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A small-molecule c-Myc inhibitor, 10058-F4, induces cell-cycle arrest, apoptosis, and myeloid differentiation of human acute myeloid leukemia

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Objective. **The protooncogene c-Myc plays an important role in the control of cell proliferation, apoptosis, and differentiation, and its aberrant expression is frequently seen in multiple human cancers, including acute myeloid leukemia (AML). As c-Myc heterodimerizes with Max to transactivate downstream target genes in leukemogenesis. Inhibition of the c-Myc/Max heterodimerization by the recently identified small-molecule compound, 10058-F4, might be a novel antileukemic strategy.**

Materials and Methods. **HL-60, U937, and NB4 cells and primary AML cells were used to examine the effects of 10058-F4 on apoptosis and myeloid differentiation.**

Results. **We showed that10058-F4 arrested AML cells at G_0/G_1 phase, downregulated c-Myc expression and upregulated CDK inhibitors, p21 and p27. Meanwhile, 10058-F4 induced apoptosis through activation of mitochondrial pathway shown by downregulation of Bcl-2, upregulation of Bax, release of cytoplasmic cytochrome C, and cleavage of caspase 3, 7, and 9. Furthermore, 10058-F4 also induced myeloid differentiation, possibly through activation of multiple transcription factors. Similarly, 10058-F4–induced apoptosis and differentiation could also be observed in primary AML cells.**

Conclusion. **Our study has shown that inhibition of c-Myc/Max dimerization with small-molecule inhibitors affects multiple cellular activities in AML cells and represents a potential antileukemic approach.** © **2006 International Society for Experimental Hematology. Published by Elsevier Inc.**

The basic helix-loop-helix leucine zipper transcription factor, c-Myc, plays a key role in cell proliferation, apoptosis, and terminal differentiation of hematopoietic cells via regulating transcription of downstream target genes [1,2]. Expression of c-Myc is rapidly upregulated upon mitogen stimulation and downregulated during hematopoietic differentiation.

The importance of c-Myc on leukemogenesis was first recognized by the finding of c-Myc mutations in aggressive Burkitt's lymphoma, and was subsequently demonstrated in the *Eμ-Myc* transgenic mouse model [3]. Ninety percent of Eμ-Myc mice developed B-cell lymphoma during the first 5 months of life [4,5]. A recent study also found that several important oncogenes in acute myeloid leukemia (AML) such as *AML1-ETO*, *PML/RARα*, and *PLZF/RARα* induce leukemogenesis by activating c-Myc [6]. In addition, c-Myc is upregulated by the activating mutations of *Flt3* receptor tyrosine kinase, the most prevalent type of mutations in AML [7]. The data suggest that c-Myc might be a common downstream molecule of these oncogenes. The importance of c-Myc in myeloid leukemogenesis is further demonstrated by the induction of myeloid leukemia using ectopically expressed c-Myc in murine bone marrow (BM) progenitors [8].

Therefore, c-Myc is an appealing target for developing novel anti-AML therapy. The potential of inducing apoptosis and terminal differentiation of leukemic cells by c-Myc inactivation was first exemplified in an animal model, in which c-Myc is conditionally expressed in hematopoietic

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progenitors [9]. Because of the requirement of c-Myc proteins to maintain cancer cells in a transformed state, even a brief inactivation would be sufficient to induce sustained tumor regression [10]. Given the success of using imatinib, a small molecule inhibitor on BCR/ABL, on chronic myeloid leukemia [11], identification of small molecule compounds that could specifically inactivate oncogenic proteins has been an attractive approach for novel therapeutics. Recently, small molecules that could inhibit c-Myc activity have been shown to inhibit c-Myc–driven transformation [12]. Because c-Myc needs to dimerize with Max, a helix-loop-helix leucine zipper protein, to bind the specific DNA sequences (called E-box with a central CACGTG motif) in the promoters of its target genes, inactivation of c-Myc could be achieved by blocking the DNA binding activity of c-Myc through inhibition of c-Myc/Max dimerization [2]. Small nonpeptide molecules, which interfere with c-Myc/Max heterodimerization, have been shown to inhibit c-Myc–induced transformation of chicken embryonic fibroblasts [13]. Likewise, other low-molecular-weight molecules, acting through similar mechanisms, have recently been identified by a yeast two-hybrid strategy, and been demonstrated to inhibit tumor growth both in vitro and in vivo [12,14]. Thus, we used the small-molecule c-Myc inhibitor, 10058-F4, to evaluate the potential of such agents on AML cells. 10058-F4 prevented the binding of c-Myc/Max dimers to its DNA targets, inhibited leukemic proliferation, and induced apoptosis through mitochondrial pathway. In the mean time, 10058-F4 also induced myeloid differentiation of AML cells, possibly through modulation of specific transcription factors. Furthermore, the observed activity of 10058-F4 in cell lines could also be extended to primary AML cells, indicating that small-molecule c-Myc inhibitors might represent a novel therapy for AML.

Materials and methods

Preparation of reagents and cell culture

Small-molecular-weight c-Myc inhibitor (10058-F4; [Z,E]-5-[4-ethylbenzylidine]-2-thioxothiazolidin-4-one, molecular weight 249.4; Calbiochem, San Diego, CA, USA) was dissolved in dimethylsulfoxide (DMSO) to 40 mM according the manufacturer's instructions and further diluted to indicated concentrations in culture medium before use. Caspase-3 inhibitor, z-DEVD-FMK in solution, was obtained from Calbiochem. HL-60 cells were maintained in RPMI-1640 culture medium supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells in exponential growth phase were used for experiments. Normal BM mononuclear cells (MNCs) from healthy volunteers or AML patients (with >90% of blasts identified by flow cytometric analysis) were isolated by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density-gradient centrifugation after obtaining informed consent. Cells were either cryopreserved in Iscove's modified Dulbecco medium with 10% DMSO and 20% FCS or immediately used for experiments. Cryopreserved MNCs were used for experiments immediately after thawing. The characteristics of primary AML cells are listed in supplemental Table 1. Use of human cells has been approved by the Institutional Review Board at Taipei Medical University.

