Quercetin and Methotrexate in Combination have Anticancer Activity in Osteosarcoma Cells and Repress Oncogenic MicroRNA-223

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ABSTRACT

Introduction Osteosarcoma (OS) is one of the most common bone neoplasms in adolescents. Notable short- and long-term toxic effects of OS chemotherapy regimens have been reported. Hence, new chemotherapeutic agents with the ability to potentiate OS chemotherapy drugs and protect non-tumorous tissues are required.

Methods Saos-2 cells were treated with Methotrexate (MTX) and Quercetin (Que) (a polyphenolic flavonoid with anti-tumor effects) alone and in combination. MTT assay was performed to investigate the cytotoxicity of the drugs. Moreover, apoptosis-involved genes, including miR-*223,* p53, BCL-2, CBX7, and CYLD expression were analyzed via qRT-PCR. Annexin V-FITC/PI kit was employed to assess the apoptosis rate.

Results The MTT results showed that Que increases MTX cytotoxicity on OS cells. The measured IC50s are 142.3 µM for QUE and 13.7 ng/ml for MTX. A decline in MTX IC50 value was observed from 13.7 ng/ml to 8.45 ng/ml in the presence of Que. Moreover, the mRNA expression outcomes indicated that the combination therapy significantly up-regulates the tumor suppressor genes, such as p53, CBX7, and CYLD, and declines anti-apoptotic genes BCL-2 and miR-223, which can lead to proliferation inhibition and apoptosis inducement. Furthermore, the apoptosis rate increased significantly from 6.03 % in the control group to 38.35 % in Saos-2 cells that were treated with the combination of MTX and Que.

Conclusion Que, with the potential to boost the anticancer activity of MTX on Saos-2 cancer cells through proliferation inhibition and apoptosis induction, is a good candidate for combination therapy.

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Introduction

Osteosarcoma (OS) is one of the most prevalent bone neoplasms in children and young adults [1–3]. OS originates in mesenchymal cells, and its most frequent subtypes include osteoblastic Osteosarcoma, chondroblastic Osteosarcoma, and fibroblastic sarcoma. With a propensity to occur in the long bones, Swellings, and pain in the affected bone, occasionally severe pain, are characteristic traits in OS patients [4–9]. Retinoblastoma hereditary mutation and p53 recessive mutation in the Li- Fraumeni syndrome are the two genes currently suggested to be associated with the disease [10].

The standard approach to treat OS consists of surgery for primary bones in addition to neoadjuvant and adjuvant chemothera-

py using a combination of MTX, Doxorubicin (DOX), Cisplatin, and Ifosfamide in a range of various doses [11–19]. In spite of the significant attempts to improve surgical procedures and chemotherapy, the survival rate for patients with metastatic disease (mostly pulmonary) is below 30 % [20–22]. Various studies have been conducted to enhance chemotherapy in patients with an inadequate histologic response or reduce therapy in those with a positive response. The results are not satisfactory, and patients' outcomes with OS have not improved [23–25].

Significant short– and long-term toxic effects are in correlation with the chemotherapy regimen of OS. Approximately 1.8 % of HDMTX-treated patients suffer renal dysfunction, and a 4.4 % mortality rate has been announced among those patients [10]. Therefore, enhanced therapeutic regimens that potentiate MTX effects and protect non-tumorous tissues are required in order to improve OS patients' treatment.

Quercetin (Que) is a polyphenolic flavonoid (3, 39, 49, 5, 7-pentahydroxyflavone) existing abundantly in vegetables, fruits, and grains. Que is a unique compound, and its reported biological activities include anti-oxidation, anti-bacterial, anti-viral, cell-cycle modulation, angiogenesis suppressing, and anti-tumor effects. Numerous studies have indicated that various Que concentrations inhibit malignant cell proliferation by regulating particular signaling pathways in many types of neoplasms, including breast, colorectal, prostate, ovarian, lung, hepatic, and gastric [26, 27, 28]. Que has gained substantial attention as a pro-apoptotic agent with specific activity on tumor cells rather than healthy cells. Que enhanced cancer cells' sensitivity to cisplatin In hepatoma. researches have also shown Que's capability to suppress cell proliferation in OS, and Synergistic effects of the combination of DOX and Que have been reported in breast cancer and leukemia [29, 30]. These results imply that Que may increase MTX cytotoxicity on OS cancer cells while reducing its toxic effects on healthy cells.

