

Anticancer effect of quinacrine on diffuse large B-cell lymphoma via inhibition of MSI2-NUMB signaling pathway

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Abstract. Diffuse large B-cell lymphoma (DLBCL) is the most common subtype of non-Hodgkin's lymphoma. Despite improvements in the clinical outcomes of DLBCL, ~30% of patients will develop relapse/refractory disease. Therefore, novel therapeutic drugs have been investigated to improve disease outcomes. Previous studies have revealed the anti-cancer effects of quinacrine (QC) on tumor cells *in vitro*, although its role in human DLBCL is yet to be identified. The present study sought to examine the cytotoxic effect of QC on DLBCL cells. QC induced G0/G1 cell cycle arrest and apoptosis in the DLBCL cell lines SU-DHL-8 and OCI-LY01, in a dose-dependent manner, in addition to the downregulation of cyclin-dependent kinase 4/6 and the upregulation of cleaved poly-ADP ribose polymerase 1. Upon exposure to QC, RNA-binding protein Musashi homolog 2 inactivation and activation of protein numb homolog were observed. In addition, QC was able to inhibit the expression of Myc proto-oncogene protein. The results of the present study indicated that QC may be a potential anti-DLBCL drug.

Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common type of lymphoma, representing 25% of all lymphoproliferative disorders (1). Despite its aggressive disease course, ~50-70% of patients may experience benefits with R-CHOP (rituximab plus cyclophosphamide, doxorubicin, vincristine and prednisone) chemotherapy (1). However,

there remain certain patients with relapse or disease which is refractory to R-CHOP, ultimately with only ~10% achieving a cure, requiring aggressive salvage chemotherapy and transplantation (2). Therefore, novel therapeutic drugs are being developed to improve the outcomes of this disease.

Quinacrine [QC; 6-chloro-9-(diethylamino-1-methylbutylamino)-2-methoxyacridine] is a 9-aminoacridine derivative clinically used as an antimalarial drug, which has additionally been observed to have anti-cancer activity (3,4). A number of studies have suggested that the anti-cancer activity of QC is not associated with its DNA-binding ability, and is mediated via the suppression of survival signaling in cancer cells (3). Simultaneous activation of cellular tumor antigen p53 (p53) and suppression of the phosphatidylinositol-3 kinase/RAC- α serine/threonine-protein kinase/protein kinase mTOR and nuclear factor (NF)- κ B pathways serve an important role in the anti-cancer activity of QC (3,5,6). Additionally, in human colon carcinoma cell lines, QC has been demonstrated to promote tumor necrosis factor ligand superfamily member 10, oxaliplatin and 5-fluorouracil cytotoxicity by inducing NF- κ B inactivation (6,7). QC is a chemosensitizer which is able to enhance chemotherapeutic drug-induced apoptosis in cancer cells (8-11). However, the effect of QC on DLBCL cells has not been reported.

The present study investigated the effects of QC on proliferation and apoptosis in DLBCL cell lines and clarified the possible target molecules of QC in DLBCL cells *in vitro*.

Materials and methods

Reagents. QC was obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany; cat. no. Q3251) and dissolved in PBS as a 10 mM stock solution. Dilutions to the required concentrations were made in RPMI-1640 medium. Rabbit polyclonal to RNA-binding protein Musashi homolog 2 (MSI2; cat. no. ab50829) antibody was purchased from Abcam (Cambridge, UK); rabbit monoclonal protein numb homolog (Numb; cat. no. 2761S), Myc proto-oncogene protein (c-Myc; cat. no. 5605), β -actin (cat. no. 8457S) antibody, rabbit polyclonal poly-ADP ribose polymerase 1 (PARP) antibody (cat. no. 9542S), rabbit monoclonal cyclin-dependent kinase (CDK)6 (cat. no. D4S8S), rabbit monoclonal CDK4 (cat. no. D9G3E) and rabbit polyclonal

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caspase-3 antibody (cat. no. 9665S) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Cell culture. DLBCL cell lines OCI-Ly01 and SU-DHL-8 were purchased from the American Type Culture Collection (Manassas, VA, USA) and passaged for <6 months following receipt or resuscitation from stocks, and were maintained in RPMI-1640 medium (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (AusGeneX, Molendinar, Queensland, Australia), 4 mM L-glutamine (Sigma-Aldrich; Merck KGaA), 100 U/ml penicillin (Hyclone; GE Healthcare Life Sciences) and 100 U/ml streptomycin (Hyclone; GE Healthcare Life Sciences). All cell cultures were performed at 37°C in a humidified atmosphere with 5% CO₂.

