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The Novel Oral BET-CBP/p300 Dual Inhibitor NEO2734 Is Highly Effective in Eradicating Acute Myeloid Leukemia Blasts and Stem/Progenitor Cells

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Abstract

Acute myeloid leukemia (AML) is a disease characterized by transcriptional dysregulation that results in a block in differentiation and aberrant self-renewal. Inhibitors directed to epigenetic modifiers, aiming at transcriptional reprogramming of AML cells, are currently in clinical trials for AML patients. Several of these inhibitors target bromodomain and extraterminal domain (BET) proteins, cyclic AMP response binding protein-binding protein (CBP), and the E1A-interacting protein of 300 kDa (p300), affecting histone acetylation. Unfortunately, single epigenetic inhibitors showed limited efficacy due to appearance of resistance and lack of effective eradication of leukemic stem cells. Here, we describe the efficacy of 2 novel, orally available inhibitors targeting both the BET and CBP/p300 proteins, NEO1132 and NEO2734, in primary AML. NEO2734 and NEO1132 efficiently reduced the viability of AML cell lines and primary AML cells by inducing apoptosis. Importantly, both NEO drugs eliminated leukemic stem/progenitor cells from AML patient samples, and NEO2734 increased the effectiveness of combination chemotherapy treatment in an in vivo AML patient-derived mouse model. Thus, dual inhibition of BET and CBP/p300 using NEO2734 is a promising therapeutic strategy for AML patients, making it a focus for clinical translation.

Introduction

Already for decades the standard treatment for patients with acute myeloid leukemia (AML) is combination chemotherapy consisting of cytarabine and an anthracycline. Although this treatment induces complete remission (CR) in most patients, only 30%-40% of patients survive 5 years after diagnosis.[1] Often a small subpopulation of leukemia cells survives chemotherapy treatment (minimal or measurable residual disease [MRD]).[2] Leukemia cells with stem cell features, referred to as "leukemic stem cells" (LSC), residing within MRD, are thought to be responsible for disease recurrence.[3] To improve the outcome of AML patients, alternative efficient and safe treatment options are warranted.

As it is increasingly recognized that therapy resistance in cancer cells can occur in the absence of a genetic cause,[4,5] and as LSC in AML are found across all genetically defined risk groups, nonmutational mechanisms are thought to play a major role in driving the development of relapse. Preclinical and clinical studies in AML patients investigating compounds targeting epigenetic regulators, either as a single agent or in combination with standard chemotherapy, are showing promising results.[6,7] Examples of epigenetic inhibitors that are clinically tested are drugs targeting bromodomain and extraterminal domain (BET) inhibitors, including I-BET762 (NCT02308761),[8] OTX015 (NCT01713582),[9] ABBV-075 (NCT02391480),[10] and FT1101 (NCT02543879).[11] Moreover, several preclinical and clinical studies of drugs targeting the cyclic AMP response binding protein-binding protein (CBP) and its paralogue E1A interacting protein of 300 kDa (p300) are ongoing, including studies with the oral p300/CBP inhibitor CCS1477 (NCT04068597).[12–14] BET family protein BRD4 and others (BRD2, BRD3, and BRDT) function as epigenetic modifiers (chromatin readers) to facilitate gene transcription through binding of acetylated lysine in histones as well as binding to transcription factors. BET inhibitors (BETi) exhibit antiproliferative effects in many different types of malignancies,[8,9,15–18] though the molecular and cellular mechanisms that govern sensitivity remain largely unknown. In cancer cells, several transcriptional programs and enhanced expression of *c-Myc* are dependent on BET proteins. Expression of *c-Myc*, under the control of a super-enhancer, is involved in the growth and survival of AML stem/progenitor cells.[19–22] BRD4 demonstrated to be a therapeutic target for AML, and inhibition of BRD4 and subsequently downregulation of *c-Myc* and *Bcl2* resulted in eradication of AML progenitor cells.[23–27] In vivo treatment with BETi led to reduced AML load and an improved survival of mice engrafted with primary AML cells.[23,25,27] Furthermore, in a phase I clinical trial (NCT01713582), treatment with the BETi OTX015 resulted in CRs in several AML

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Supplemental digital content is available for this article.