MicroRNAs(miRNAs) are small (18–22 nucleotides in length), non-coding RNAs that regulate post-transcriptional gene expression. MiRNAs play a crucial role in cancer progression through repressing oncogenes or tumor-suppressor genes' expression levels. Thus microRNAs' dysregulation may be an essential factor in tumorigenesis [31]. The miR-223 sequence is profoundly conserved throughout the evolution process, which implies that miR-223 has a vital part in physiological processes. Moreover, miR-223's indispensable role in carcinogenesis has been announced in various tissues. It may function as an oncogene or a tumor suppressor, or both in different types of malignancies [32–34].

Located on chromosome 17p13, p53 is the most regularly mutated gene in cancer (about 90 % in OS), which was first recognized as a tumor suppressor in 1989. p53 plays a vital part in regulating DNA repair, apoptosis, and the cell cycle and plays an essential role in human tumor prognosis and development, including Osteosarcoma [20, 35–37].

The BCL-2(B-cell lymphoma-2) family proteins are decisive apoptosis regulators, which are found mainly on the outer membrane of mitochondria. Based on the Structure, the family is classified into two main sub-groups, the anti-apoptotic proteins (BCL-XL, BCL-W, MCL-1, BFL-1, BCL-W, BCL2L10, and BCL-2) and the pro-apoptotic proteins(BOK, BAK, and BAX or BIM, PUMA, NOXA), which are distinguished by BCL-2 homology (BH) domains that have been conserved in the evolution process [38, 39].

Encoding a polycomb group protein, CBX7, is an essential factor in controlling genes involved in developmental regulation. CBX7 loss has been linked to progressed malignancy grade in various carcinomas, while CBX7 up-regulation has been associated with extended survival [40–42].

First discovered in 2000, the CYLD gene(chromosome 16q12.1) controls multiple cellular and signaling pathways through coding a cytoplasmic deubiquitinating (DUB) enzyme. Following studies exhibited CYLD roles in immunoregulation, inflammation, cell cycle progression, osteoclastogenesis, and tumorigenesis [43–45].

In this study, we examined the potential of Que in enhancing MTX anticancer effects on human Osteosarcoma by performing an MTT assay and analyzing the expression of the genes involved in apoptosis, including miR-223, p53, BCL2, CBX7, and CYLD, in addition to investigating the apoptosis rate using annexin V-FITC/PI kit and flow cytometry device.

Material and method

Cell culture

The Saos-2 cell line was acquired from the American Type Culture Collection (ATCC, USA). The cells were cultured in RPMI 1640 medium enriched with 10 % fetal bovine serum (FBS), 100 mg/ml streptomycin, and 100 IU/ml penicillin and incubated at 37 °C in an atmosphere with 5 % carbon dioxide (CO2). Trypan blue dye exclusion test was performed to define the number of viable cells [46].

MTT assay

MTT assay was performed to evaluate the MTX Cytotoxicity effect in saos-2 cells. Shortly after washing with PBS, using 0.25 % trypsin, cells were detached and then centrifuged at 1500 rpm for 7 minutes. The cells were seeded in 96 well plates with a concentration of 10^4 cells per well in 200 µl of culture medium. 24 hours later, the medium was discarded and replaced with a new medium containing the chemotherapeutic drugs (MTX, Que, and the combination of them in a variety of different doses). after 48 hours of incubation, 20 µl of 5 % MTT was added to each well, and after additional 4 hours of incubation, the supernatant was removed, and 200 µl of DMSO was added to dissolve the Formazan crystals. The absorbance was measured using the microplate reader (Bio-Rad, California) at 570 nm, and the viability of the Saos-2 cells was determined employing the following formula: [(treated sample OD/untreated sample OD) × 100]. The IC50 (half maximal inhibitory concentration) was assessed via GraphPad Prism 9 software.