Cell viability analysis. The cell viability of DLBCL cell lines was measured using the MTS method (CellTiter 96[®] Aqueous One Solution; cat. no. 207284; Promega Corporation, Madison, WI, USA). A total of 2×10⁴ cells/well were incubated in quadruplicate in a 96-well microculture plate, in the presence of different concentrations of QC in a final volume of 0.1 ml for 48 h at 37°C. Subsequently, each well was treated with MTS (20 μl MTS/100 μl) for 4 h, and the absorption values at 590 nm were determined using an automatic ELISA plate reader (iMark; Bio-Rad Laboratories, Inc., Hercules, CA, USA). Values were normalized to untreated (control) samples.

Cell cycle analysis. Cells (1.0×10⁵/ml) were treated with 0, 1, 1.5 and 2 μmol/l QC for 48 h, and subsequently fixed with 100% cold ethanol at -20°C for 48 h, followed by staining with a Cell Cycle Staining kit [propidium iodide (PI); MultiSciences Biotech Co., Ltd., Hangzhou, China; cat. no. CCS012] in the presence of RNase for 15 min at room temperature. Cell-cycle distribution was assessed using a FACScan instrument (BD FACSCanto[™] II; BD Biosciences, Franklin Lakes, NJ, USA). Data were analyzed using FlowJo 7.6.1 software (FlowJo LLC, Ashland, OR, USA).

Analysis of apoptosis. Cells (1.0×10⁵/ml) were treated with 0, 0.8, 1.6 and 3.2 μmol/l QC for 24 h. Staining was performed using annexin V-fluorescein isothiocyanate (Multisciences Biotech Co., Ltd.; cat. no. 4100546) in conjunction with PI, according to the manufacturer's protocol, and was assessed using a FACScan instrument (BD FACSCanto[™] II; BD Biosciences). Data were analyzed using BD FACSDiva software version 3.3.11. Apoptosis was validated via PARP cleavage and analyzed through western blotting.

Protein extraction and western blot analysis. Cells were lysed using SDS buffer (BBI Solutions, Cardiff, UK) containing proteinase inhibitors (phenylmethylsulfonyl fluoride). Cell extracts containing 50 μg of proteins, determined by the bicinchoninic acid method, were separated by SDS-PAGE on a 12% gel, and transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories, Inc.). The membrane was blocked in 5% nonfat milk (Shanghai Bright Diary Group Co., Ltd, Shanghai, China) at room

temperature for 2 h and incubated with specific antibodies (1:1,000) overnight at 4°C. Primary antibodies were detected by incubating the membrane in anti-rabbit IgG, HRP-linked antibody (cat. no. 7074; Cell Signaling Technology, Inc.) for 2 h at room temperature, using enhanced chemiluminescence (PerkinElmer, Inc., Waltham, MA, USA). Densitometry quantification of immunoblot analyses was performed using Image Lab software (version 5.2.1; Bio-Rad Laboratories, Inc.).

Statistical analysis. All statistical analyses were performed using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). Data are presented as the mean ± standard deviation. The statistical significance of the differences observed between experimental groups was determined using one-way analysis of variance and a post hoc LSD test. P<0.05 was considered to indicate a statistically significant difference.

Results

QC inhibits the growth of SU-DHL-8 and OCI-Ly01 cells. The present study investigated whether QC leads to the inhibition of DLBCL cell growth. The two DLBCL cell lines (OCI-Ly01 and SU-DHL-8) were cultured with varying concentrations of QC (0, 2.5, 5 and 10 μM) for 24 h, and it was observed that the cells exhibited green fluorescence, and that the fluorescence intensity gradually weakened with the increase in QC concentration (Fig. 1A). Cell viability was assessed by MTS assay. As the dose of QC increased from 1 to 8 μM, cell growth inhibition increased in a dose-dependent manner in the two DLBCL cell lines (Fig. 1B and C). The half-maximal inhibitory concentrations (IC₅₀) for SU-DHL-8 and OCI-Ly01 were 2 and 1.8 μM, respectively. The two cell lines were treated with a variety of different concentrations of QC for 96 h, and cell growth was inhibited in a dose- and time-dependent manner (Fig. 1D and E).

QC arrests the cell cycle of SU-DHL-8 and OCI-Ly01 cells at the G0/G1 phase. In order to understand whether the growth inhibitory effect of QC contributed to cell cycle arrest, the effects of QC on the cell cycle were evaluated. It was observed that QC (2 μM) induced apparent G0/G1 phase arrest in OCI-Ly01 compared with control cells (P=0.00022; Fig. 2A and B). QC (1, 1.5 and 2 μM) was able to decrease the protein expression of CDK4 compared with control cells (P=0.002, P<0.001, P<0.001), and consistent results were observed with CDK6 (P<0.001) in OCI-Ly01 cells (Fig. 2C and D).

