

C-Myc inhibitor 10058-F4 increases the efficacy of dexamethasone on acute lymphoblastic leukaemia cells

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Abstract. The long-term survival rate in paediatric acute lymphoblastic leukaemia (ALL) exceeds 80%; however, the outcome of adult ALL remains to be poor. Glucocorticoids (GCs) are the preferred drugs in the traditional treatment of ALL patients. In the anti-leukaemia molecular mechanisms of GCs, c-Myc inhibition serves a critical role. In the present study, a c-Myc inhibitor that increased the sensitivity to GCs in NALM6 cells of the B-cell-ALL cell line and CEM cells of the T-cell-ALL cell line was investigated. The data demonstrated that 10058-F4, a c-Myc inhibitor, increased the growth inhibition, G₀/G₁ phase arrest and apoptosis of the NALM6 and CEM cells as induced by dexamethasone (DXM), a type of GC. Additionally, 10058-F4 reinforced the decreased expressions of c-Myc, cyclin-dependent kinase (CDK)-4 and CDK6 in the NALM6 and CEM cells treated with DXM. These findings indicated that DXM in combination with the c-Myc inhibitor 10058-F4 may be a novel, potent therapeutic strategy for the treatment of ALL.

Introduction

Acute lymphoblastic leukaemia (ALL) is the most common malignancy in children; the 5-year disease-free survival probability of children with ALL has been improved to approximately 80%. While ALL is less prevalent in adults, the long-term survival rate is only 35-50% even with the adoption of allogeneic haematopoietic stem cell transplantation (allo-HSCT) treatment (1-3). Recently, chimeric antigen receptor (CAR) T-cell therapy was induced to relapsed and refractory B-ALL patients, resulting in remission rates of 67-90% (4). However, the incidence of relapse after CAR T-cell treatment exceeds

40% (5). Thus, novel strategies and drugs for ALL treatment need further exploration.

c-Myc, an oncogene that encodes a 64-67 kDa transcription factor, has been known for a few decades. c-Myc plays an important role in cell-fate decisions, including proliferation, differentiation and apoptosis. The abnormal expression of the c-Myc gene is critically involved in tumourigenesis (6,7). c-Myc expression increases in primary T-ALL and B-ALL cells and indicates a poor prognosis (8). Interestingly, c-Myc inhibition by small hairpin RNA or BET bromodomain have been shown to prevent leukaemia initiation in mice by eliminating leukaemia-initiating cell (LIC) activity and suppressing the growth of relapsed of paediatric T-ALL cells (9). Thus, c-Myc inhibition combination with other traditional chemotherapy agents could more effectively eliminate ALL cells.

Glucocorticoids (GCs) can induce G₁ cell cycle arrest and the apoptosis of lymphoid cells; GCs are the preferred drugs in the traditional regimens of ALL patient treatments, such as VP (Vincristine, VCR + Prednison, P), VDP (Vincristine, VCR + Daunorubicin, DNR + Prednison, P), and VDLP (Vincristine, VCR + Daunorubicin, DNR + L-asparaginase, L-ASP + Prednison, P) (10-12). The direct binding of GCs to cytosolic glucocorticoid receptors (GRs) can induce the release of the latter from its protein complex and subsequent dimerization and translocation to the nucleus. The dimerization of GRs by binding to GC response elements regulates a series of gene expression inducing apoptosis and cycle arrest (13). Among these genes, c-Myc inhibition plays a critical role in the apoptosis and cell cycle arrest of ALL cells induced by GCs (14,15). Thus, we speculated that the combination of GCs with c-Myc inhibitors could synergistically kill ALL cells by down-regulating further c-Myc expression.

In this study, we investigated the inhibition of cell viability and the induction of cell cycle and apoptosis in ALL cell lines treated with dexamethasone (DXM) alone or in combination with c-Myc inhibitor 10058-F4. Our results for the first time demonstrated that 10058-F4 increased the growth inhibition of ALL cells induced with DXM by cell cycle arrest and the promotion of apoptosis.