MTT assays

Cells, plated in 96-well plates (10^5/mL for cell lines and 5×10^5/mL for primary leukemic cells), were treated in triplicate with indicated concentrations of 10058-F4. At various time points, 20 μL 5 mg/mL MTT (Sigma) was added to each well. After incubation at 37°C for 3 hours, the MTT medium was removed and 100 μL DMSO lysis buffer was added. The number of viable cells was assessed by the percentage of absorbance of treated cells relative to that of solvent controls, using 570-nm wavelength on a spectrophotometer.

Electrophoretic mobility shift assay

Nuclear protein extraction and electrophoretic mobility shift assay (EMSA) were carried out as described previously [15]. For antibody supershifts, anti-Myc antibody (Cell Signaling Technology, Boston, MA, USA) at a 1:100 dilution was preincubated with nuclear extracts for 30 minutes before adding oligonucleotide to the binding reactions. Electrophoresis gels were transferred to positively charged nylon membrane and detected by the streptavidin-horseradish peroxidase kit.

Measurements of cell cycle and myeloid differentiation

For measurement of cell cycle, cells were prepared by the hypotonic method as described previously [16]. Data were acquired with a FACSCalibur using CellQuest software and analyzed using ModFit LT program (Verity Software House Inc., Topsham, ME, USA). For phenotyping, 1×10^5 cells were incubated with 5 μL indicated phycoerythrin (PE)-conjugated CD11b, CD11c, or CD14 antibodies (Becton Dickinson, Mountain View, CA, USA) on ice for 30 minutes and washed twice with ice-cold phosphate-buffered saline before phenotypic analysis. Granulocytic or monocytic differentiation was also assessed by nitroblue tetrazolium (NBT)

Supplemental Table 1. Information on primary acute myeloid leukemia specimen

Sample no.	FAB classification	Phenotype	Cytogenetics
3	AML-M1	CD13(+) CD14(−) CD19(+) CD33(+) CD34(+) HLA-DR(+) CD61−) CD117(−)	46XY
4	AML-M5	CD13(+) CD14(+) CD19(−) CD33(+) CD34(+) CD61(−) CD117(−)	NA
5	AML-M0	CD11b(−) CD13(−) CD14(−) CD19(−) CD33(+) CD34(+) CD117(+) HLA-DR(+)	NA
6	AML-M2	CD13(+) CD14(−) CD19(−) CD33(+) CD34(−) CD61(−) CD117(−)	46, XY, t(8:21)(q22,q22)
7	AML-M2	CD13(+) CD14(−) CD33(+) HLA-DR(+) CD34(++) CD61(−) CD117(−)	45XY –15
8	AML-M5	CD13(+) CD14(+) CD19(−) CD33(+) CD34(−) CD64(+) CD117(−)	NA

NA = not available.

reduction tests (Sigma, St. Louis, MO, U SA) and α-naphthyl acetate esterase (NAE) stains (Sigma), respectively. Cells were treated for the measurement of NBT reduction activity as described [17]. Cytospin slides were stained by means of the NAE kit following the manufacturer's protocol.

Western blot analysis

Antibodies against c-Myc, p21, p27, cyclin D1, cyclin D2, cyclin D3, Bcl-2, Bcl-xL, Bax, Bak, cytochrome-C, caspase 3, 7, 8, and 9 (all from Cell Signaling Technology) and β-actin (US Biologicals, Swampscott, MA, USA) were used in this study. The concentration for each antibody was titrated according to manufacturer's instructions. Whole-cell extracts were prepared by lysing the cells with NETN buffer (50 mM Tris-HCl [pH 7.9], 150 mM NaCl, 0.5 mM ethylenediamine tetraacetic acid, and 0.5% Nonidet P-40), containing 1× Pro-Pure protease inhibitor cocktail (Amresco, Solon, OH, USA). Cytosolic proteins were prepared using a nuclear/cytosol fractionation kit (Pierce, Rockford, IL, USA). Total proteins were separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and electroblotted on polyvinylidene difluoride membrane. Western blots were performed using previously described antibodies at a 1:1000 dilution, followed by incubation with horseradish peroxidase-conjugated secondary antibody (Sigma) at a 1:5000 dilution. Blots were developed using enhanced chemiluminescence kit (NEN).

Quantitative real-time PCR

Cells treated with solvent or 60 μM 10058-F4 for 3 days were harvested at indicated time points and total RNA was extracted with a RNA extraction kit (Protech, Palm Beach Gardens, FL, USA). One microgram total RNA was first transcribed into cDNA in 25 μL reagent mix, and 1/25 of the cDNA was subjected to semi-quantitative polymerase chain reaction (PCR) for c-Myc transcripts or to quantitative real-time PCR for indicated genes. After semi-quantitative PCR, PCR products were resolved by electrophoresis using 2% agarose gel. Real-time PCR was performed in triplicate for each sample using SYBR Green PCR kit (ABI, Applied Biosystems, Foster City, CA, USA) on an ABI 9700HT machine following the manufacturer's protocols. Primers and the annealing temperature for each primer set are listed in supplemental Table 2. Control reactions lacking cDNA template were included to assess specificity, and showed no appreciable amplification (data not shown). The fluorescence intensity is related to the initial numbers of RNA copies, which can be assessed by determining the threshold cycle (C_T). Relative expression of individual genes at indicated time points was calculated based on comparative C_T method [18]. The serial changes of individual genes were plotted against the data derived from solvent-treated cells.

Clonogenic assays

Primary AML MNCs adjusted to 5×10^5/mL first cultured with 10% 5637 bladder cancer cell-conditioned medium and indicated concentrations of 10058-F4 for 2 days were plated in 35-mm dishes with methylcellulose culture medium containing 20% 5637-conditioned medium, 20% FCS, 5 ng/mL granulocyte-macrophage colony stimulating factor, and 3 U/mL erythropoietin (Amgen, Thousand Oaks, CA, USA) for 7 days. Leukemic colonies (>30 cells) were scored visually under an inverted microscope.