Consistent results were also observed in SU-DHL-8 cells. QC (1.5 and 2 μM) induced apparent G0/G1 phase arrest compared with the control cells (P=0.002, P=0.0002) (Fig. 3A and B), the protein expression of CDK4 decreased following treatment with QC (1, 1.5 and 2 μM) compared with control cells (P<0.001, respectively) and the same was observed for CDK6 (P<0.001) (Fig. 3C and 3D).

QC induces apoptosis of SU-DHL-8 and OCI-Ly01 cell lines. In order to study the induction of apoptosis, SU-DHL-8 and OCI-Ly01 cells were treated with four different QC concentrations, 0, 0.8, 1.6 and 3.2 μM, for 24 h. Compared

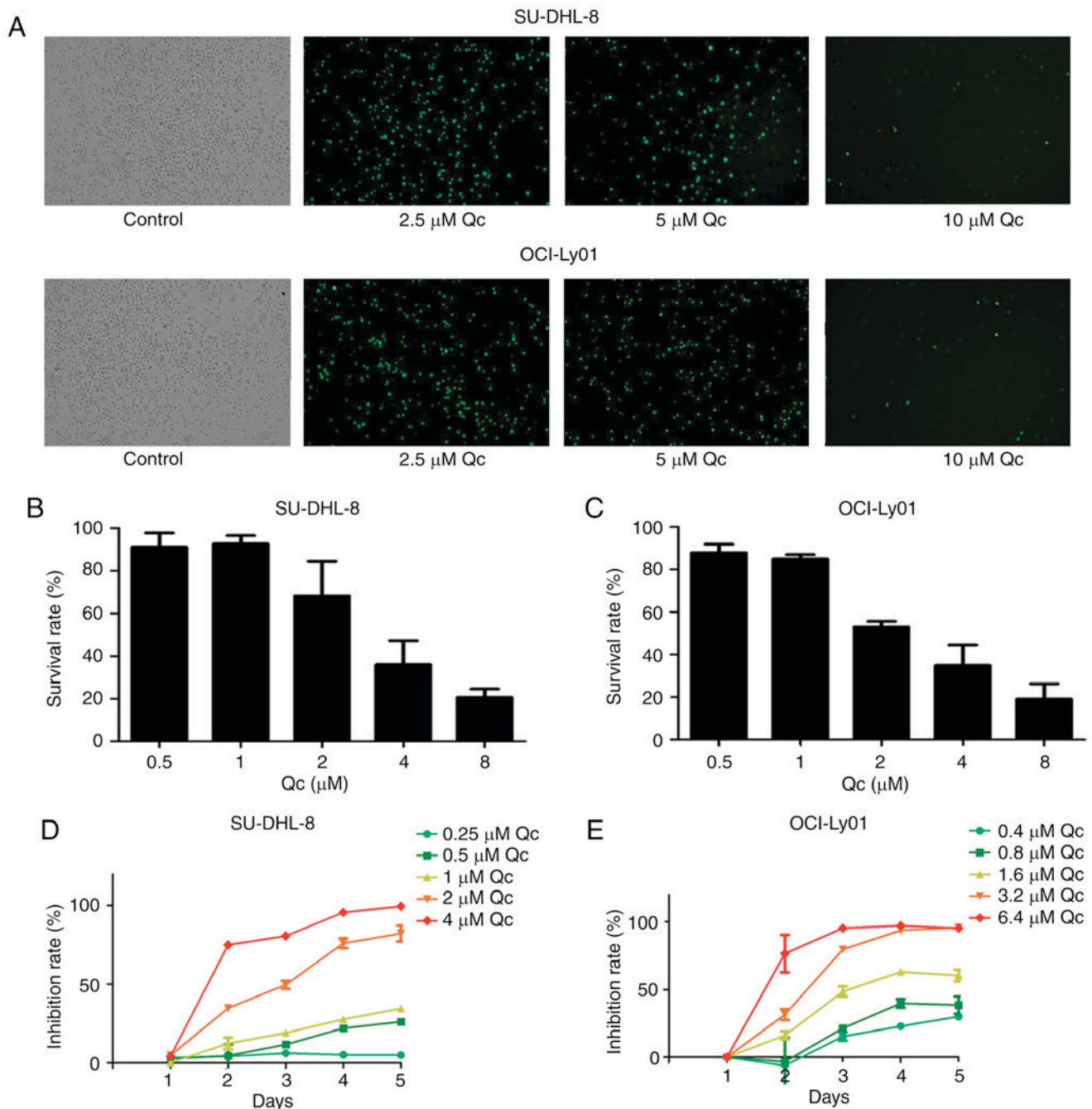


Figure 1. QC inhibits the proliferation of diffuse large B-cell lymphoma cells. (A) DLBCL cell lines (SU-DHL-8 and Ly01) were incubated with QC and exhibited green fluorescence. Fluorescence intensity gradually weakened with the increase in drug concentration o. Magnification, $\times 100$. (B) Viability of SU-DHL-8 cells and (C) OCI-Ly01 cells following treatment with QC for 48 h. The effect of QC on the growth of the (D) SU-DHL-8 and (E) OCI-Ly01 cell lines is presented. QC, quinacrine.