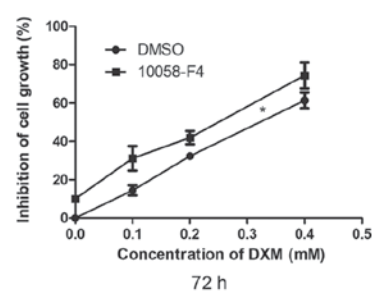
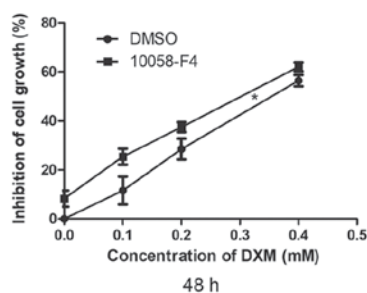
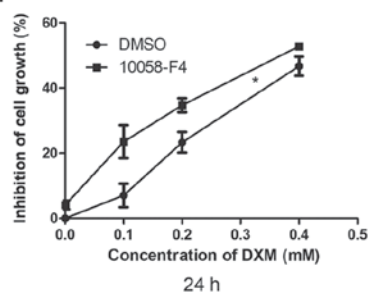
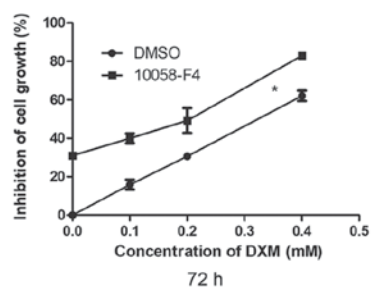
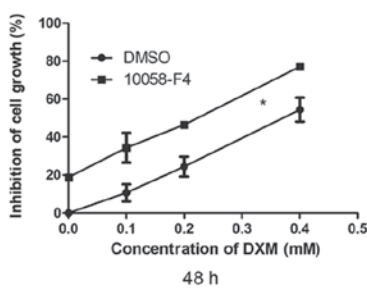
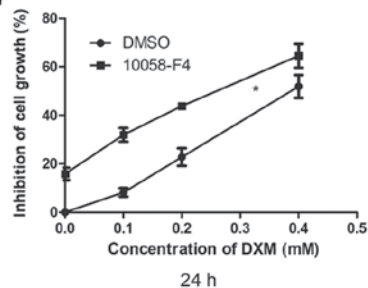
Materials and methods

Cell lines and reagents. NALM-6 (a B-ALL cell line) and CEM cells (a T-ALL cell line) were purchased from the

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Key words: acute lymphoblastic leukaemia, c-Myc inhibitor, glucocorticoids, cell cycle, apoptosis

NALM6

30 μ M 10058-F460 μ M 10058-F4

CEM

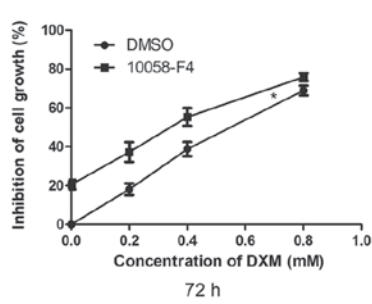
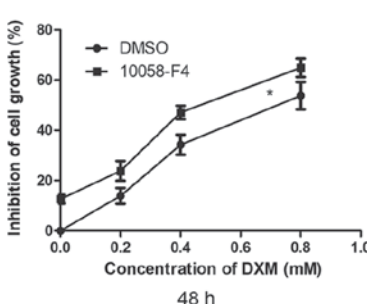
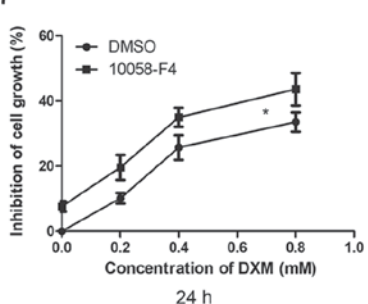
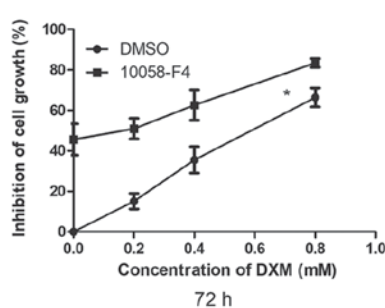
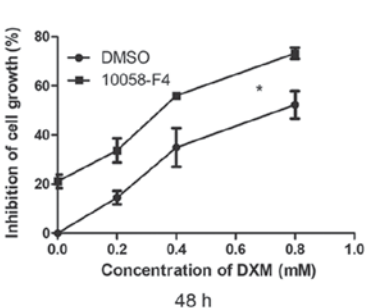
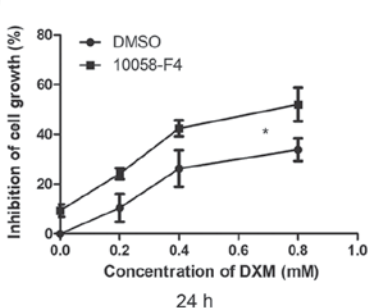
30 μ M 10058-F460 μ M 10058-F4

Figure 1. Growth inhibition rates of NALM6 and CEM cells exposed to DXM combined with 10058-F4 (30 and 60 μ M) for 24, 48 and 72 h. Data are presented as the mean \pm standard deviation. * P <0.05 for all concentrations of DXM vs. 10058-F4 plus DXM. DXM, dexamethasone.

