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Anticancer drugs with chemotherapeutic interactions with thymoquinone in osteosarcoma cells

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Abstract. – OBJECTIVE: Osteosarcoma (OS) is the most common primary malignant tumor of the bone. Treatment options include surgery, chemotherapy, and radiotherapy. Following surgery, multi-agent chemotherapy drugs are effective but are associated with significant side effects and toxicity. Thymoquinone (TMQ) is a pharmacological component of black cumin that has multiple anti-tumorigenic effects. The goal of this study was to determine the effect of TMQ in combination with chemotherapy drugs on the growth inhibition of osteosarcoma and the potential clinical utility of TMQ in the treatment of OS.

MATERIALS AND METHODS: We evaluated the effects on the MG63 OS cell line when TMQ, 5-fluorouracil, and oxaliplatin combinations were applied that MG63 OS cell line viability was measured with the cell proliferation and apoptosis assay according to dose and time-dependent effects.

RESULTS: Application of 10 μM TMQ combined with 5FU and OXA at a low concentration of 1 μM that was discovered an ineffective dose of the used drugs as anticancer decreased cell viability and increased apoptosis in cells at a significant rate at 48 and 72 h.

CONCLUSIONS: TMQ has potential benefits in preventing the onset and progression of chemotherapy drug-induced toxicity and side effects, and may reduce resistance to chemotherapy drugs. We consider that TMQ may be a potential therapeutic drug for OS and/or other cancers.

Key Words:
Osteosarcoma, Chemotherapy, Nigella sativa, Thymoquinone, 5-fluorouracil, Oxaliplatin, Low dose effective, Low toxicity.

Introduction

Osteosarcoma (OS), involving histological findings of osteoid production in association with mesenchymal cells, is the most common primary malignant solid tumor of the bone[1-4]. Despite significant progress in developing OS therapies, the incidence of OS-related deaths is not declining, and 5-year survival rates are still not greater than 70-80%[1-3,5]. Treatment options include local control surgical procedures, such as limb-salvage surgery, tumor resection, tumor resection arthroplasty, amputation, metastasectomy, high-dose chemotherapy (using agents such as methotrexate, doxorubicin, cisplatin, oxaliplatin (OXA), 5-fluorouracil (5FU), bleomycin, taxol, and etoposide), and radiotherapy. These treatments are largely effective in many patients and have improved overall patient survival over the last several years[2-4,6,7]. Current treatment management includes preoperative (neoadjuvant) chemotherapy and postoperative (adjuvant) chemotherapy, preferably within the context of clinical assays[4]. Single-drug regimes at high doses may not be effective for many patients and are associated with various toxicities[3,4]. Thus, although multi-agent chemotherapy regimens are often considered to be effective, these medications are associated with various significant side effects and toxicities[3,4]. Additionally, there are patients who are inherently resistant to chemotherapy agents or may become unresponsive to these drugs during chemotherapeutic treatment (seen in 35-45% of patients)[4]. There have been few promising developments with respect to improving the survival rate of chemotherapy patients with localized disease, which has reached a plateau over the last two or three decades[3]. However, some new agents, such as the immune modulator liposomal muramyl-tripeptidephosphatidyl ethanolamine, have shown efficacy, with an ~8% improvement in overall survival at 6 years[8].

Alternative and/or herbal medicines have attracted attention in recent years and some are