with the control group, the percentages of apoptotic cells of groups treated with QC increased significantly in a dose-dependent manner in SU-DHL-8 and OCI-Ly01 cells (Fig. 4A and B). In addition, the expression levels of cleaved PARP protein increased in groups treated with QC compared with control cells in the OCI-Ly01 cell line ($P < 0.001$), and the same results were noted in the SU-DHL-8 cell line ($P < 0.001$, $P = 0.007$ and $P < 0.001$, respectively) (Fig. 4C and D).

QC regulates the MSI2-NUMB-c-Myc signaling pathway. It was previously reported that MSI2 served an important role

in hematopoietic stem cell function and cell fate determination (12). MSI2 inhibits the expression of Numb, which is an evolutionarily well-conserved protein (13,14). Therefore, the present study examined whether QC may affect the MSI2-Numb signaling pathway. SU-DHL-8 and OCI-Ly01 constitutively expressed MSI2 and Numb. When SU-DHL-8 and OCI-Ly01 were treated with 0, 1.6, 3.2 and 6.4 μ M QC for 24 h, the expression of MSI2 decreased, whereas that of Numb increased (Fig. 5A and B), suggesting that the inhibition of MSI2 may lead to the accumulation of Numb protein. Previous studies have demonstrated that c-Myc

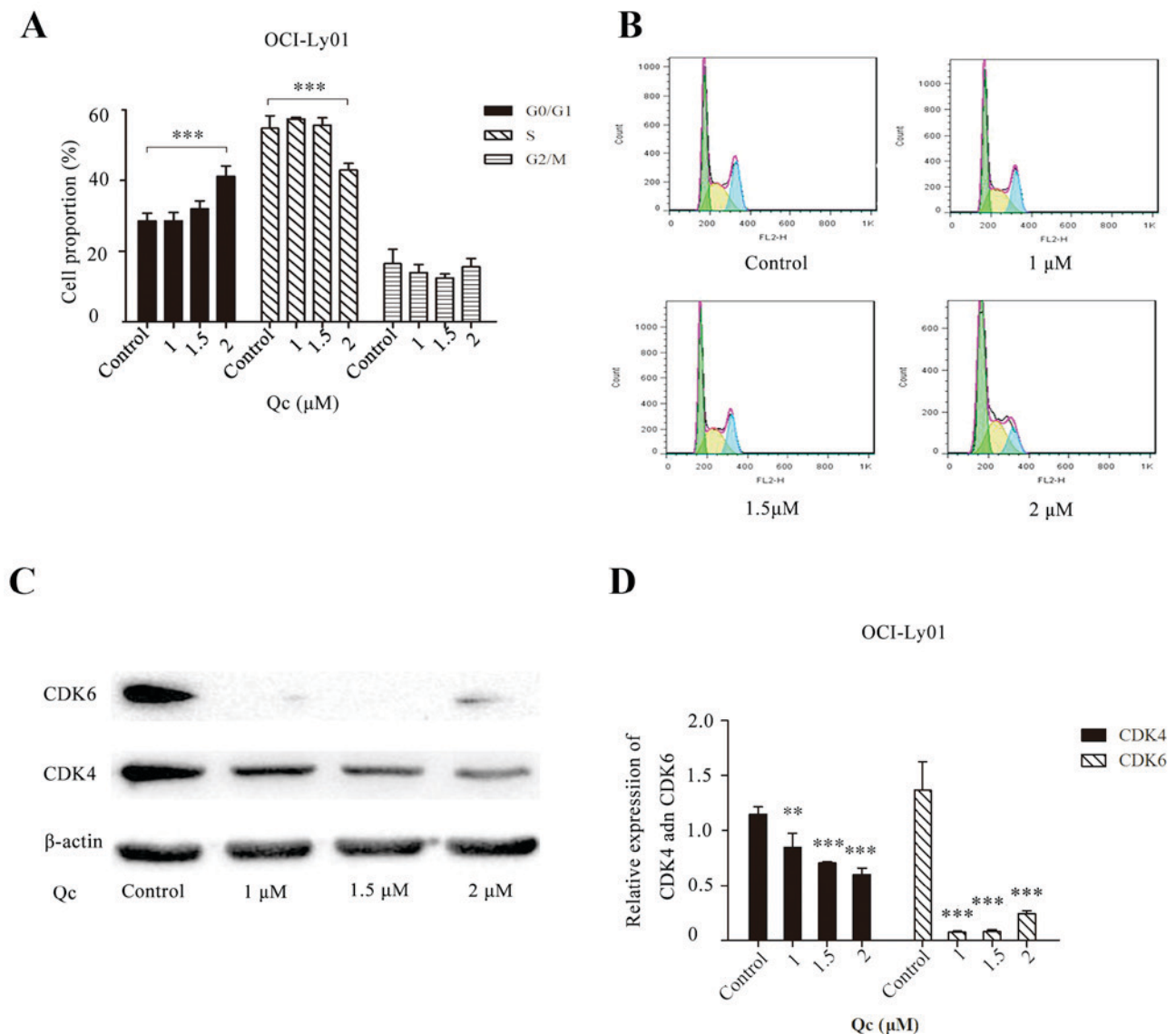


Figure 2. QC induces cell cycle arrest of OCI-Ly01 cells at the G0/G1 phase. (A) Analysis of the (B) flow cytometry data demonstrated cell cycle arrest at the G0/G1 phase. The protein expression of CDK4/CDK6 decreased in OCI-Ly01 cells following treatment, as demonstrated by (C) western blotting and (D) densitometric analysis. Each experiment was performed in triplicate, and error bars represent the mean \pm standard deviation. ** $P < 0.01$; *** $P < 0.001$ vs. control. QC, quinacrine; CDK, cyclin-dependent kinase.