American Type Culture Collection (Manassas, VA, USA). These cells were maintained in RPMI-1640 culture medium (GE Healthcare, Chicago, IL, USA) supplemented with 10% heat-inactivated foetal bovine serum (PAN-Biotech GmbH, Aidenbach, Germany), 100 units/ml penicillin, and 100 units/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). All cell cultures were carried out at 37°C in a humidified atmosphere with 5% CO₂. According to manufacturer instructions, DXM

and 10058-F4 (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) were dissolved in dimethylsulphoxide (DMSO) at 10 and 20 mM, respectively. Reagents were stored at -20°C and further diluted to indicated concentrations in culture medium before use.

Cell viability assay. Cell viability was measured by MTS method (CellTiter 96® Aqueous One Solution, cat. no. 207284; Promega Corporation, Madison, WI, USA). Cells were seeded

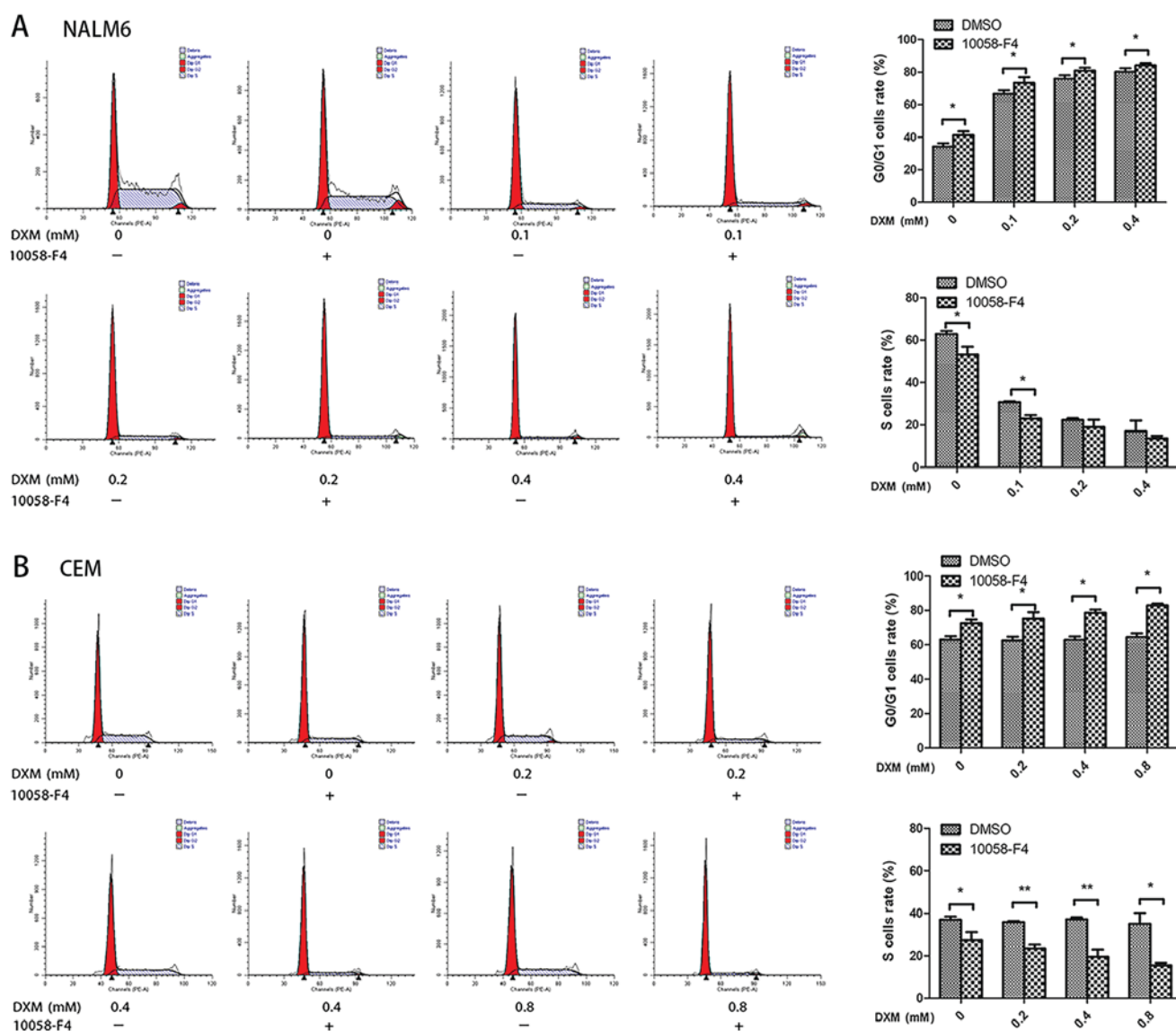


Figure 2. Cell-cycle arrest of NALM6 and CEM cells is induced by DXM combined with 10058-F4. (A) Cell cycle distribution was assessed by FACScan in NALM6 cells treated with DXM (0, 0.1, 0.2 and 0.4 mM) alone or in combination with 10058-F4 (60 μ M) for 24 h. (B) Cell cycle distribution was assessed by FACScan in CEM cells treated with DXM (0, 0.2, 0.4 and 0.8 mM) alone or in combination with 10058-F4 (60 μ M) for 24 h. Data are presented as the mean \pm standard deviation (n=3). *P<0.05 and **P<0.01, as indicated. DXM, dexamethasone.

