viability, which we observed in all three groups, showed us that proliferation was suppressed. Apoptosis increased when the samples



**Figure 1:** Flow-cytometry images of TQ, ARA-C and TQ+ARA-C groups during 48 hours of incubation. UL: Necrotic cells; UR: Late apoptotic cells; LL: Live cells; LR: Proapoptotic cells.



**Figure 2:** Flow-cytometry images of TQ, ARA-C and TQ+ARA-C groups during 72 hours of incubation. UL: Necrotic cells; UR: Late apoptotic cells; LL: Viable cells; LR: Proapoptotic cells.

**Table 1:** Comparison of apoptosis and viable cell counts with experimental groups and controls at different concentrations and incubation times.

Experimental Group/ Incubation Time 10000 cells /ml	Applied Concentration	AML Apoptosis N: cell	AML Control(DMSO) Apoptosis N: cell	P value	AML Viability N: cell	AML Control (DMSO) Viability N: Cell	P value
AML-M2+TQ	25 μM	7400	6500	< 0.001	1200	1600	< 0.001
	50 μM	8500	6800	< 0.001	800	2000	< 0.001
	100 μM	8300	7800	< 0.001	1000	1300	< 0.001
AML-M2+TQ	25 μM	7200	6400	< 0.001	1600	1800	< 0.001
	50 μM	6900	6000	< 0.001	1700	2100	< 0.001
	100 μM	6300	7400	< 0.001	1400	1700	< 0.001
AML-M2+ARA-C	0.1 μmol	8400	8100	< 0.001	800	1000	< 0.001
	0.5 μmol	8400	7700	< 0.001	600	1100	< 0.001
	1 μmol	8900	8000	< 0.001	400	1200	< 0.001
AML-M2+ARA-C	0.1 μmol	7400	5900	< 0.001	1400	2000	< 0.001
	0.5 μmol	6400	5700	< 0.001	2400	2800	< 0.001
	1 μmol	7500	7000	< 0.001	1700	2100	< 0.001
AML-M2+TQ+ARA-C	25 μM/0.1 μmol	8200	8000	< 0.001	700	900	< 0.001
	50 μM/0.5 μmol	8000	7100	< 0.001	1300	1600	< 0.001
	100 μM/1 μmol	8600	7700	< 0.001	600	1400	< 0.001
AML-M2+TQ+ ARA-C	25 μM/0.1 μmol	7200	6000	< 0.001	1900	2100	< 0.001
	50 μM/0.5 μmol	7100	6800	< 0.001	1800	2000	< 0.001
	100 μM/1 μmol	7100	6400	< 0.001	1300	2200	< 0.001



were compared with their control groups at 48 and 72-hour incubation periods when Ara-C (0.1, 0.5, 1 µmol) and TQ (25, 50, 100 µM each) were applied at three different concentrations. ( $p < 0.001$ ) viability decreased ( $p < 0.001$ ) (Figure 1). In addition, cell proliferation was suppressed. When the 48 and 72-hour incubation times and three different concentrations applied for the TQ, Ara-C, and TQ+Ara-C combined groups of AML cell lines were compared, it was observed that the number of apoptotic cells increased more and the viability decreased at the 48<sup>th</sup> hour compared to the 72<sup>nd</sup> hour ( $p < 0.001$ ) (Table 1 and Figures 1,2).