Quantitative RT-PCR

To ascertain mRNA expression levels, total RNA was extracted from cells employing a TRIzol® reagent, following the supplier's protocol (Invitrogen). The RNA concentration was assessed by NanoDrop (Thermo Scientific, USA). Complementary DNA (cDNA) was synthesized using an RT reagent Kit (QIAGEN) following the supplier's protocol. The PCR amplification was conducted at 94 °C for 10 min(initial denaturation) succeeding by 45, 94 °C cycles for 10 s and 60 °C for 30 s and 72 °C for 20 seconds, in the ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA). The expression levels of genes were analyzed and expressed as relative

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mRNA expression of CT (threshold cycle) value. ($2^{-(\Delta\Delta CT)}$ method). The primers that were used are included in ▶ **Table 1**.

Apoptosis assay via annexin V-FITC/PI

We have seeded The Saos-2 cancer cells at a concentration of 10^5 cells per well in a six-well plate, and they were incubated for 24 hours in standard conditions. We treated the cells with MTX, Que, and their combination. Next, we detached the cells and resuspended them in binding buffer, and they were incubated at room temperature and in a dark room for 15 minutes with 400 µL of the binding buffer,5 mL of annexin V-FITC (fluorescein iso- thiocyanate), and 10 mL of PI(propidium iodide). Eventually, we used the Becton Dickinson Bioscience's flow cytometry device in order to ascertain the cancer cells' apoptosis.

Statistical analysis

All conducted tests were repeated 3 times, and collected data is presented as mean ± standard deviation (SD). The one-way analysis of variance, the Tukey (post-hoc), and the Student's t-test were utilized for statistical analysis employing the Graph Pad Prism software in which the p-value < 0.05 was regarded as significant.

▶ **Table 1** The primers.

Target gene	Sequences (5' 3')		Annealing temperature
CBX7	Forward	CATGGAGCTGTCAGCCATC	59.0 °C
	Reverse	CTGTACTTTGGGGGCCATC	
BCL2	Forward	CCTCCAGGTAGGCCCGTTTT	57.5 °C
	Reverse	GGGCCTCTGTTCCTTCCCTC	
p53	Forward	GCGTGTGGAGTATTTGGATG	61.0 °C
	Reverse	GTACAGTCAGAGCCAACCTC	
CYLD	Forward	CCTTTATGTCAAGAGGTGGTG	59. °C
	Reverse	GAGTAATGATTGGAAAGAAG	
β-actin	Forward	TCCCTGGAGAAGAGCTACG	59.0 °C
	Reverse	GTAGTTTCGTGGATGCCACA	
miR-223	Forward	CGTGTATTTGACAAGCTG	58.5 °C
	Reverse	GAACATGTCTGCGTATCTC	
U6	Forward	CTCGCTTCGGCAGCACATA	58.5 °C
	Reverse	GTGCAGGGTCCGAGGTCG	

Results

Que increases MTX cytotoxicity in OS cancer cells

Various Que and MTX doses were employed to determine their effects on OS cancer cells (saos-2 cell line). We used Dimethylsulfoxide (DMSO; 0.1 %) as a negative control. After 48 hours, a dose-dependent decline in cancer cell viability in the presence of both drugs was observed (▶ **Fig. 1**). The measured IC50s are 142.3 µM for Que and 13.7 ng/ml for MTX. We used 140 µM Que combined with the same MTX doses, which increased MTX cytotoxicity on cancer cells and decreased cancer cells' viability. Moreover, a decrease in the IC50 value of MTX was observed (from 13.7 ng/ml to 8.45 ng/ml). These results suggest that Que can have a potentiation effect on MTX-mediated cytotoxicity in OS cancer cells.