was regulated by Numb (15). Therefore, the present study assessed the expression level of c-Myc by western blotting. As hypothesized, QC decreased c-Myc expression with the increase of drug concentration in SU-DHL-8 and OCI-Ly01 cells (Fig. 5C and D).

Discussion

QC is a well-known antimalarial drug, and its anticancer effects have been demonstrated (16-19). It has been demonstrated that QC may induce apoptosis and arrest the cell cycle at the S phase via inhibition of topoisomerase activity, and induction of p53 and CDK inhibitor 1 in breast cancer and colon cancer cells (4,20), and inhibition of NF- κ B and Wnt-T-cell factor signaling via the adenomatous polyposis coli gene in breast cancer (21). However, to the best of our knowledge, the inhibition of QC in DLBCL cells has been not reported. The present study investigated

the antiproliferative potential of QC in SU-DHL-8 and OCI-Ly01 cells. It was observed that QC inhibited cell growth. The IC_{50} of QC in SU-DHL-8 and OCI-Ly01 cells was 1.8 and 2 μ M, respectively, approximately equal to the IC_{50} of QC in other cancer cells, including non-small cell lung cancer (22), breast cancer (4) and leukemia K562 cells (23). The increased protein expression of cleaved PARP following treatment with QC indicated that QC was able to induce cellular apoptosis in OCI-Ly01 and SU-DHL-8 cell lines, which was consistent with previous reports (4,5,11). QC additionally induced cell cycle arrest at the G0/G1 phase and apoptosis in human DLBCL cell lines SU-DHL-8 and OCI-Ly01 in a dose-dependent manner, and the decreased expression of CDK6 and CDK4 confirmed G0/G1 cell cycle arrest. However, previous reports indicated that QC induced cell cycle arrest at the G1/S and G2/M (22), and sub-G1 and S phases (24), which is not consistent with the results of the present study. A possible reason for this is that the