Statistical analysis

The significance of differences between experimental and control conditions was determined by Student's *t*-test. A p value < 0.05 was regarded as statistically significant.

Results

c-Myc inhibitor, 10058-F4, inhibits growth of leukemic cells and dimerization of Myc and Max

We first examined the levels of c-Myc protein in AML cell lines and in normal BM MNC. All AML cells (HL-60, U937, and NB4), but not normal BM MNC expressed high levels of c-Myc (Fig. 1A). Because 10058-F4 has been shown to inhibit cell growth of solid tumors, we next determined its activity on AML cell by MTT assays [14]. All three cell lines were sensitive to 10058-F4 in a dose-dependent manner (Fig. 1B). Effects on AML cells

Supplemental Table 2. Polymerase chain reaction primers, and annealing temperatures used in this study

Genes	Primer sequences	T_{anneal}
c-Myc	Forward: 5′-GCCACGTCTCCACACATCAG-3′ Reverse: 5′-TCTTGGCAGCAGGATAGTCCTT-3′	55
C/EBP_β	Forward: 5′-GAACAGCAACGAGTACCGGGTG-3′ Reverse: 5′-CCCATGGCCTTGACCAAGGAG-3′	55
C/EBP_β	Forward: 5′-ATCTATATTTTGCCAACCAA-3′ Reverse: 5′-AGATTCCCAAAATATACAGA-3′	50
Egr-1	Forward: 5′-CAACTACCCTAAGCTGGAGGAG-3′ Reverse: 5′-GTGGGTTGGTCATGCTCACTAG-3′	55
GATA-1	Forward: 5′-CAAGAAGCGCCTGATTGTCAG-3′ Reverse: 5′-AGTGTCGTGGTGGTCGTCTG-3′	55
c-JUN	Forward: 5′-GGAAACGACCTTCTATGACGATGCCCTCAA-3′ Reverse: 5′-GAACCCCTCCTGCTCATCTGTCACGTTCTT-3′	58
JUN-B	Forward: 5′-CCAGTCCTTCCACCTCGACGTTTACAAG-3′ Reverse: 5′-GACTAAGTGCGTGTTTCTTTTCCACAGTAC-3′	55
β-Actin	Forward: 5′-GGACTTCGAGCAAGAGATGG-3′ Reverse: 5′-AGCACTGTGTTGGCGTACAG-3′	50

C/EBP_β = CCAAT/enhancer binding protein-β.

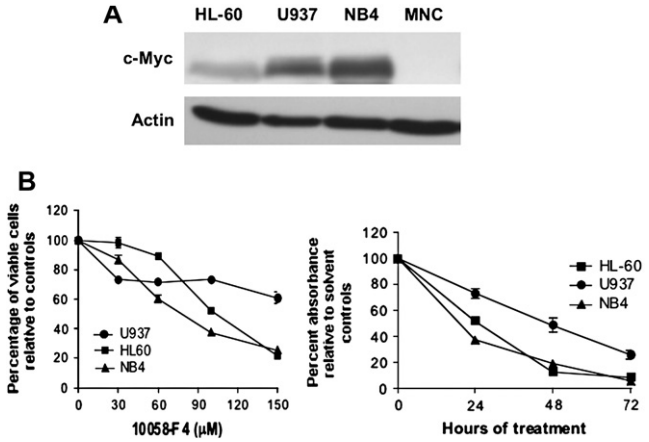

Figure 1. Expression of c-Myc in acute myeloid leukemia (AML) cell lines and in normal bone marrow (BM) mononuclear cells (MNCs) and the growth inhibition by 10058-F4. (**A**) Levels of endogenous c-Myc proteins in HL-60, U937, and NB4 cells and in normal BM MNC were determined by Western blotting. (**B**) Growth inhibition of HL-60 (solid square), U937 (solid circle), and NB-4 (solid triangle) cells by 10058-F4 were assessed by MTT assay after exposure to indicated concentrations of 10058-F4 for 24 hours (left panel) and to 100 μM of 10059-F4 for up to 72 hours (right panel). Data represent the mean ± standard error of mean of experiments in quadruplicate.