Quantitative RT-PCR results

Effects OF Que on miR-223, p53, BCL2, CBX7, and CYLD gene expression

Que up-regulated p53, CBX7, and CYLD genes expression considerably and down-regulated miR-223 and BCL2 gene expression, dramatically and slightly, respectively (▶ **Fig. 2**).

Effects OF MTX on miR-223, p53, BCL2, CBX7, and CYLD gene expression

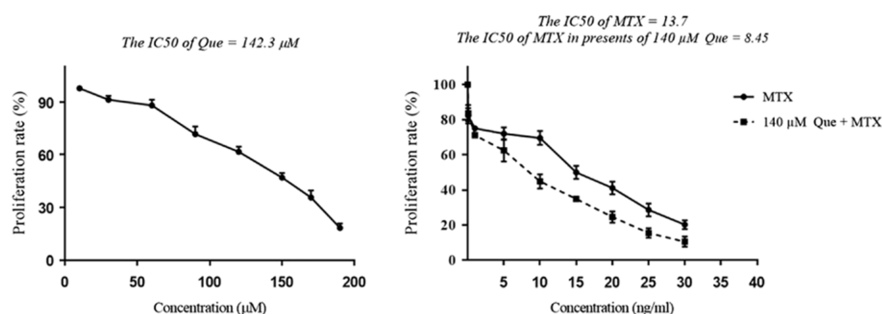
MTX increased CYLD gene expression significantly without having a significant effect on p53 and BCL2 gene expression. Moreover, MTX down-regulated Bcl-2 gene expression substantially while slightly down-regulating miR-223 (▶ **Fig. 2**).

Effects OF Que AND MTX combination on miR-223, p53, BCL2, CBX7, and CYLD gene expression

Que AND MTX combination up-regulated p53, CBX7, and CYLD gene expression dramatically while down-regulating miR-223 and anti-apoptotic BCL2 gene expression substantially (▶ **Fig. 2**).

The Apoptosis Results

It was revealed by the apoptosis assay (annexin V-FITC/PI) that the rates of early apoptosis, as well as the late apoptosis, were heightened meaningfully in Saos-2 cells that were treated with MTX and the combination of MTX and Que comparing to the control group which was containing untreated cells. The collective apoptosis was



▶ **Fig. 1** Proliferation Inhibition of Saos-2 Cells Treated with Quercetin or MTX. Cell viability was determined by MTT assay 48 after treatment. The results are expressed as mean ± SD (n = 3).