in 96-well plates (100 μ l per well at 2×10^5 /ml) and were treated with DXM (0-0.8 mM) alone or in combination with 30 and 60 μ M 10058-F4 for 24, 48 and 72 h and the concentration of 10058-F4 was used in our previous study (16), which produced a better sensitization effect in combination with valproic acid. Besides, this concentration of 10058-F4 was also chosen in another study (17). In each well, 20 μ l MTS solution were added, and the cells were incubated at 37°C for 3 h. The absorbance values of each well were measured by spectrophotometry (iMark; Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 490 nm.

Cell cycle analysis. Cell cycle stage was detected by Cell Cycle Staining kit [propidium iodide (PI); cat. no. CCS012; MultiSciences Biotech Co., Ltd., Hangzhou, China]. Cells were treated with DXM (0-0.8 mM) with or without 60 μ M 10058-F4 for 24 h, collected in a tube using pipet tips, washed twice in 4°C PBS solution and resuspended in 1 ml DNA

staining solution. Subsequently, 10 μ l permeabilization solution were added, and these samples were incubated in the dark at room temperature for 30 min. Cell cycle distribution was assessed by FACScan (BD FACSCanto™ II; BD Biosciences, Franklin Lakes, NJ, USA) analysis. The data were analysed by ModFit LT programme (Verity Software House Inc., Topsham, ME, USA).

Apoptosis detection by flow cytometry. Apoptotic cells were detected on a FACScan flow cytometer (BD FACSCanto™ II; BD Biosciences) using Annexin V-fluorescein isothiocyanate (FITC) and PI (BD Pharmingen, San Diego, CA, USA) staining. Cells treated with DXM (0-0.8 mM), alone or in combination with 60 μ M 10058-F4 for 24 h, were collected, washed twice in 4°C PBS and resuspended in 50 μ l Annexin V binding buffer. Next, 5 μ l Annexin V-FITC and 5 μ l PI were added, and these samples were incubated in the dark at room temperature for 15 min. Finally, 450 μ l Annexin V binding