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gradually being introduced as alternatives to, or are being used in combination with, chemical drugs[9]. *Nigella sativa* (NS), known as black cumin or black seed, is used frequently used in folk medicine in the Middle East, some Asian countries, Southern Europe, and North Africa[9-11]. Various pharmacologically active components have been isolated from NS, including thymoquinone (TMQ), thymohydroquinone, dithymoquinone, thymol, carvacrol, nigellimine-N-oxide, nigellicine, nigellidine, and alpha-hederin. Long-term studies have shown that NS, its oil, and TMQ – which represent the most widely studied forms – are quite safe, particularly when given orally[10,12]. TMQ has been analyzed and anti-oxidant, anti-inflammatory, anti-diabetic, and anti-cancer effects have been seen, both in *in vivo* and *in vitro* models[12,13]. The treatment of many conditions with NS or TMQ has been described, including fever, the common cold, microbial infections, immune stimulation, hypertension, asthma, pain, headaches, encephalomyelitis, diabetes mellitus, rheumatic diseases, and cancer[9-13]. The antitumor effects of NS were recognized by Ibn Sina (980-1037). He generally used NS for the treatment of tumors, mainly hard splenic masses, as stated in his book Al-Qanoon fi el-Tibb[10,14]. Modern medicine has shown that using TMQ can arrest cell cycle progression in different cancers[12], such as lung cancer[15,16], breast cancer[17-19], ovarian cancer[20,21], prostate cancer[22], colon cancer[23], fibrosarcoma[24,25], and OS[26,27]. The effects of TMQ in OS are listed in Table I. Many studies have shown that TMQ affects the mechanism of cancer. However, we are not aware of any reported study (*in vivo* or *in vitro*) on the effects of TMQ on drug interactions in OS.

The purpose of this study was to outline recent developments in the field of OS therapies. We have investigated the questions that need answering to assess the potential benefits of the use of TMQ in combination with chemotherapy drugs for OS, and the potential clinical utility of TMQ in the treatment of OS.

Materials and Methods

Dulbecco's modified Eagle's medium/nutrient mixture F-12 Ham (DMEM/F12), antibiotics (penicillin/streptomycin/amphotericin-B), and fetal bovine serum (FBS) were purchased from Pan-Biotech (Aidenbach, Germany). Drugs such as OXA, 5FU, and TMQ were purchased from Sigma-Aldrich (St. Louis, MO, USA). The MTT cell proliferation kit was from Roche Diagnostics (Indianapolis, IN, USA). The Muse Annexin V and Dead Cell Assay and Cell Cycle Assay kits were purchased from EMD Millipore (Billerica, MA, USA).

Cell Culture

In this study, the MG63 cell line was used for human OS cells. Cells were grown in DMEM/F12, supplemented with 10% fetal bovine serum and 1% antibiotics in a 5% CO_2 atmosphere at 37°C. At 75% confluence, proliferated cells were passaged using trypsin/EDTA (0.25% trypsin, 0.02% EDTA; Pan-Biotech, Aidenbach, Germany).

Preparation and Treatment of Drugs

A stock solution of OXA was prepared in ultrapure water (UPW) at 10 mM and 5FU was prepared in dimethyl sulfoxide (DMSO) at 200 mM. TMQ was prepared in DMSO at 20 mM. During the experimental period, the stock solutions of the drugs were freshly prepared and diluted in DMEM/F12. The final DMSO concentration in cell culture was kept at 0.5%. At the beginning of the experiment, cells were seeded at 5×10^3 cell/wells in 96-well plates. The cells were left for 1 day to attach. For the determina-

Table I. How effective TMQ was shown impact on OS.

Pathways	Result in impact
Lack of p53 augments	To induced apoptosis and caspase activation[26,28]
p21 (WAF1) up regulation and expression	To induce G1 and G2/M phase cell cycle arrest[26]
Blockade of NF-KB DNA binding and the downregulation of its target gene products (XIAP, surviving, CD34 and VEGF)	Inhibitory effects on in vitro cell proliferation and *in vivo* growth[27]
↑ Expression of cleaved caspase-3 and Smac	Antiproliferative[28]

tion of toxic doses, cells were exposed to increasing concentrations of the drugs for 48 and 72 h. Effects of TMQ on cells were investigated by gradually increasing the dose (Figure 1). In the following experiment, 10 and 20 μM TMQ were separately combined with six different doses of OXA and 5FU (Figure 2, 3). The alone- and combined-treatment groups were observed for toxicity under a phase-contrast inverted microscope (CKX41, Olympus Life Science, Hamburg, Germany) and then cytotoxicity assays were started. All of the experiments were repeated three times (n = 3).