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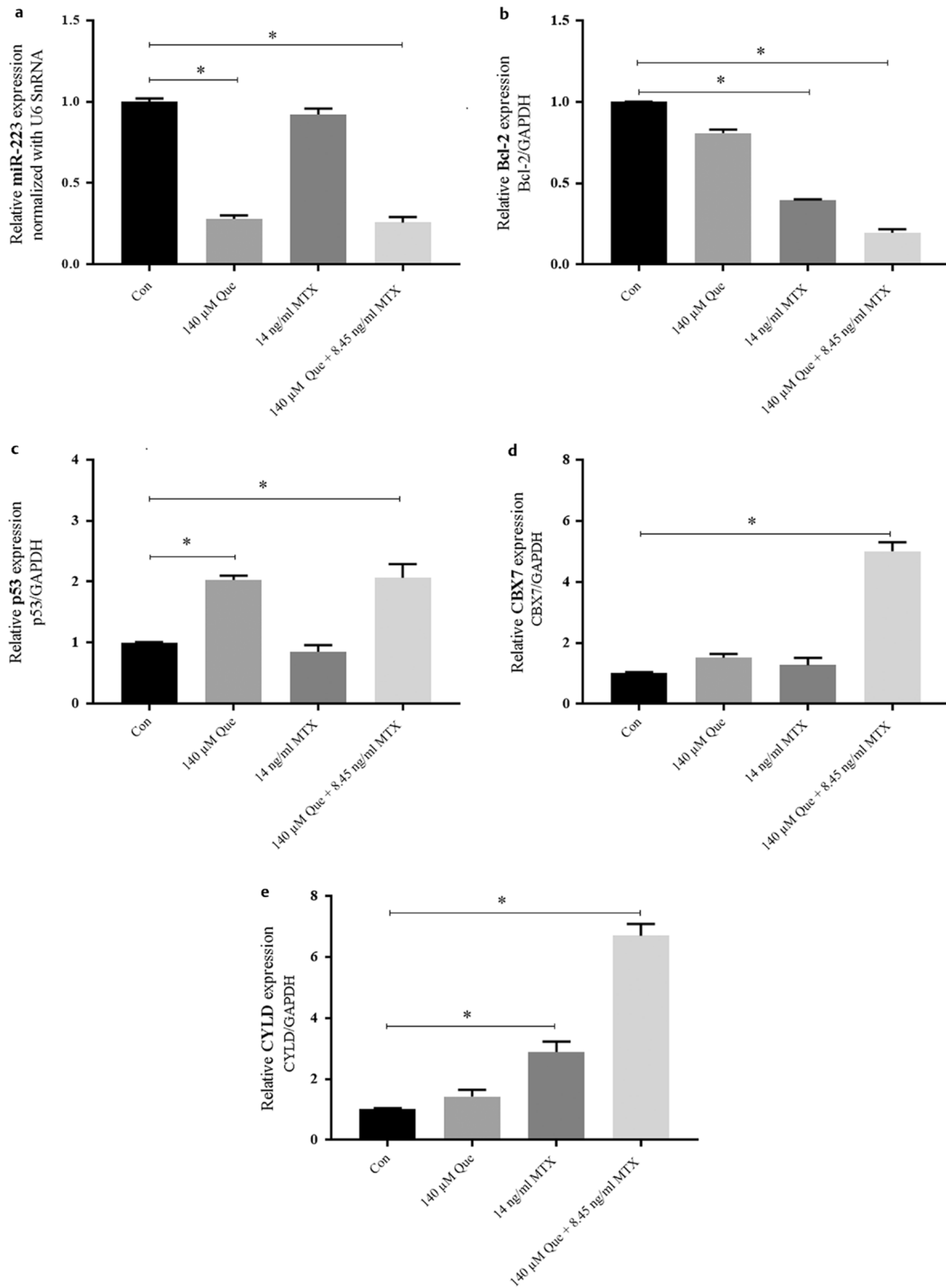

▶ **Fig. 2** The expression levels of, **a**) miR-233, **b**) BCL-2, **c**) p53, **d**) CBX7, and **e**) CYLD after 48 hours of treatment, in Saos-2 cells. The results are stated as mean ± SD (n = 3), * P < 0.05, #P < 0.01.