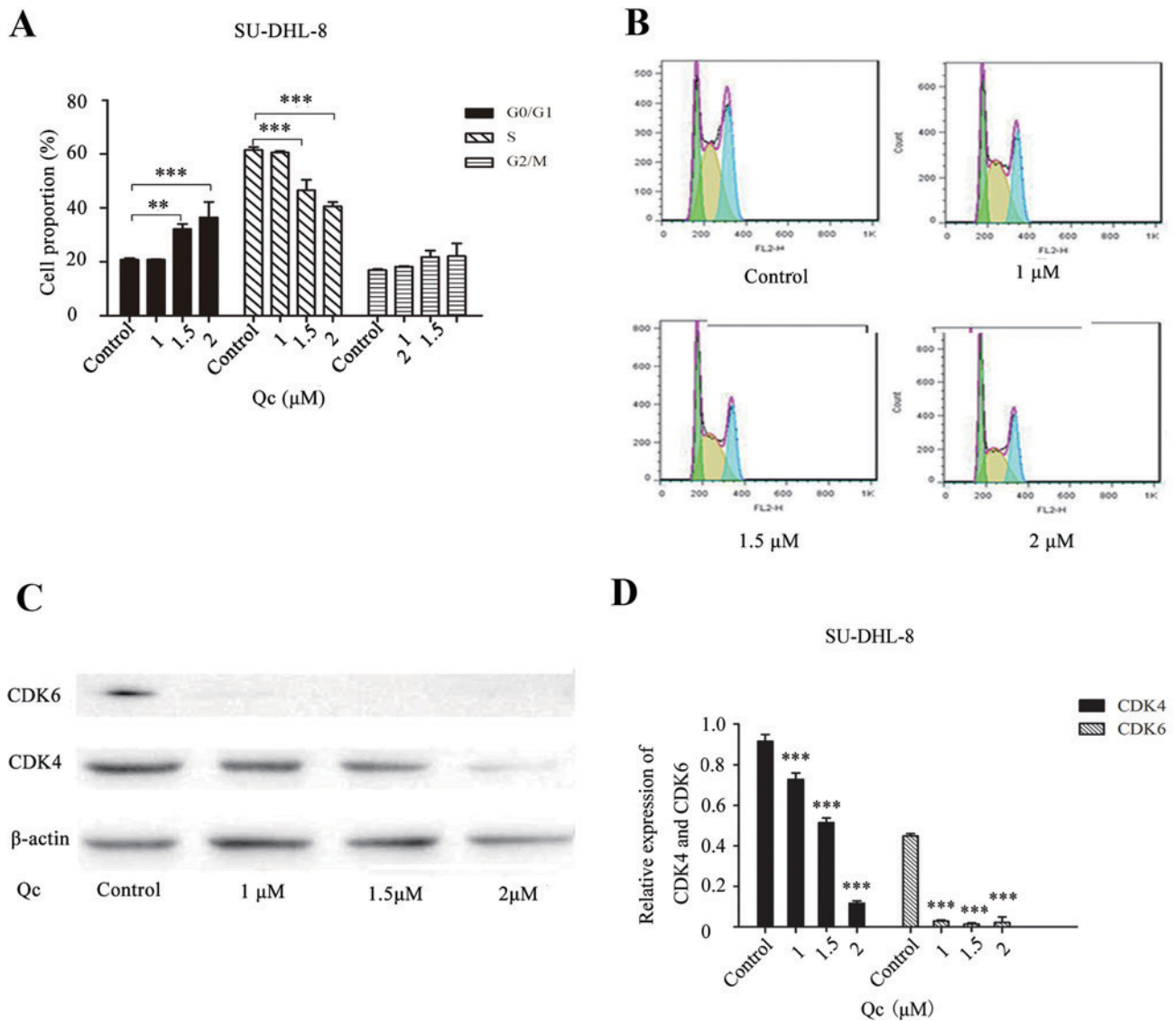


Figure 3. QC induces cell cycle arrest of SU-DHL-8 cells in G0/G1 phase. (A) Analysis of the (B) flow cytometry data demonstrated cell cycle arrest at the G0/G1 phase. The protein expression of CDK4/CDK6 decreased in SU-DHL-8 cells following treatment, as demonstrated by (C) western blotting and (D) densitometric analysis. Each experiment was performed in triplicate, and error bars represent the mean \pm standard deviation. ** $P < 0.01$; *** $P < 0.001$ vs. control. QC, quinaquine; CDK, cyclin-dependent kinase.

molecular mechanism of QC is heterogeneous in different types of cells.

The Musashi proteins are RNA-binding proteins which are encoded by two translational regulatory genes, MSI1 and MSI2, located on chromosomes 12 and 17 (25,26). They distinctively regulate transcriptional events and act as cell cycle regulators (27). The Musashi gene family is highly expressed in stem cells (28,29). MSI1 and MSI2 expression has been associated with an unfavorable prognosis in several types of tumor, including glioma, pediatric brain tumor, breast cancer and colorectal cancer (30,31). In previous studies, high MSI2 expression indicated a poor prognosis and facilitated risk and treatment stratification in adult and pediatric patients with B-cell acute lymphoblastic leukemia (32,33), indicating that MSI2 was able to serve an important role in B-cell neoplasm. In the present study, MSI2 expression was detected in SU-DHL-8 and OCI-Ly01 cells, which further supported this hypothesis. Notably, the results

of the present study demonstrated that QC was able to down-regulate MSI2 expression and upregulate Numb expression. Previous studies demonstrated that MSI2 was able to inhibit Numb mRNA translation, promoting the development and progression of pancreatic cancer (34).

In addition, the knockdown of Numb has been demonstrated to increase c-Myc expression (15). c-Myc is the master transcription factor of cell proliferation and is involved in numerous hematological and solid types of cancer (35), including DLBCL (36). In the present study, it was observed that QC induced cell cycle arrest at the G0/G1 phase. Consistent with the results of the present study, downregulation of MSI2 and c-Myc has been observed to induce cell cycle arrest at the G0/G1 phase (37) and Numb overexpression may inhibit cell cycle progression at the G0/G1 phase (38,39). Additionally, CDK4 and CDK6 are two classical cell cycle-associated proteins which are involved in cell cycle transformation between the G0/G1 and S phases. It