## Discussion

Different strategies are used in the treatment of AML. Apoptosis in cancer cells is carried out with the use of certain chemical drugs that are compulsorily administered [7]. Significant problems have been observed in patients during treatment with anti-carcinogenic chemical drugs. The anti-carcinogenic potential of natural products attracts the attention of clinicians and scientists [8]. The application of natural herbal products on humans is more advantageous than anti-carcinogenic drugs. It has been observed that phytochemicals used in cancer treatment today are a good alternative to synthetic drugs. In recent years, it has been found that TQ, an anti-carcinogenic and anti-mutagenic herbal product taken with diet, suppresses cell proliferation in studies [9]. The antitumor effect of TQ has been demonstrated in many studies covering lung [10] pancreatic [11] prostate [12] and colon [13] cancers. In our study, the effects of Ara-C and TQ, a natural herbal product, on apoptosis, viability, and cell proliferation in acute myeloid leukemia (Kasumi-6 cell line) cell lines were investigated. Uncontrolled growth and proliferation of cancer cells is an important feature in carcinogenesis and causes an increase in tumor size and problems in treatment. It has been shown that the expression and/or activity of regulators of cell cycle progression and cell proliferation can be inhibited by TQ [4-19].

In our study, three different doses of TQ (25, 50, 100 µM) and Ara-C (0.1, 0.5, 1 µmol each) were applied to AML (Kasumi-6) cell lines in 48 and 72 hours incubation periods. The effects of TQ and Ara-C on apoptosis and cell proliferation were investigated when used alone or together. When the three different concentrations of active ingredients were applied, apoptosis increased ( $p < 0.001$ ), and viability decreased ( $p < 0.001$ ) when the samples were compared with their control groups in 48 and 72-hour incubation periods (Table 1). In addition, cell proliferation was also suppressed. In some studies, different drug combinations were used together with TQ. The combination of TQ and doxorubicin has been shown to have a greater effect than the use of these agents individually [20]. A similar finding has been reported for combinations of TQ and 5-fluorouracil [21]. TQ and cisplatin [10]. In a study investigating the effect of TQ on prostate cancer cells, it was observed that TQ was effective on regulatory proteins that provide the transition from G1 to S phase and blocked cells that transitioned from G1 to S phase [12]. Similarly, the suppressive effect of TQ in the G1 phase of the cell cycle was observed in

previous studies with cancer cell lines [22]. In studies with the anti-carcinogenic Ara-C used in the treatment of AML, it has been reported that Ara-C inhibits DNA polymerase and impairs DNA replication and repair, depending on the dose; in the samples, it is used. The incorporation of Ara-C into the DNA structure occurs only in the synthesis phase (S phase) of DNA synthesis [23-26]. In a study, TQ was investigated in Jurkat lymphoblastic cell line and showed that it has a synergistic effect in combination with DOX. This combination strategy can be an alternative way for more powerful anticancer effects [27]. Almajali, et al. examined the effects of TQ in HL60 leukemia cells and they reported that TQ significantly induced cycle arrest at G0-G1 phase [4]. Anti-apoptotic proteins (eg, Bcl-2 and survivin) have also been reported to regulate the release of cytochrome by mitochondria [28,29] also found that TQ had a significant apoptotic effect in the first 48 hours, unlike other incubation times, similar to our findings. TQ has been shown to be extremely unstable in aqueous solutions, with pronounced effects of both pH and light. Although we used 100% DMSO to dissolve TQ, it may not have shown its apoptotic effect for as much as 48 hours in the 72-hour incubation period due to the aqueous phase of the medium.

In this study, it was observed that apoptosis increased and viability decreased in AML cell lines induced by TQ and Ara-C. However, cell proliferation was decreased. The results of our study are consistent with other studies, for example, other studies such as human myeloblastic leukemia [14] human colon cancer [30] liver cancer [31] cell line. TQ has been shown to induce apoptosis in some cell lines. In our previous studies, we applied *in vivo* animal experimental study with antiepileptic drugs and we measured apoptosis and genotoxicity [32,33]. By novel studies and methodologies, the study results should be confirmed [34-36].

## Conclusion

In conclusion, our findings showed that TQ can be used as an anti-carcinogenic phytochemical. However, it has been observed that it does not have an effective complementary effect when used with Ara-C. In further studies, observing the possible effects of anti-apoptotic and anti-carcinogenic treatment with TQ and Ara-C in AML cell lines *in vitro* in AML-produced experimental animals will provide the reliability and beneficial effect of such studies.

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