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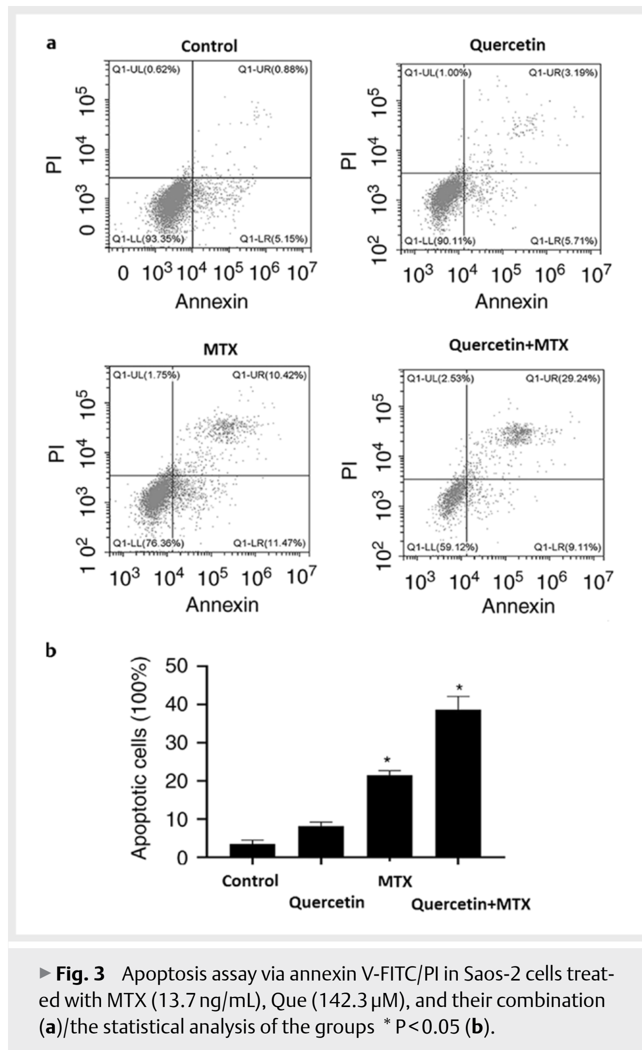

▶ **Fig. 3** Apoptosis assay via annexin V-FITC/PI in Saos-2 cells treated with MTX (13.7 ng/mL), Que (142.3 μM), and their combination (**a**)/the statistical analysis of the groups * $P<0.05$ (**b**).

8.9 % for the cells that were treated with Que, 21.89 % for the cells that were treated with MTX, and 38.35 % for the cells treated with the combination of MTX and Que (▶ **Fig. 3**).

Discussion

Osteosarcoma (OS) is the most prevalent primary bone malignancy, which generally affects adolescents and young adults and can be lethal if untreated. 8.9 % of cancer-related deaths in children are associated with OS, and the overall 5-year survival rate for OS patients is 60–70 % [47, 48].

MTX acts through repressing the generation of metabolites crucial for nucleotides synthesis, and it is utilized to treat OS combined with other chemotherapy agents such as DOX and Cisplatin. In the majority of chemotherapy regimens, a combination of two or more drugs helps to decrease cytotoxicity and enhance chemotherapy agents' potency [49, 50].

Que exhibits its anticancer effects in various ways, including proliferation inhibition, apoptosis inducement, cell cycle arrest, and repressing mitosis through modulating cyclins and pro-apoptotic agents [51]. Que's ability to repress in vitro proliferation and its capacity to induce apoptosis and autophagy has been shown in various studies in numerous cell lines [51–55] including a study that reported that Que can suppress cell growth and metastasis in Osteosarcoma and increase Os' Cells Sensitivity to Cisplatin [56]. Besides, several studies have stated that Que up-regulates the p53 gene [57, 58] while downregulating anti-apoptotic BCL2 [59, 60]. Moreover, it has been reported that Que can efficiently repress the miR-223-3p expression and activate the autophagy pathway [61].

Mutated in more than 50 % of neoplasms, p53 is a tumor-suppressor gene that has been linked to numerous human tumors, including OS. p53 stimulates specific DNA response agents; consequently, the expression of genes is linked to key processes such as cell cycle arrest, apoptosis, and DNA repair. Osteosarcoma's growth hindrance due to p53 up-regulation has been acknowledged, and Various studies have indicated that p53 is related to apoptosis, and A mutant p53 does not repress growth, nevertheless boosts proliferation and tumorigenic potential [62–65] employing the combination of MTX and Que up-regulated p53 significantly compared to using MTX alone, which can, as shown in the apoptosis assay, result in tumor suppression through apoptosis inducement.