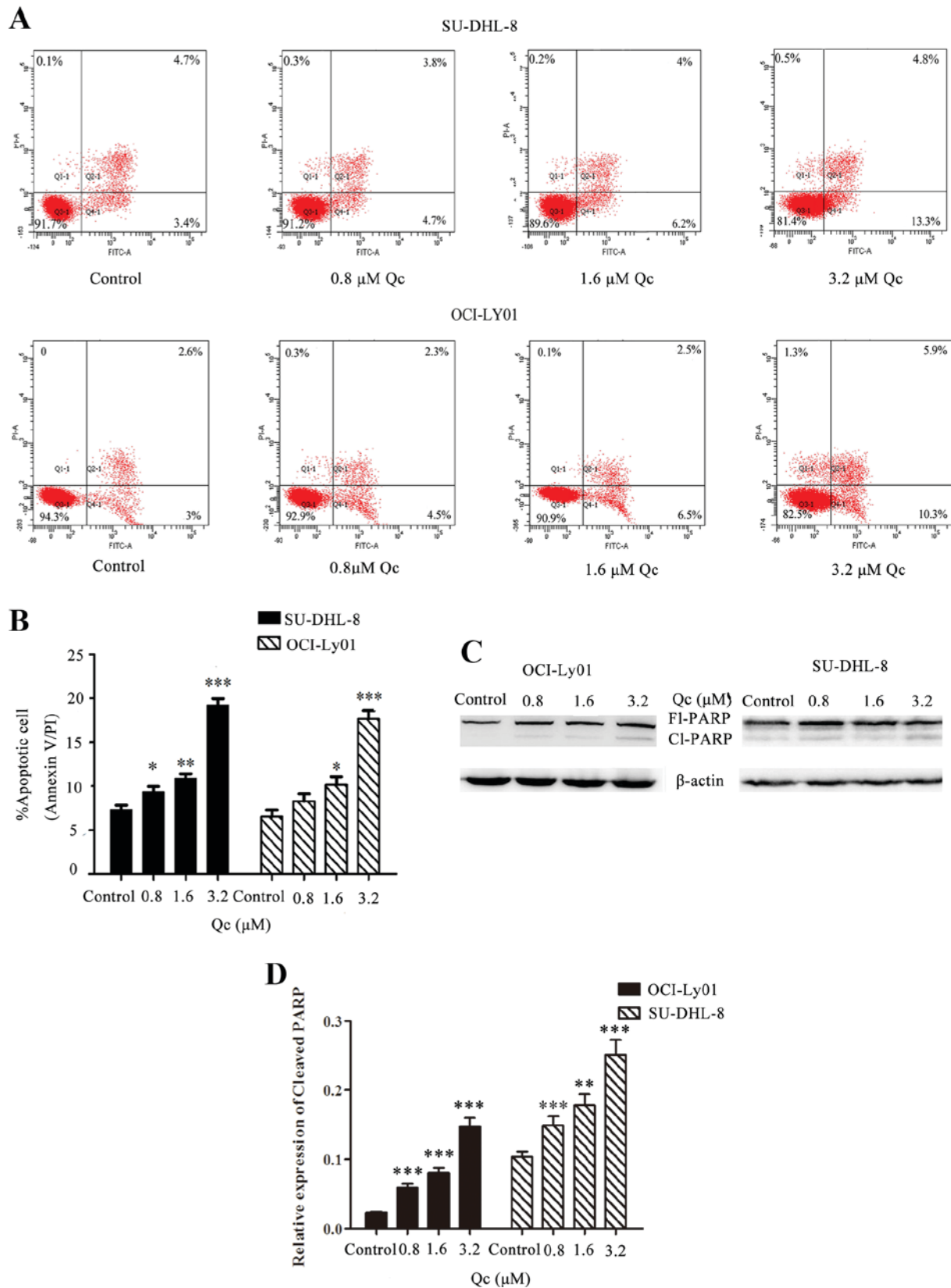


Figure 4. QC induces apoptosis *in vitro* in diffuse large B-cell lymphoma cells. SU-DHL-8 and OCI-LY01 cells were treated with QC for 24 h, and subjected to (A) flow cytometry and (B) statistical analysis. CI-PARP expression following treatment with QC for 24 h in SU-DHL-8 and OCI-LY01 cells was examined via (C) western blotting and (D) densitometric analysis. Each experiment was performed in triplicate, and error bars represent the mean \pm standard deviation. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs. control. QC, quinacrine; PARP, poly-ADP ribose polymerase 1; FI, full-length; CI, cleaved.

was observed that QC decreased CDK4 and CDK6 expression in the OCI-LY01 and SU-DHL-8 cell lines, consistent

with the observed G0/G1 cell cycle arrest. Therefore, it was hypothesized that QC may inhibit MSI2 expression, increase