Cell Viability Assay

MG63 cell viability was measured with the MTT assay after 48 and 72 h of treatment. MTT-1 solution (10 μL, 5 mg/mL) in the kit was added at 1/10 final concentration into each well. After 4 h, a 1:1 ratio of MTT-2 solution in the kit was added to each well to dissolve the formed purple formazan product. Absorbance was measured using a microplate ELISA reader (Epoch, BioTek Instruments, Inc., Winooski, VT, USA) at 570 nm. The percentage of the cell viability was calculated according to the control.

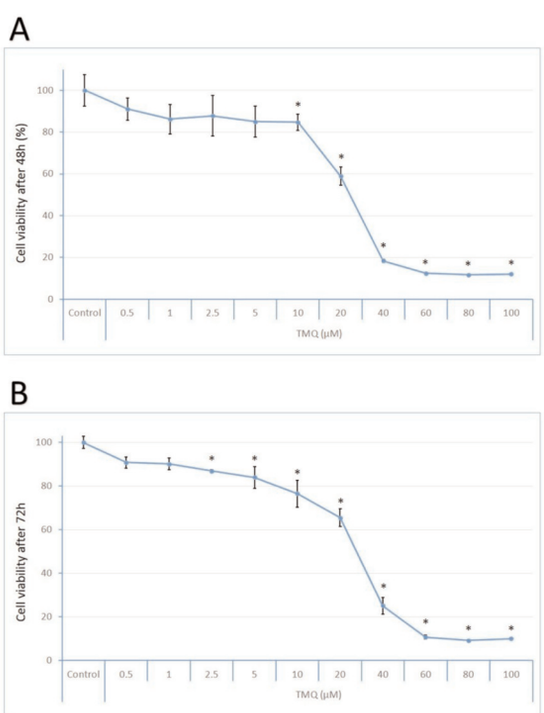

Figure 1. Effects of thymoquinone (TMQ) application on cell viability in a dose- and time-dependent manner. *p< 0.001, vs. control group. ***A,*** 48 h application, ***B,*** 72 h application.

Annexin V and Dead Cell Assay

Cells were grown in 6-well plates and treated with 5FU, OXA, and TMQ alone and combined (72 h for the apoptosis assay) before assaying using the Muse Cell Analyzer (Merck Millipore, Billerica, MA, USA). Cells were collected after various treatments, washed twice with 1× phosphate buffered saline (PBS) and re-suspended in 1× PBS for cell viability assays. For the apoptosis profiling assay, cells were re-suspended in 1% fetal bovine serum (FBS). Apoptotic cell counts were obtained with the Muse Annexin V and Dead Cell Assay kit (Merck Millipore). The assays were conducted in triplicate and in accordance to the manufacturer's instructions.

Statistical Analysis

All data were expressed as mean±SD for each group and analyzed in SPSS 15.0 software (SPSS, Inc., Chicago, IL, USA). Intergroup comparisons were performed using ANOVA or paired Student's t-test. Values of $p < 0.05$ and $p < 0.001$ were considered to indicate statistical significance.

Results

Effects of TMQ on MG63 Cell Viability

Figure 1 shows that treatment of MG-63 cells with TMQ increased toxicity in a dose- and time-dependent manner (Figure 1A, B). Toxicity was evident at 10 μM after 48 h and 72 h; 10 μM TMQ decreased the cell viability at a rate of 15 ± 4% at 48 h, and 23 ± 6% at 72 h. Also, 20 μM TMQ decreased the cell viability at a rate of 41 ± 4% at 48 h, and 35 ± 4% at 72 h.