BCL2 is an integral protein found particularly on the external membrane of mitochondria and acts as an anti-apoptotic agent; thus, its overexpression inhibits cells from undergoing apoptosis. Overexpression and mutations in the BCL2 gene are correlated with cancer development, and BCL2 upregulation has been reported in several types of cancers [65–67]. Furthermore, the correlation between BCL2 inhibition and apoptosis inducement has been reported by various studies [68–70]. The combination of MTX and Que down-regulated BCL2 significantly, accelerating apoptosis inducement.

CBX7, a member of Polycomb-group proteins, has been determined as a tumor suppressor in various cancers. Multiple studies have proclaimed that CBX7 may perform a crucial role in the initiation and progression of various human neoplasms, and CBX7 under-expression has been linked to a more advanced stage of the tumor and a low survival rate in some carcinomas, including pancreatic and colon [71, 72]. Bao. z. et al. have reported that glioma cells' invasive potency was diminished due to CBX7 over-expression [73]. Moreover, cell growth inhibition and apoptosis inducement in cervical cancer cells were observed in another study [74]. Also, CBX7's significant downregulation in OS cell lines has been reported [75]. The combination of MTX and QUE up-regulated CBX7 significantly compared to using MTX alone, leading to apoptosis inducement, as the mentioned studies have reported.

The CYLD gene product, which is a deubiquitination enzyme, regulates cell survival or proliferation and several signaling pathways. Many studies have confirmed the CYLD gene tumor suppressor role, and loss or down-regulation of the CYLD gene has been reported in several types of malignancies [76–79]. Additionally, the link between CYLD upregulation and apoptosis promotion has been reported in various studies [80–82].

MiRNAs are a class of small, non-coding RNAs that have gained loads of attention due to their ability to regulate gene expression. microRNA-223(miR-223) has been determined to be a potential prognostic and diagnostic marker for various cancers. However MiR-223,s role in cancer is yet to be illuminated since contradicting observations not only in various cancers but in the same type of cancer have been reported [83–85]. Some studies proclaimed that the ex-

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pression of miR-223 in OS was significantly decreased [83, 85] while another study claimed that miR-223 is up-regulated in OS [86]. Studies have also reported that miR-223 functions as an oncogene in human colorectal cancer [87] and gastric cancer [88], and over-expression of miRNA-233 showed a substantial reduction in cellular apoptosis and increased proliferation and invasion vitro [88].

Employing chemotherapeutic agents to induce apoptosis is an essential mechanism to treat various cancers. The present study investigated the Saos-2 cells' apoptosis after being treated with MTX, Que, and their combination. The results have shown that the combination of MTX and Que elevates the apoptosis rates in Saos-2 cancer cells more than treating the cells by MTX alone; Up-regulation of p53, CBX7, and CYLD and down-regulating BCL2 and miR-223 seems to be playing a crucial role in the raised apoptosis rates.

Conclusion

Saos-2 cells were treated with Que AND MTX alone and in combination, and MTT assay was conducted to investigate the cell viability and the cytotoxicity of the drugs. Besides, the mRNA expression levels of miR-223, p53, BCL2, CBX7, and CYLD were evaluated. The mRNA expression results indicated that the Que AND MTX combinations result in a significant up-regulation of tumor suppressors such as p53, CBX7, and CYLD, and a noteworthy reduction in anti-apoptotic BCL2 and miR-223 that may act as an oncogene in OS, which can lead to proliferation inhibition and apoptosis inducement. Overall, these findings may assist with a better understanding of the molecular mechanisms underlying the anticancer properties of Que AND MTX and suggesting Que as an effective chemotherapy agent with the ability to Enhance the anticancer potential of MTX on Saos-2 cancer cells through proliferation inhibition and apoptosis induction.

Ethical approval

All experimental procedures were applied in accordance with the approval from the Ethics Committee of Tabriz University of Medical Sciences (IR.TBZMED.VCR.REC.1398.265).

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Conflict of Interests

The authors declare that they have no conflict of interest.

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