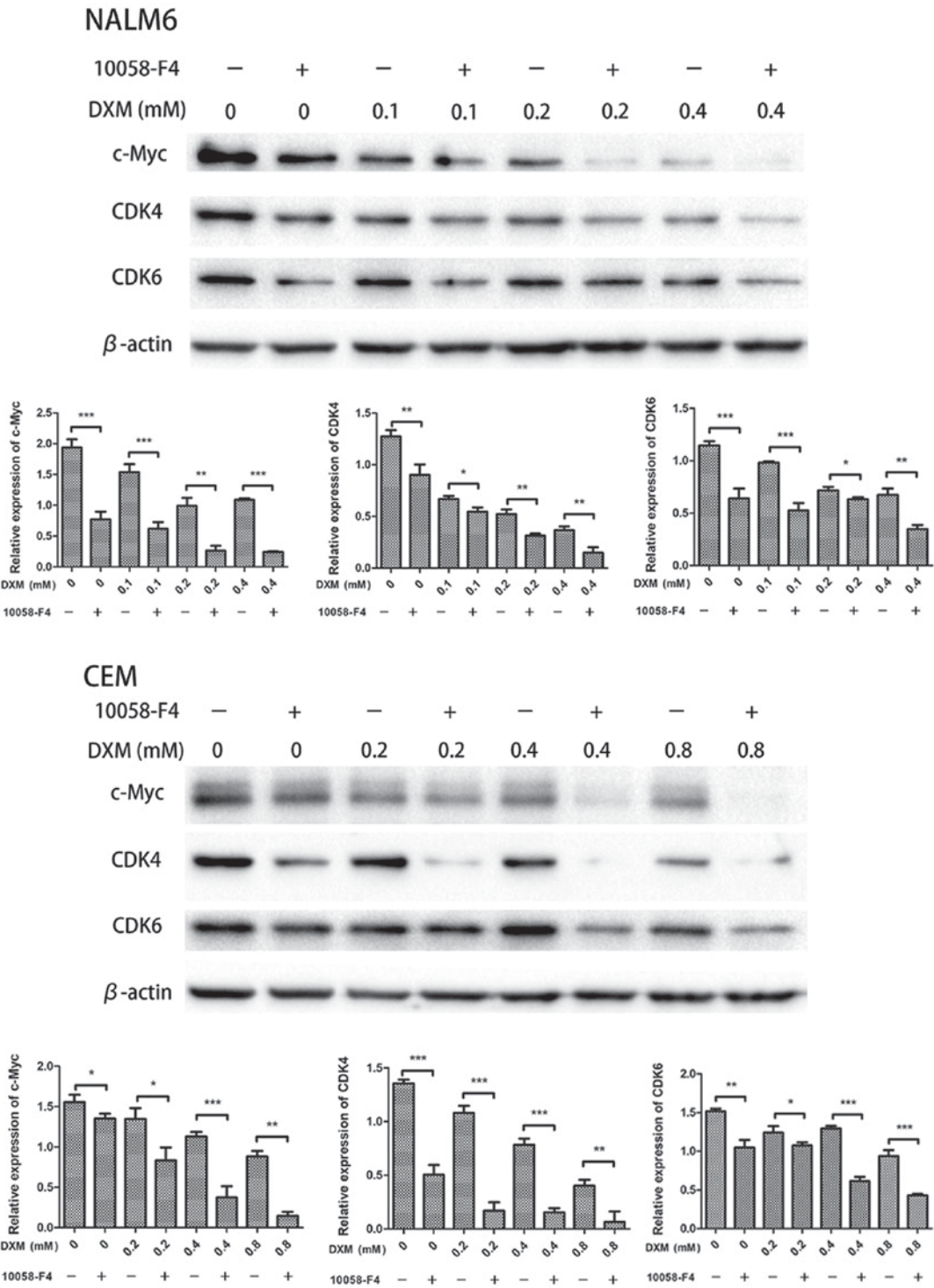


Figure 3. Protein expression of c-Myc, CDK4 and CDK6 decreases in NALM6 and CEM cells following exposure to DXM combined with 60 μ M 10058-F4. Bar graphs were plotted according to the densitometry of the CDK4/CDK6 and β -actin band densities. Data are presented as the mean \pm standard deviation (n=3). *P<0.05, **P<0.01 and ***P<0.001, as indicated. DXM, dexamethasone; CDK, cyclin-dependent kinase.

buffer were added, and cell death was measured by flow cytometry.

Western blot analysis. Cells were treated with DXM (0-0.8 mM), alone or in combination with 60 μ M 10058-F4 for 24 h, and lysed using sodium dodecyl sulphate (SDS) buffer containing proteinase inhibitors (PMSFs). The total protein concentrations of the cells were determined by BCA Protein Assay kit (Beyotime Institute of Biotechnology, Haimen,

China). The samples were separated on 12% SDS polyacryl- amide gel electrophoresis (SDS-PAGE) gels and transferred onto polyvinylidene fluoride (PVDF) membranes (Bio-Rad Laboratories, Inc.). The PVDF membranes were blocked with 5% non-fat milk for 2 h at room temperature and incubated with primary antibodies, i.e., rabbit anti-human polyclonal c-Myc, rabbit anti-human monoclonal cyclin-dependent kinase (CDK)-4, rabbit anti-human monoclonal CDK6, rabbit anti-human monoclonal cleaved PARP, rabbit anti-human