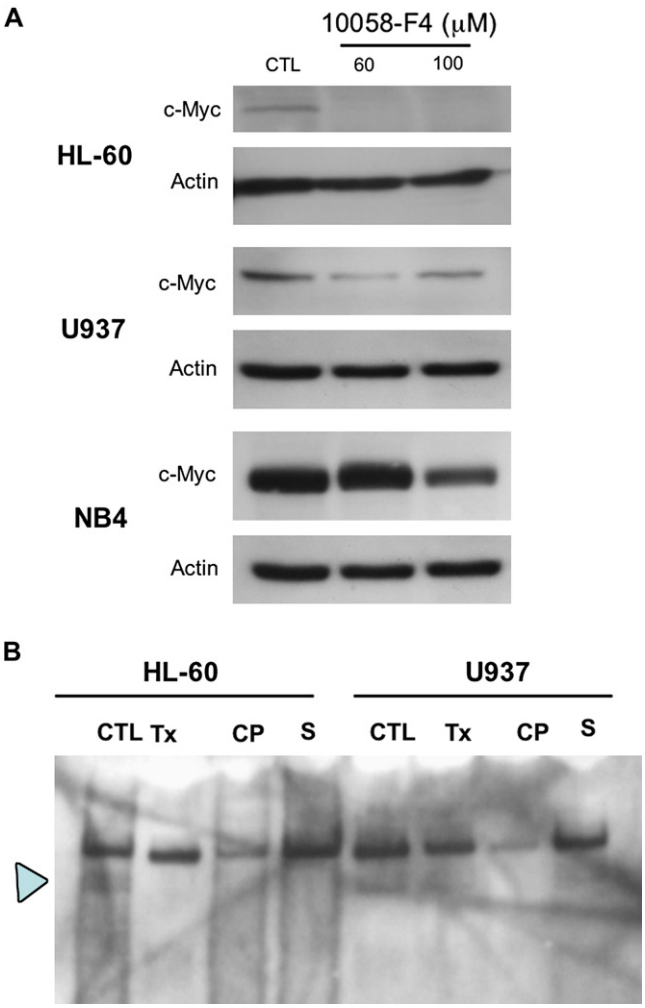

Figure 2. Effects of 10058-F4 on the levels of c-Myc protein and its DNA binding ability. (**A**) c-Myc expression of HL-60, U937, and NB4 cells before and after treatment with 60 and 100 μM 10058-F4 for 24 hours was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotting. Levels of β-actin were used as loading control. (**B**) HL-60 and U937 cells were treated with 100 μM 10058-F4 for 24 hours before harvesting for nuclear extracts. Nuclear extracts were incubated with biotinylated oligonucleotide before electrophoretic mobility shift assay (EMSA) experiments as described in Materials and Methods. For competing assays, the binding of biotinylated probes was competed with 10-fold excess of cold probes. For supershift assays, nuclear extracts were preincubated with anti-Myc before EMSA experiments. CTL = solvent control, Tx = treated cells, CP = cold probes, S = supershift. Arrowhead indicates the Myc/Max complexes.

became more evident when treatment with 100 μM 10058-F4 extended to 72 hours (Fig. 1C). But its effect did not correlate with the levels of c-Myc shown in Figure 1A.

Treatment with 10058-F4 decreased levels of c-Myc proteins in all three cell lines (Fig. 2A). Levels of c-Myc in cells treated with 100 μM 10058-F4 were lower than those treated with 60 μM. As 10058-F4 has been previously reported to inhibit the dimerization of c-Myc and Max in solid tumor cell lines, we next investigated whether 10058-F4 blocks the DNA binding c-Myc/Max dimers to their DNA target sites by using EMSA and gel supershift assays. Nuclear extracts from HL-60 and U937 cells treated with DMSO control or 100 μM 10058-F4 for 24 hours were incubated with biotinylated oligonucleotides containing a consensus c-Myc–binding E-box site before being subjected to EMSA assays. Treatment with 10058-F4 prevented the binding of the Myc/Max heterodimers to their DNA targets (Fig. 2B) [19]. The binding of c-Myc/Max complex was blocked by the excess of competitive cold probes, and specific antibodies in the supershift studies.

10058-F4 induces cell-cycle arrest and apoptosis of AML cells

We next examined whether the reduced cell numbers by 10058-F4 observed in MTT assays were caused by growth inhibition or by apoptosis. Treatment with 100 μM 10058-F4 for 24 hours significantly arrested cell cycle at G_0/G_1 phase (Fig. 3A; 87.02% ± 1.69% vs 67.67% ± 1.28% in the controls, $p < 0.05$, data on U937 and NB4 not shown) in a dose-dependent manner as higher concentrations of 10058-F4 led to significantly higher percentage of cells in G_0/G_1 phase (Fig. 3B). To elucidate the mechanisms underlying the cell-cycle arrest, we examined the changes of p53, cyclin-dependent kinase (CDK) inhibitors, p21 and p27, because previous studies have shown that c-Myc is recruited to the p21 promoter and blocks the p53-induced p21 activation [20–22]. Treatment with 10058-F4 upregulated levels of p21 and p27 as early as 12 hours after treatment (Fig. 3C). But p53 was not detected in all cell lines tested (data not shown), suggesting that upregulation of p21 is p53-independent.

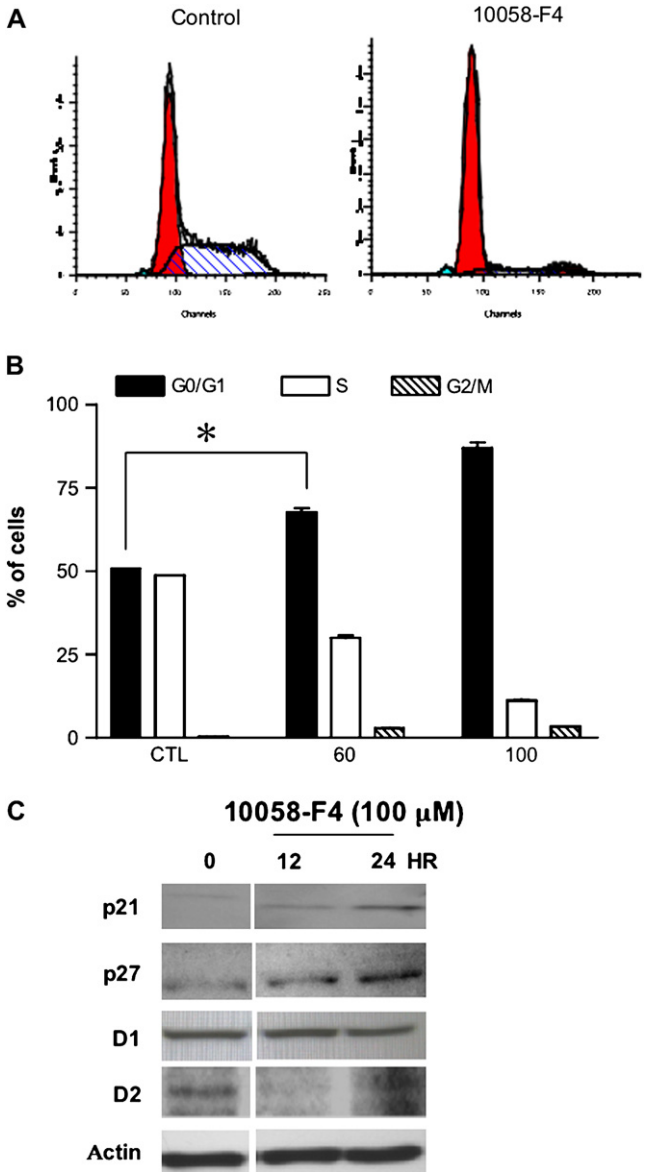

Figure 3. 10058-F4 induces cell-cycle arrest and cell-cycle related proteins. (**A**) HL-60 cells treated with 100 μM 10058-F4 for 24 hours were harvested for cell-cycle analysis by flow cytometry. (**B**) Percentage of HL-60 cells in different phases of cell cycle after treatment with 60 and 100 μM 10058-F4 for 24 hours. Error bars represent SEM. *$p < 0.05$. CTL = solvent control. (**C**) Expression of p21, p27, cyclin-D1, and cyclin D2 in HL-60 cells after exposure to 100 μM 10058-F4 for 0, 12, and 24 hours. β-actin was used as loading control. Results are representative of two independent experiments.

c-Myc has been shown to upregulate cyclin D2 through direct binding to the E-box sequences in the promoter [23].We next investigated whether 10058-F4 inhibits cell proliferation through downregulation of cyclins. We found that 10058-F4 did not affect levels of cyclin D1 and cyclin D3 (data not shown), but markedly decreased levels of cyclin D2 (Fig. 3C), suggesting that 10058-F4 is likely to arrest cell cycle through inhibition of specific binding of c-Myc to cyclin D2 promoter.