Decrease in MG63 cell Viability with Combination 5FU Plus TMQ

When applied alone, 5FU decreased MG63 cell viability in a dose and time-dependent manner. At 48 and 72 h, the toxicity of 5FU was evident from the dose of 5 μM (Figure 2A1, B1). The 10 and 20 μM dosages of TMQ were combined with 5FU. The cell viability percentage differences between the group with 5FU alone and the groups with the combinations are shown in Figure 2 A2 and B2. Application of 10 μM TMQ combined with 1 μM 5FU decreased cell viability at a statistically significant rate (29% and 26%) at 48 and 72 h. However, it was found that the combination of 10 μM TMQ and 5 μM 5FU affected cell viability most at 48 and 72 hours (Figure 2A2, B2). At 48 and 72 h, the most

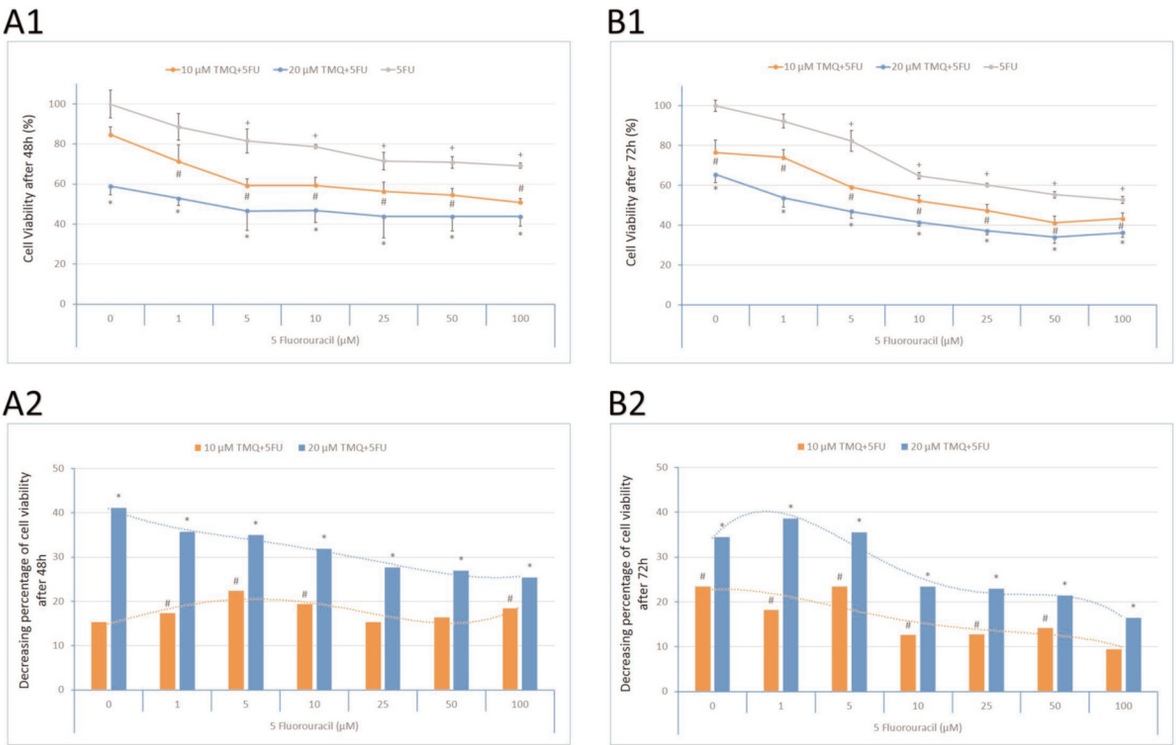

Figure 2. Effects of TMQ combined with 5-fluorouracil (5FU) on cell viability in a dose- and time-dependent manner. A1. Combined application for 48 h. The control (nothing applied; 100%), in comparison with the group receiving 5FU alone +, the control group in comparison with the group receiving 10 μM TMQ+5FU $^{\#}$, and the control group in comparison with the group receiving 20 μM TMQ + 5FU. $^{*}p < 0.05$. A2. Combination group vs. the 5FU-alone group. The group receiving 5FU alone in comparison with the group receiving 10 μM TMQ + 5FU $^{\#}$, and the group receiving 5FU alone in comparison with the group receiving 20 μM TMQ + 5FU. $^{*}p < 0.05$. B1. Combined application for 72 h. The control group (100%) in comparison with the group receiving 5FU +, the control group in comparison with the group receiving 10 μM TMQ + 5FU $^{\#}$, and control group in comparison with the group receiving 20 μM TMQ + 5FU. $^{*}p < 0.05$. B2. Combination group and the group receiving 5FU alone. The group receiving 5FU alone in comparison with the group receiving 10 μM TMQ + 5FU $^{\#}$, and the group receiving 5FU alone in comparison with the group receiving 20 μM TMQ + 5FU. $^{*}p < 0.05$.

effective 5FU dosages combined with 20 μM TMQ, with respect to cell viability, were 1 and 5 μM (Figure 2A2, B2).

Combination of OXA and TMQ Decreased MG63 cell Viability