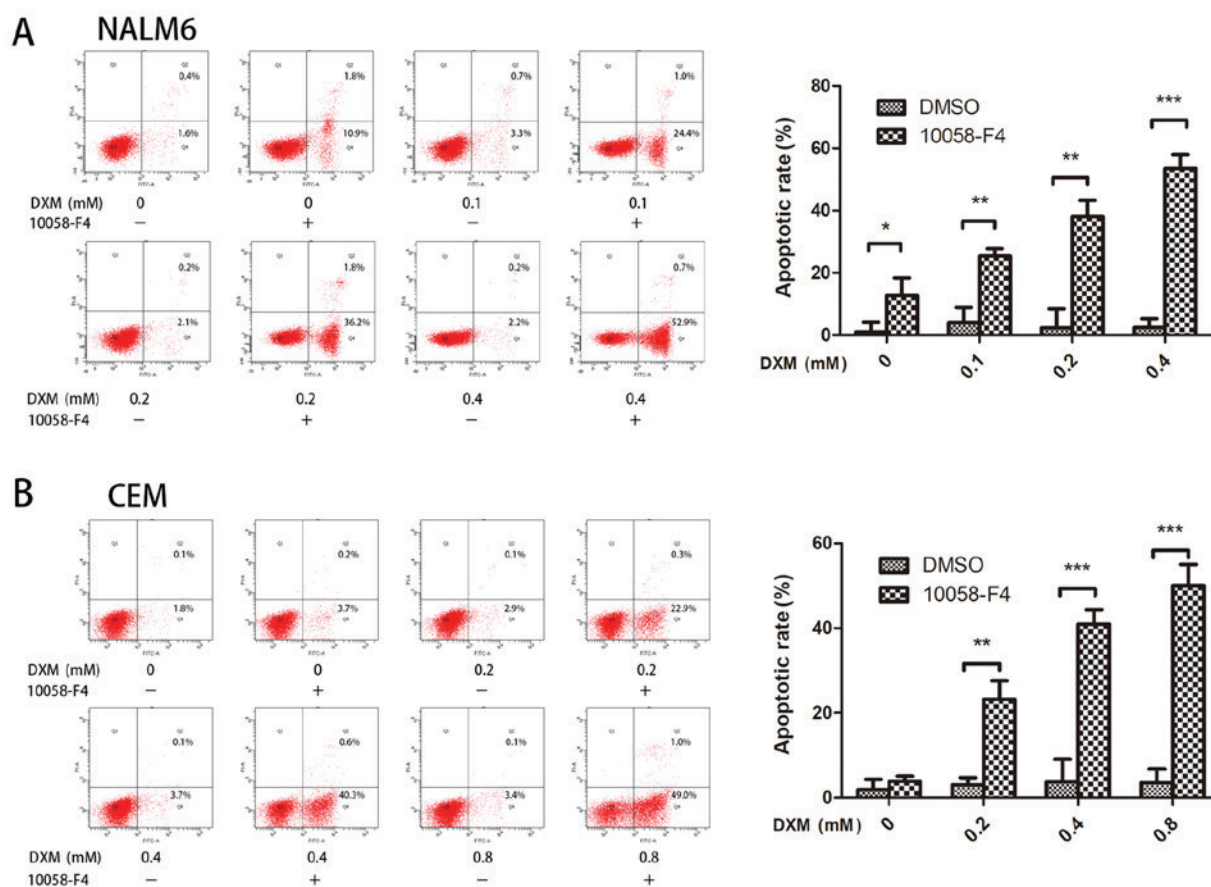


Figure 4. Cell deaths of NALM6 and CEM cells is induced by DXM combined with 10058-F4. (A) Cell death was detected by dual staining with Annexin V/PI in NALM6 cells treated with DXM (0, 0.1, 0.2 and 0.4 mM) alone or combined with 10058-F4 (60 μ M) for 24 h. (B) Cell death was also examined by dual staining with Annexin V/PI in CEM cells treated with DXM (0, 0.2, 0.4 and 0.8 mM) alone or combined with 10058-F4 (60 μ M) for 24 h. Data are presented as the mean \pm standard deviation (n=3). *P<0.05, **P<0.01 and ***P<0.001, as indicated. PI, propidium iodide; DXM, dexamethasone; DMSO, dimethylsulfoxide.

monoclonal cleaved caspase-3 and rabbit anti-human monoclonal β -actin (dilution, 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA), overnight at 4°C. After washing three times for 10 min each time with TBST solution, these PVDF membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (dilution, 1:5,000; Cell Signaling Technology, Inc.) for 2 h at room temperature. The membranes were washed three times again with TBST solution, and protein bands were visualized with an enhanced chemiluminescence detecting kit. Densitometry quantification of immunoblot analyses was performed using Image lab software (v. 5.2.1; Bio-Rad Laboratories, Inc.).

Statistical analysis. Significant differences between the means \pm standard deviation of experimental and control groups were compared by Student's t-test. Two-way analysis of variance and Tukey's post hoc test were performed for multiple comparisons. P<0.05 was considered to indicate a statistically significant difference. All statistical analyses were performed using SPSS Statistics 18.0 software (SPSS, Inc., Chicago, IL, USA).

Results

10058-F4 increases the growth inhibition of NALM6 and CEM cells induced by DXM. Cell growth was analysed by

MTS assay. The growth inhibition rates of the NALM6 cells treated with DXM (0, 0.1, 0.2 and 0.4 mM) combined with 10058-F4 were higher than those treated with corresponding concentrations of DXM (P<0.05 for all). In the CEM cells, the growth inhibition rates of DXM (0, 0.2, 0.4 and 0.8 mM) plus 10058-F4 were also higher than those of the corresponding concentrations of DXM alone (P<0.05 for all; Fig. 1).

10058-F4 increases the cell-cycle arrest of NALM6 and CEM cells induced by DXM. To explore whether the combination of 10058-F4 with DXM induced further cell cycle arrest, we compared the cell cycle distribution of NALM6 and CEM cells treated with DXM alone or in combination with 10058-F4. As shown in Fig. 2A, the rates of G₀/G₁ cells in the groups treated with DXM (0, 0.1, 0.2 and 0.4 mM) in combination with 60 μ M 10058-F4 for 24 h were higher than those in the corresponding groups treated with DXM alone (P<0.05 for all). The same results were observed in the CEM cells (P<0.05 for all; Fig. 2B). Additionally, compared with those in the groups treated with DXM alone, the protein expressions of c-Myc, CDK4 and CDK6 in the groups treated with 10058-F4 and DXM decreased in the two cell lines (P<0.05 for all; Fig. 3). These findings indicated that 10058-F4 dramatically increased the cell-cycle arrest induced by DXM.