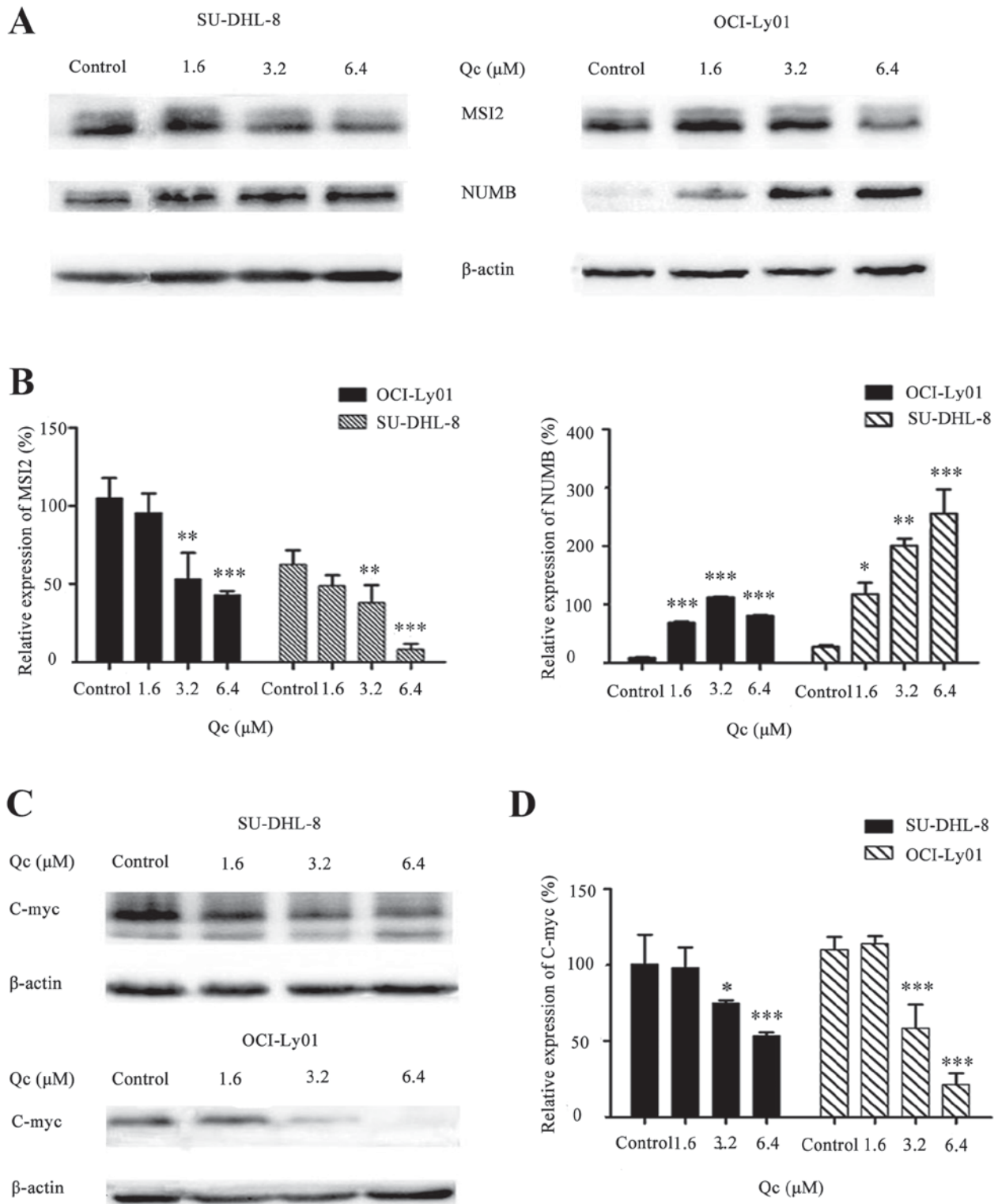


Figure 5. QC regulates the expression of MSI2, Numb and c-Myc proteins in SU-DHL-8 and OCI-Ly01 cells. The expression of MSI2 declined and the expression of Numb increased, as demonstrated by (A) western blotting and (B) densitometric analysis. The expression of c-Myc decreased following treatment with QC, as demonstrated by (C) western blotting and (D) densitometric analysis. β -actin was used as the loading control. Each experiment was performed in triplicate, and error bars represent the mean \pm standard deviation. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs. control. QC, quinacrine; MSI2, RNA-binding protein Musashi homolog 2; Numb, protein numb homolog; c-Myc, Myc proto-oncogene protein.

NUMB expression, suppress c-Myc expression, and decrease CDK4 and CDK6 expression.

In conclusion, the results of the present study indicated that QC may inhibit the growth of DLBCL cells, possibly

via the MSI2-NUMB signaling pathway, and is a potential drug for the treatment of DLBCL. However, further studies *in vivo* are required to confirm the clinical effects of QC in DLBCL.

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