OXA alone decreased the viability of MG63 cells in a dose- and time-dependent manner. At 48 h, the toxicity induced by OXA was evident at the dosage of 1 μM (Figure 3A1). At 72 h, the 5 μM dose of OXA significantly decreased cell viability; however, this effect was not observed at 1 μM. Next, TMQ doses of 10 and 20 μM were combined with OXA. The cell viability percentage differences between the group with OXA alone and the groups with the combinations are shown in Figure 3A2 and B2. The application of 10 μM TMQ combined with 1 μM OXA decreased cell viability at a statistically significant rate (42% and 42%) at 48 and 72 h. The most effective dose of OXA combined with TMQ for cell viability was 1 μM (Figure 3A2, B2). The combinations with 10 and 20 μM TMQ decreased cell viability at rates of 28% and 41% *vs.* the application of OXA alone at 48 h, and by 38% and 51% at 72 h, respectively (Figure 3A2, B2).

Apoptosis was Aggravated by TMQ in Combination with 5FU or OXA

According to the results of Annexin V and Dead Cell Assay, while 10 μM TMQ combined with 5FU or OXA increased apoptosis in MG63 cells at 48 h, the application of TMQ alone did not induce apoptosis (Figure 4). The percentage of early apoptosis in the TMQ alone-treated

MG63 cells was 1.15% (Figure 4D), whereas with the combined treatments with 5FU and OXA, the percentages of early apoptosis increased from 35 and 40.2% (Figure 4B, C) to 60.35 and 61.65%, respectively (Figure 4 DB, DC).

Discussion

TMQ targets different sites in cancer cells by different pathways. TMQ inhibits inflammation, tumor angiogenesis, migration-invasion, tumor cell proliferation, and carcinogen activation, and increases apoptosis, carcinogen detoxification, antioxidant effects, tumor suppressor, cell cyclic arrest, and enhancement of chemo-drugs in cancer cells[5,12]. In cell lines and experimental studies, it has been shown that a combination of TMQ, platinum drugs, and 5FU significantly reduced tumor cell proliferation, tumor size, angiogenesis, and side effects, and increased apoptosis in such cancers[6,15,16,19-21,27-32]. Patients with cancer are known to use herbal products – sometimes along with directed therapy – although there is little information about the effects in OS of such drug combinations. In the current study, it was shown that the combination of TMQ with OXA or 5FU has effects on OS cells; different drug doses were also investigated. The results of this study indicate that, when TMQ is used with OXA or 5FU, it is a promising agent against OS as part of a low dose chemotherapeutic regime. In addition, when TMQ was used alone, it killed the OS cells. Thus, TMQ may be a potential future drug treatment for OS.