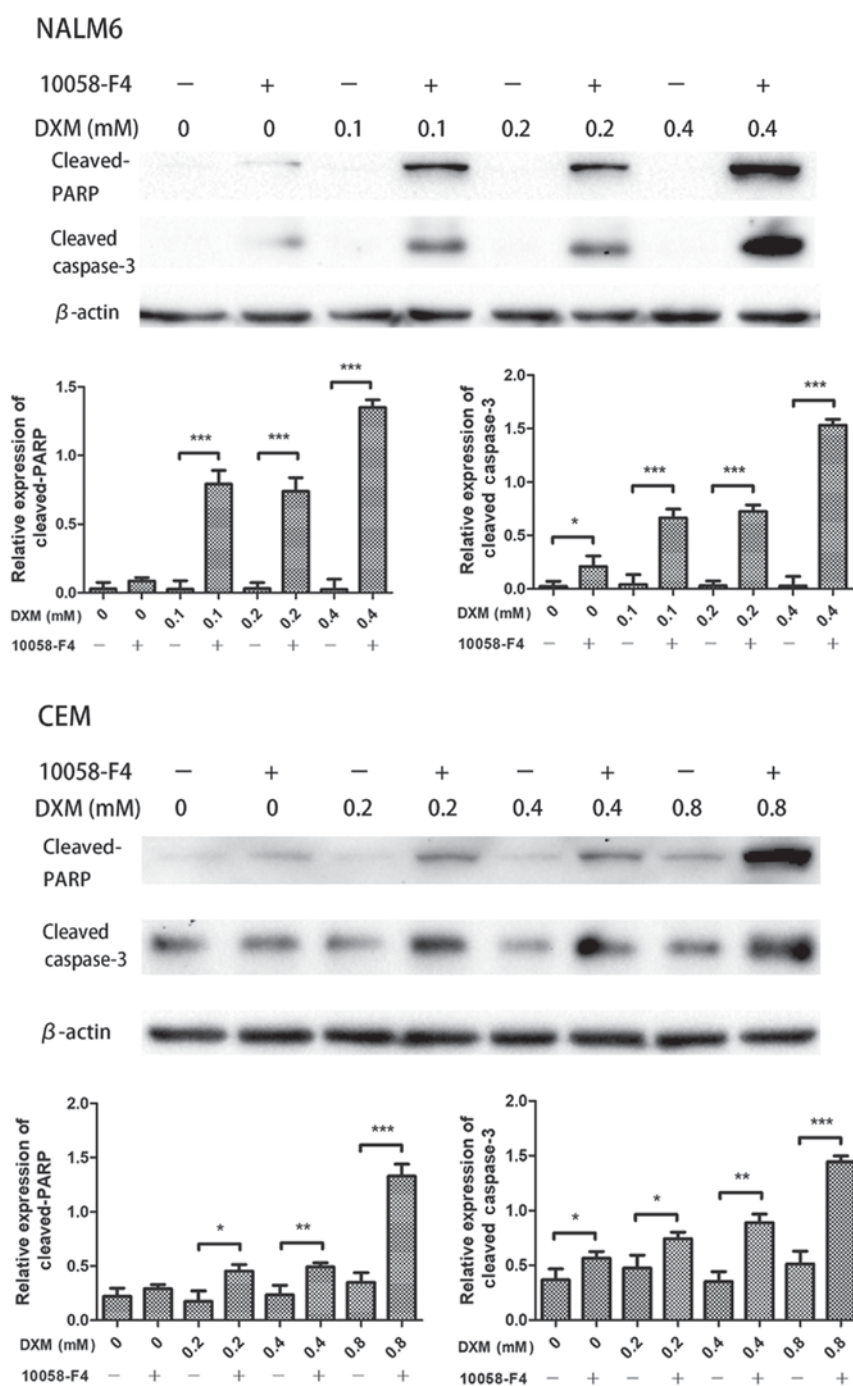


Figure 5. Protein expressions of cleaved PARP and cleaved caspase-3 increases in NALM6 and CEM cells following exposure to DXM combined with 60 μ M 10058-F4. Bar graphs were plotted according to the densitometry of the cleaved caspase-3/cleaved PARP and β -actin band densities. Data are presented as the mean \pm standard deviation (n=3). *P<0.05, **P<0.01 and ***P<0.001, as indicated. DXM, dexamethasone; PARP, poly-(adenosine diphosphate ribose) polymerase.