We also examined whether the decreased cell numbers observed in MTT assays were caused by increased apoptosis. Treatment with either 60 or 100 μM 10058-F4 for 24 hours induced marked apoptosis shown by the increased numbers of apoptotic cells (Fig. 4A, 6.0% in the controls, 11.7% at 60 μM, and 21.3% at 100 μM). Concomitant with induction of apoptosis, 10058-F4 downregulated expression of antiapoptotic Bcl-2 and upregulated expression of proapopototic Bax in the treated cells (Fig. 4B). However, the levels of other members of proteins in Bcl-2 family including antiapoptotic Bcl-xL and proapoptotic Bak were not affected (data not shown).

As apoptosis can go through either intrinsic (mitochondrial) or extrinsic (fas receptor-mediated) pathways, we next investigated through which pathway 10058-F4 induces apoptosis. The activation of intrinsic pathway by 10058-F4 was demonstrated by release of cytoplasmic cytochrome C from mitochondria, and cleavage of caspase-3, -7, and -9 (Fig. 4C). The specificity of activation of caspase-3 was further validated by the decrease of apoptotic cells after addition of caspase-3 inhibitor, z-DEVD-FMK (Fig. 4D). The partial reduction in apoptosis in z-DEVD-FMK–treated cells suggests that 10058-F4 induces apoptosis through both caspase-3–dependent and –independent pathways. In contrast to the activation of intrinsic pathway, treatment with 10058-F4 for 24 or 48 hours did not affect expression of fas receptor (CD95) or the level of cleaved caspase-8 (data not shown), indicating that intrinsic pathway is the major route for 10058-F4–induced apoptosis.

10058-F4 induces myeloid differentiation of AML cells

Because c-Myc proteins regulate not only cell proliferation but also terminal differentiation of hematopoietic cells, we next examined whether 10058-F4 induces myeloid differentiation in AML cells. Because treatment with 100 μM of 10058-F4 for 3 days led to massive apoptosis of cells and resulted in insufficient cell numbers for additional analysis, cells were treated with 60 μM 10058-F4 for 3 days before phenotypic analysis. While treatment with 10058-F4 increased expression of CD11c and CD14, and slightly decreased CD11b expression in HL-60 cells (Fig. 5A, upper panel), it increased CD14 in U937 cells and CD11b and CD14 in NB4 cells (Fig. 5B middle and lower panel). These results indicate that 10058-F4 could induce myeloid differentiation of AML cells. As CD14 is a marker for monocytic differentiation, we further validated myeloid differentiation by morphology and NAE stains. HL-60 cells treated with 60 μM 10068-F4 for 3 days displayed morphologic features of differentiation and were positive for NAE stain (Fig. 5B). The granulocytic differentiation in HL-60 cells by 10058-F4 was not evident as measured by NBT test (data not shown), suggesting that 10058-F4 might preferentially induce monocytic differentiation.