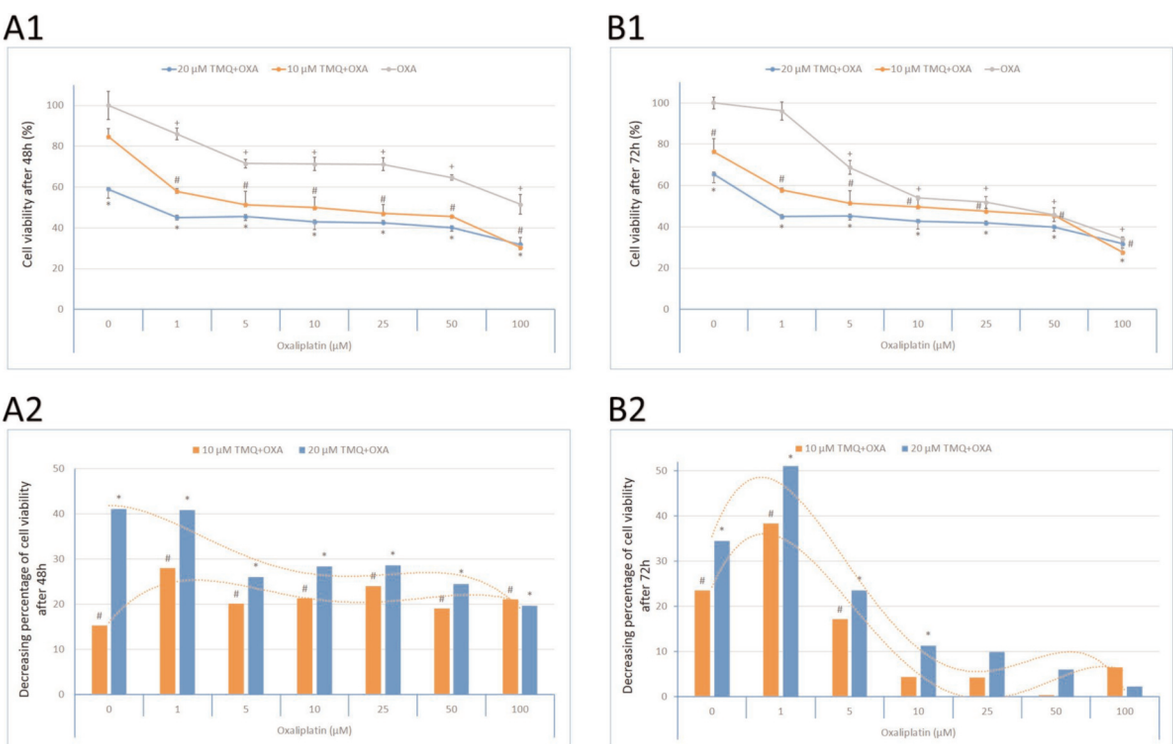

Figure 3. Effects of TMQ combined with oxaliplatin (OXA) on cell viability in a dose- and time-dependent manner.
A1. Combined application for 48 h. The control group (100%) in comparison with the group receiving OXA alone +, the control group in comparison with the group receiving 10 μM TMQ + OXA $^{\#}$, and the control group in comparison with the group receiving 20 μM TMQ + OXA. $^{*}p < 0.05$. A2. Combination group vs. OXA-alone group. The group receiving OXA alone in comparison with the group receiving 10 μM TMQ + OXA #, and the group receiving OXA alone in comparison with the group receiving 20 μM TMQ + OXA. $^{*}p < 0.05$. B1. Combined application for 72 h. The control group (100%) in comparison with the group receiving OXA alone +, the control group in comparison with the group receiving 10 μM TMQ + OXA $^{\#}$, and the control group in comparison with the group receiving 20 μM TMQ + OXA. $^{*}p$ <0.05. B2. Combination group and the group receiving OXA alone.The group receiving OXA alone in comparison with the group receiving 10 μM TMQ + OXA $^{\#}$, and the group receiving OXA alone in comparison with the group receiving 20 μM TMQ + OXA. $^{*}p < 0.05$.