Combined 10058-F4 and DXM treatment greatly induces apoptosis of NALM6 and CEM cells. Previous studies have demonstrated 10058-F4 as an effective agent in inducing cell death in myeloma and AML cells (17,18). Based on these reports, we determined whether 10058-F4 increased the apoptosis of ALL cells induced by DXM. In the NALM6 and CEM cells, the apoptotic rates of groups treated with DXM and 10058-F4 significantly increased when compared with those of groups treated with DXM alone. As shown in Fig. 4A, compared with treatments with corresponding concentrations of DXM (0, 0.1, 0.2 and 0.4 mM) alone, the cell death rates

(Annexin V+/PI+ and Annexin V+/PI-) of NALM6 cells treated with 10058-F4 combined with DXM significantly increased (P<0.05 for all). Similarly, the cell death rates of the CEM cells treated with DXM (0, 0.2, 0.4 and 0.8 mM) in combination with 60 μ M 10058-F4 for 24 h were significantly higher than those treated with corresponding concentrations of DXM alone (P<0.05 for all; Fig. 4B). The Western blot results also showed that 10058-F4 further promoted the cleavages of caspase-3 and cleaved-PARP in NALM6 and CEM cells induced by DXM (P<0.05 for all; Fig. 5), suggesting that 10058-F4 significantly increased the apoptosis of ALL cells induced by DXM.

Discussion

c-Myc, a helix-loop-helix-leucine zipper (HLH-ZIP) onco-protein, dimerizes with another HLH-ZIP protein, Max, and subsequently binds DNA. This c-Myc-Max interaction regulates target gene expression (19). In T-ALL, 55% of the patients harbour Notch1 mutations that activate Notch signalling and promote c-Myc expression (20). Additionally, 10 to 20% of T-ALL patients have FWB mutations, an E3 ubiquitin ligase responsible for the degradation of c-Myc; this also increases c-Myc expression (21). Hence, c-Myc deregulation is a common phenomenon in T-ALL. Furthermore, an increase in c-Myc expression has been observed in B-ALL cells (8). A c-Myc inhibitor (e.g., 10058-F4) can inhibit c-Myc-Max association, decrease c-Myc expression and prevent transactivation by c-Myc-Max heterodimers (22). Previous studies have implied that c-Myc inhibition eliminated both B-ALL and T-ALL cells (16,23). Our results demonstrated that 10058-F4 alone could suppress the growth of B-ALL cell line NALM6 cells and T-ALL cell line CEM cells.

Because GCs decrease c-Myc expression, we speculated that c-Myc inhibitors enhanced the growth suppression of ALL cells induced by GCs. In our study, 10058-F4 increased significantly inhibited growth, G₀/G₁ arrest and especially the apoptosis of NALM6 and CEM cells induced by DXM. Furthermore, c-Myc downregulation induced by DXM was reinforced by 10058-F4 and was accompanied by decreased CDK4/CDK6 expression. c-Myc has been demonstrated to regulate CDK6 activity; furthermore, CDK4 has been considered a target of c-Myc (24,25). Hence, our results indicated that the c-Myc-CDK4/CDK6 axis could play an important role in G₀/G₁ arrest induced by combined DXM and 10058-F4 treatment and its exact mechanism need to be further explored.

GCs are the basic drugs in the treatment of ALL; novel combinations have been focused on these drugs. LEE011, a CDK4/CDK6 inhibitor, is synergistic with the GC DXM *in vitro*. Their combination has been shown to prolong the survival of T-ALL mice model cells (26). Liu *et al* (27) reported that low-dose anisomycin promoted apoptosis and cell cycle arrest induced by GCs in GC-resistant T-ALL CEM-C1 cells via the activation of GRs and p38-MAPK/JNK. The constitutive activation of NOTCH1 signalling plays a vital role in the pathogenesis of T-ALL. Thus, PF-03084014, a γ -secretase inhibitor, was found to have a synergistic antileukaemic effect on T-ALL cells when combined with GCs *in vitro* and *in vivo* (28). However, to the best of our knowledge, we are the first to show that c-Myc inhibitors increased the sensitivity of B-ALL cell line NALM6 cells and T-ALL cell line CEM cells to the anti-neoplastic effects of GCs.

The role of the therapeutic targeting of c-Myc on antineoplastic activity *in vivo* has been debated due to reported rapid metabolism, inadequate target site penetration and possible liver and kidney toxicity (29,30); however, small molecule c-Myc inhibitors conjugated to integrin-targeted nanoparticles have been shown to overcome these defects (31). Notably, a recent study showed that the dual targeting of p53 and c-Myc selectively eradicated leukaemic stem cells in chronic myeloid leukaemia (CML) in the treatment of refractory haematological malignancies (32). The present study demonstrated that the c-Myc inhibitor 10058-F4 promoted G₀/G₁ arrest and

cell death induced by DXM in the ALL cell lines NALM6 and CEM. These findings indicated that the combination of GCs with c-Myc inhibitors may be a novel potent therapeutic strategy for ALL. However, further clinical investigations on their combination are necessary.

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Availability of data and materials

All data generated or analysed during this study are included in this published article.

Authors' contributions

QM and GO designed the experiments and revised the manuscript. ML performed the experiments and wrote the manuscript. YW, WW, SY, HZ, BH, YC, CS and YZ performed the experiments. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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