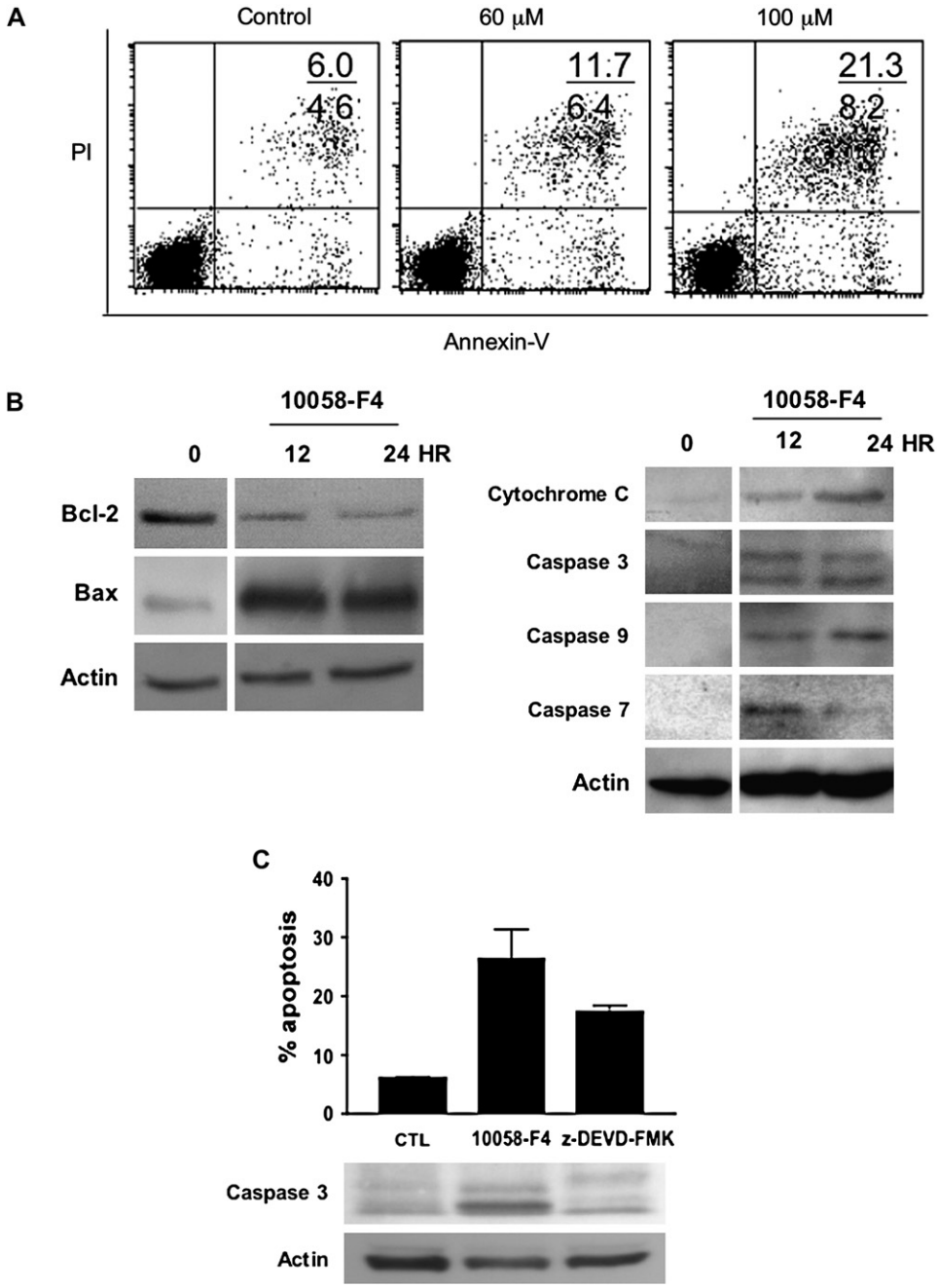

Figure 4. Induction of apoptosis by 10058-F4 and expression of apoptosis-related proteins. (**A**) HL-60 cells were either untreated or treated with 60 or 100 μM 10058-F4 for 24 hours before staining with Annexin-V and propidium iodide (PI) and analyzed by flow cytometry. Numbers in the plots represent percentage of apoptotic (Annexin-V^+/PI^+) and preapoptotic (Annexin+/PI^-) cells. (**B**) Expression of Bcl-2 and Bax and cytoplasmic cytochrome C, cleaved caspase 3, cleaved caspase 9, and cleaved caspase 7 after exposing 100 μM 10058-F4 for 0, 12, and 24 hours. (**C**) Changes in the percentage of apoptotic cells and in the levels of cleaved caspase 3 after exposing to solvent control (CTL) or 100 μM 10058-F4 for 24 hours with our without the inclusion of the inclusion of 20 μM caspase 3 inhibitor, z-DEVD-FMK in the culture.

As lineage specification of hematopoietic cells is determined by activation of unique sets of transcription factors, we next used quantitative real-time reverse transcription PCR to examine whether 10058-F4 activates specific transcription factors during myeloid differentiation of HL-60 cells. Treatment with 10058-F4 for 24 hours increased the transcript levels of c-JUN (5.7 ± 1.3-fold) and C/EBP$_\beta$ (8.9 ± 0.3-fold); and after 48 hours of treatment, the expression of c-JUN, JUN-B and Egr-1 further increased to 21.1 ± 4.3-fold, 19.7 ± 3.2-fold, and 39.3 ± 6.3-fold (Fig. 5C). In contrast, the changes on C/EBP$_\beta$ (2.6 ± 0.5-fold) and GATA-1 (1.8 ± 0.3-fold), genes responsible for