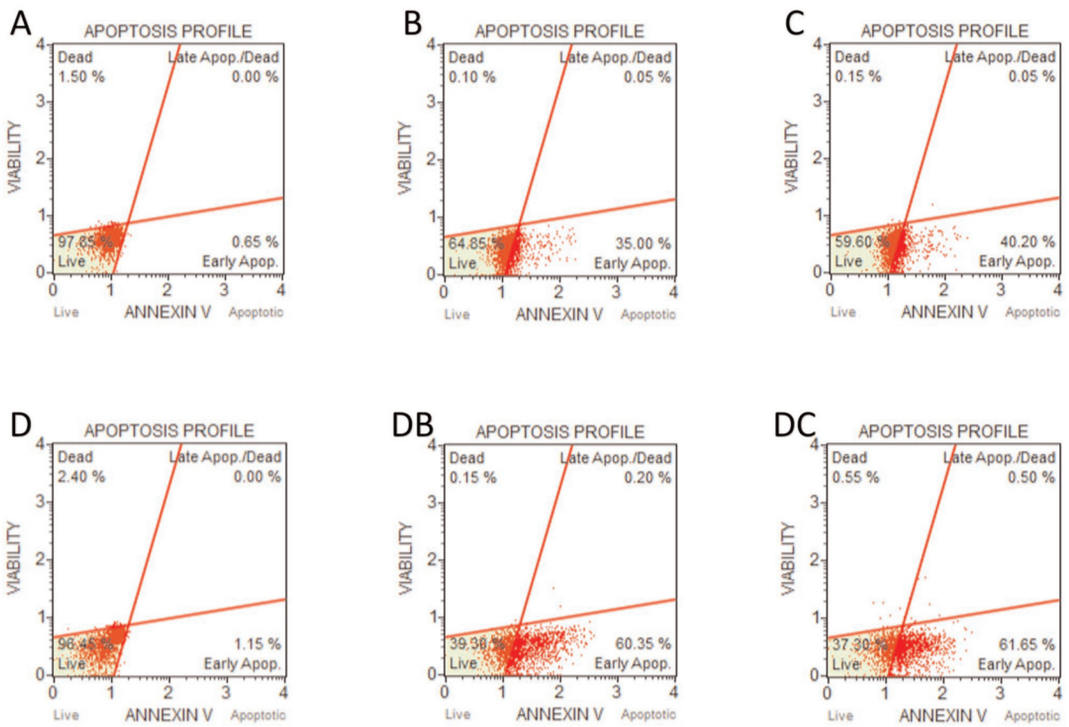

Figure 4. Evaluation of Annexin V and Dead Cell Assay. ***A,*** Untreated group, ***B,*** 5 μM 5FU- treated group, ***C,*** 1 μM OXA-treated group, ***D,*** 10 μM TMQ-treated group, DB. 10 μM TMQ combined with 5 μM 5FU-treated group, DC. 10 μM TMQ combined with 1 μM OXA-treated group.

The development of drug resistance remains a major problem in cancer chemotherapy[33]. Inherent resistance to chemotherapy agents is seen in 35-45% of OS patients[4]. Although the combination of surgery with chemotherapy has markedly improved the survival rates of OS patients, the application of anticancer drugs is still associated with acquisition of drug-resistant phenotypes and significant adverse reactions. Thus, new chemotherapeutical agents are needed and alternative treatments have been investigated[2-5,8,9,13,16,34]. In this study, the use of TMQ in combination with low doses of OXA and 5FU indicated that the combinations were as effective as higher doses of the drugs. We believe TMQ may reduce drug resistance and adverse reactions.

Conclusions

Our present study demonstrates that TMQ is able to inhibit cell proliferation, and induce apoptosis of human osteosarcoma MG-63 cells. TMQ has potential benefits in preventing the onset and progression of chemotherapy drug-induced toxicity and side effects, and may reduce inherent resistance to chemotherapy drugs. We consider that TMQ may have potential as a therapeutic for OS and/or other cancers.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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