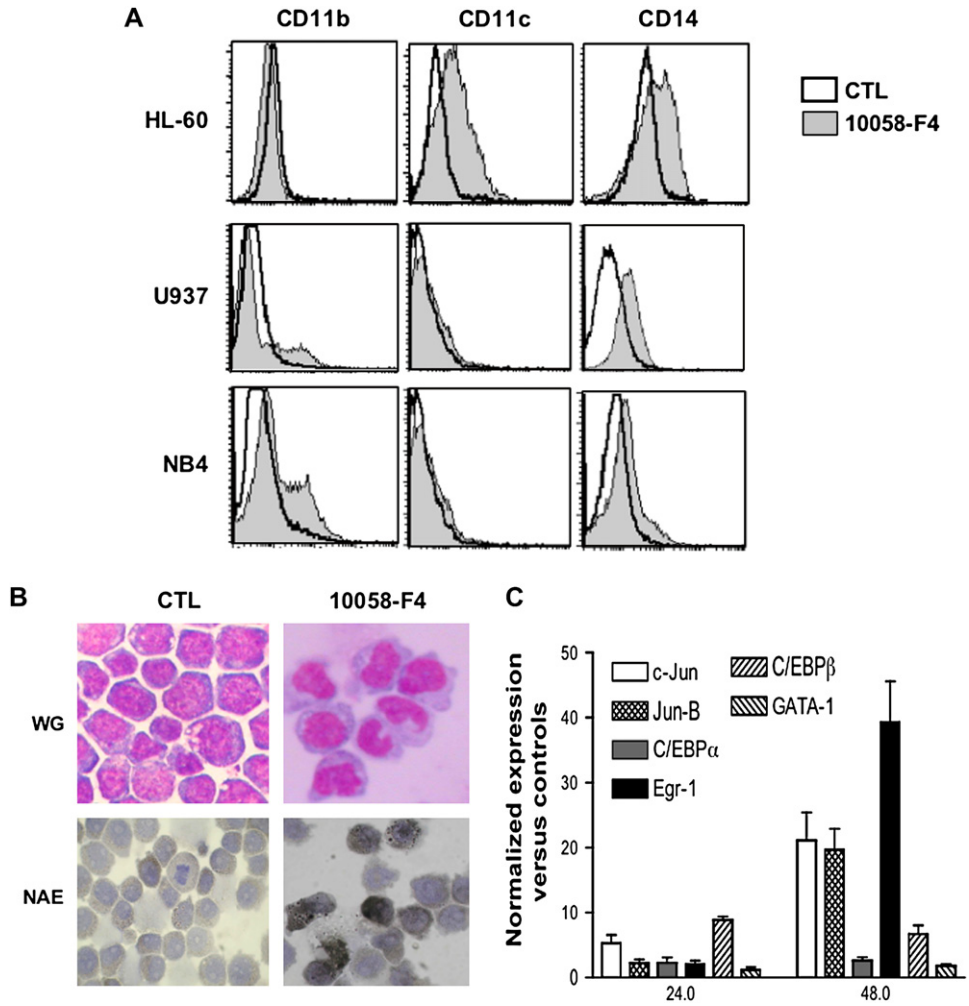

Figure 5. Myeloid differentiation induced by 10058-F4. (**A**) HL-60, U937, and NB4 cells were treated with solvent (CTL, open area), or with 60 μM 10058-F4 (gray area) for 3 days and examined for the expression of myeloid markers, CD11b, CD11c, and CD14 by flow cytometry. (**B**) Morphologic features of HL-60 before and after treatment with 60 μM 10058-F4 for 3 days were examined by Wright-Giemsa (WG) stain and α-naphthyl acetate esterase (NAE) strain. (**C**) Transcript levels of c-JUN, JUN-B, C/EBP$_{\beta}$, Egr-1, C/EBP$_{\beta}$, and GATA-1 in HL-60 cells were measured 24 and 48 hours after treatment with 60 μM 10058-F4. Serial changes in gene expression following treatment with 10058-F4 are plotted against results derived from solvent-treated cells. Symbols represent different gene transcripts are shown in the graph. The results represent one of two independent experiments.

directing neutrophilic and erythroid differentiation, were less remarkable. As c-JUN, C/EBP$_{\beta}$, and Egr-1 have been previously implicated in monocytic differentiation, these results are consistent with the morphologic and phenotypic studies and suggest that 10058-F4 induces myeloid differentiation through activation of specific transcription factors [24–27].

10058-F4 induces apoptosis and myeloid differentiation of primary leukemic cells

To further demonstrate the activity of 10058-F4 on AML cells, we next tested its effects on primary AML cells from six patients and on two normal BM MNC. Primary cells were first examined by MTT assays after exposing to 10058-F4 for 2 days. Compared to normal BM MNC, AML cells were, in general, more sensitive to 10058-F4 (Fig. 6A). In agreement with the results from cell lines, the c-Myc levels in primary AML cells decreased after exposing to 100 μM 10058-F4 for 24 hours (Fig. 6B, patient nos. 5 and 8); but different from the results in cell lines, p21 was not detected in primary AML cells (data not shown). As shown in Figure 6C, 10058-F4 caused apoptosis of primary AML cells from patient no. 5 in a dose-dependent manner. At the same time, the percentage of $CD34^{+}$ and $CD117^{+}$ leukemic blasts in this patient also decreased when exposed to 60 μM 10058-F4 for 2 days (Fig. 6D). Treatment with 60 μM 10058-F4 for 2 days decreased the numbers of leukemic colonies, and with 100 and 120 μM

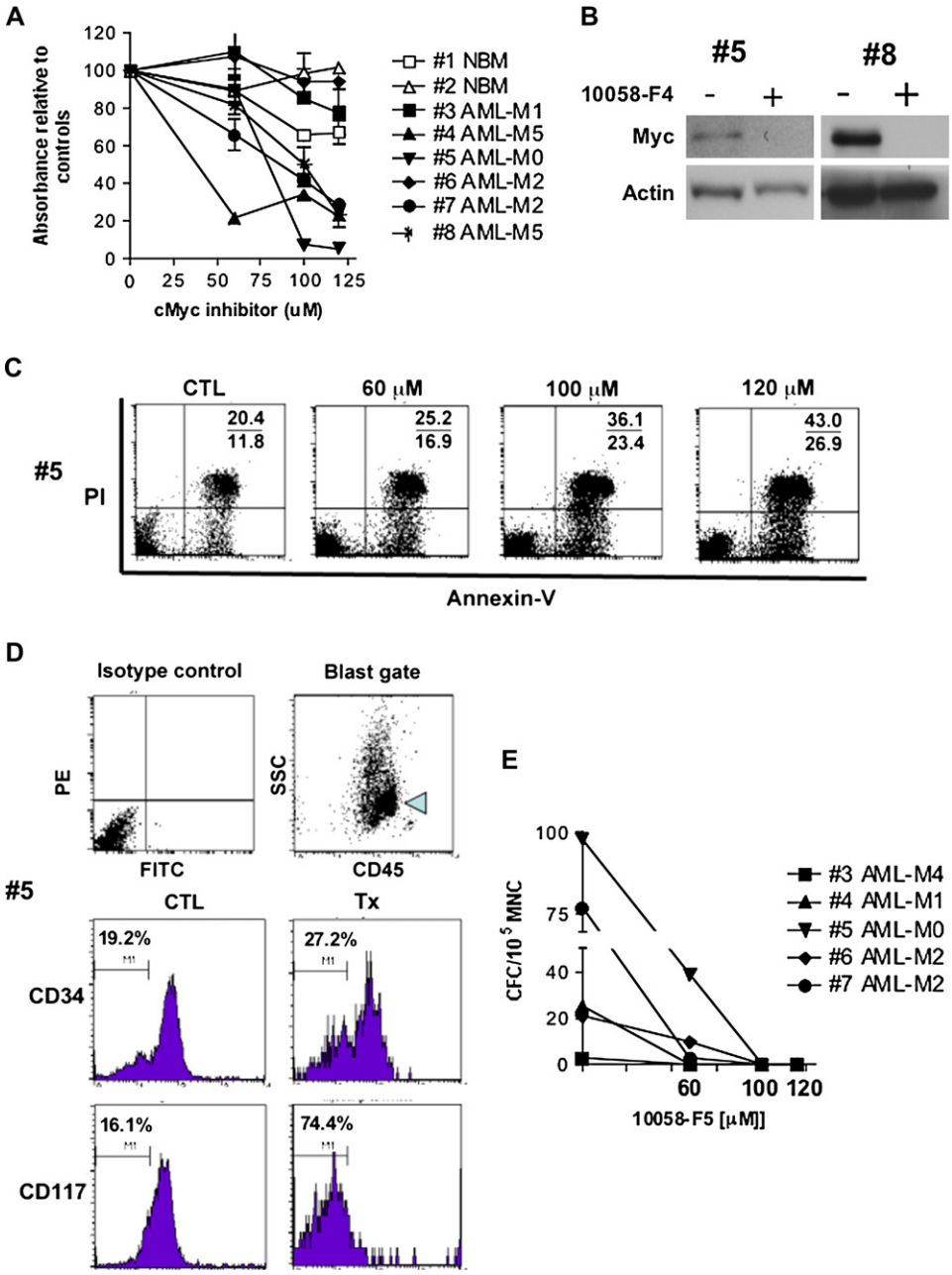

Figure 6. Effects of 10058-F4 on primary acute myeloid leukemia (AML) and normal bone marrow (BM) mononuclear cells (MNC). (**A**) Primary MNC cells (two normal and six AML patients) were treated with solvent (CTL), 60, 100, or 120 μM 10058-F4 for 48 hours before MTT assays. NBM = normal bone marrow. (**B**) Immunoblotting of c-Myc protein before (−) and after (+) treatment with 100 μM 10058-F4 for 24 hours in patient nos. 5 and 8. β-actin was used as loading control. (**C**) AML blasts from patient no. 5 were treated with solvent (CTL) or indicated concentrations of 10058-F4 for 24 hours before detecting apoptosis by annexin-V and PI staining. Numbers in each plot represent the percentage of Annexin-V^+/PI^+ (upper) and Annexin-V^+PI^- (lower) cells. (**D**) AML blasts in patient no.5 were treated with solvent (CTL) or 60 μM 10058-F4 (Tx) for 2 days before phenotypic analysis by flow cytometry. Blast cells (arrowhead) were first gated by forward scatter and CD45 staining and examined for CD34 and CD117. (**E**) Primary AML cells were treated with indicated concentrations of 10058-F4 for 2 days before being subjected to clonogenic assays. The numbers of colony-forming cells were visually scored after 7-day culture.

completely abolished the colony-forming ability in the samples tested (Fig. 6E). The treated cells also produced colonies much smaller in size (data not shown). Therefore, 10058-F4 is active not only in cell lines but also in primary AML cells.

Discussion

In this study, we tested the concept of treating AML cells by targeting c-Myc using a recently identified small molecule c-Myc inhibitor, 10058-F4, as this compound has been shown to inhibiting c-Myc transactivating activity by

blocking the dimerization of c-Myc and Max. We have shown that 10058-F4 is very effective in inhibiting the proliferation of leukemic cells, and in inducing apoptosis and myeloid differentiation.

c-Myc has been reported to increase the expression of positive cell-cycle regulators, including cyclin D1, cyclin D2, CDK4, and decrease the expression of p21 and p27 [28]. c-Myc regulates p21 expression at the transcription level through several mechanisms. One of them is by sequestering the transcription factor Sp1 from binding to p21 promoter [20,29]. The decreased c-Myc levels seen in AML cells treated with 10058-F4 might have freed up Sp1, which in turns transactivates p21. We also show that upregulation of p21 by 10058-F4 is p53-independent, consistent with the loss of p53 function in these cell lines [30,31]. Since p53 can repress c-Myc transcription and the loss of p53 synergistically enhances the c-Myc–induced tumorigenesis, it is not surprising to find that p53 is not required for the activity of 10058-F4 [32,33].

In agreement with prior studies that c-Myc directly activates cyclin D2 by binding to the E-box in its promoter, we show that 10058-F4 specifically downregulated cyclin D2 but not cyclin D1 or D3 [23]. These results support the hypothesis that 10058-F4 inhibits c-Myc activity through specific inhibition of its DNA binding activity.

The release of cytoplasmic cytochrome C, upregulation of Bax, and downregulation of Bcl-2 indicate that 10058-F4 activates intrinsic pathway of apoptosis. Activation of mitochondrial pathway supports the c-Myc's role in glucose metabolism [2]. Overexpressing c-Myc in the liver increased levels of hepatic glycolytic enzymes and lactate production in vivo [34]. Genes responsible for glucose metabolism have been identified as the c-Myc target genes, including LDH-A [35]. LDH-A, which participates in normal anaerobic glycolysis, is frequently upregulated in cancer cells, and can cause tumors when overexpressed [36]. LDH-A overexpression is required for c-Myc–mediated transformation, because lowering its expression reduced the clonogenicity of Burkitt's lymphoma.

We also show that 10058-F4 might induce myeloid differentiation preferentially to monocytic lineage in AML cells [37]. Despite that c-Myc is conventionally regarded as a transcription activator, it also functions as a transcription repressor [38]. c-Myc has been shown to repress differentiation-related p21 transcription through interaction with Miz-1, a zinc-finger transcription factor. When c-Myc level is lowered, Miz-1 activates p21 promoter, leading to myeloid differentiation, irrespective of its cell-cycle inhibitory activity [39]. 10058-F4 is likely to induce myeloid differentiation by relieving the repressive activity of c-Myc on p21. By contrast, in in vivo model using conditionally expressed c-Myc under the control of immunoglobulin heavy chain enhancer and the SRα promoter, downregulation of c-Myc leads to granulocytic differentiation [9]. The difference in differentiation between our study and the in vivo model might be explained by the expression of c-Myc from a cell-specific promoter in the in vivo model, leading to the myeloid differentiation in a more lineage-specific manner.

Treatment with 10058-F4 also activates multiple transcription factors. Genes such as Egr-1, C/EBPs, and JUN/AP1 family kinases have been well-documented for monocytic differentiation [24,25,40]. The preferential induction of monocyte-specific genes also explains the phenotypic changes in HL-60 cells and the relative increase of C/EBP$_\beta$ over C/EBP$_\beta$. Whether induction of specific transcription factors is caused by mechanisms similar to what has been described for p21, or presents a downstream signaling of p21 activation requires further investigation.

In summary, our study has demonstrated the concept that the small-molecule c-Myc inhibitor, 10058-F4, could affect multiple cellular c-Myc–related activities, and represent a potentially useful strategy for antileukemia therapy.

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