HemaSphere (2021) 5:8(e610).
http://dx.doi.org/10.1097/HS9.0000000000000610.
Received: 14 October 2020 / Accepted: 2 June 2021

patients; however, a response biomarker could not be identified.[9] Potential drug resistance associated with BETi, which can arise from suppression of the PRC2 complex thereby restoring *Myc* transcription and increasing Wnt signaling,[28] is one of the problems hampering clinical applications of these drugs. AML LSC showed to be the origin of BETi resistance by maintaining stable Myc levels and enhanced Wnt signaling activity.[4] BETi resistance can also be caused by enhanced MCL1.[29] In nuclear protein in testis (NUT) midline carcinoma, upregulation of cell cycle genes hampered BETi-induced growth arrest, and CDK4/6 inhibition could overrule BETi resistance.[30,31]

CBP and its paralogue p300 are highly homologues BRD-containing transcriptional coactivators ("writers") that regulate transcriptional activity through their acetyltransferase activity, and their interaction with transcription factors, chromatin remodeling complexes, and the basal transcriptional machinery.[32] In AML, both p300 and CBP are occasionally rearranged into fusion genes, mainly with the MLL gene, driving efficient leukemogenesis.[32,33] Furthermore, blocking the interaction between CBP/p300 and c-Myb can prevent transformation induced by oncogenes.[34–36] Preclinical data showed that targeting both CBP and p300 had antitumor activity in AML cells,[12,13,37] and also clinical trials targeting CBP/p300 are ongoing (NCT04068597).

The combination of BETi and CBP/p300 inhibitors showed synergistic activity in AML cell lines,[13] and provides a rationale for targeting both epigenetic readers (such as BET proteins) and writers (such as CBP/p300) at the same time. A novel class of compounds, developed by Epigene Therapeutics (Montreal, Canada), bind BET and CBP/p300.[38,39] Two of these compounds are NEO1132 (patent WO2017024408A1) and the structurally unrelated molecule NEO2734 (patent US20180237417A1), of which the synthesis steps are described previously.[31] NEO2734 binds with high affinity to the BRD domains of both BET and CBP/p300, and its affinity for BET proteins was higher than the single BETi molibresib. Compared with NEO2734, NEO1132 had a slightly different pattern of binding to BRD domains, with reduced affinity for both the BRD domains of CBP/p300 and a subset of BET.[39] The in vitro antitumor activity of NEO2734 was tested against a panel of cancer cell lines, showing the strongest antiproliferative activity in leukemia, lymphoma, and prostate cancer cell lines. NEO2734 has stronger antilymphoma activity than inhibitors targeting either BET or CBP/p300 alone, and showed also in vivo tumor activity in diffuse large B-cell lymphoma (DLBLC).[39] In speckle-type pox virus and zinc finger protein mutant and wild-type prostate cancer, which exhibit high-level resistance to BETi, the effect of NEO2734 was superior over the combination of the BETi JQ1 and CBP/p300 inhibitor CPI-637.[38] Moreover, NEO2734 potently inhibited the growth and induced the differentiation of NUT midline carcinoma cells.[31] Here, we characterized the biological activity of NEO1132 and NEO2734 in AML cell lines as well as in primary AML patient cells ex vivo and in an in vivo AML patient-derived xenograft (PDX) mouse model. We demonstrate that NEO2734 efficiently eradicated leukemic blasts and leukemic progenitors from AML samples, reduced engraftment of leukemic cells in the AML PDX mouse model, and had an additive in vivo effect on chemotherapy treatment.

Methods

Cell lines and culturing

HL60, THP-1, MV4;11, KG1, and KG1a were purchased from American Type Culture Collection. HL60, THP1, MV4;11, and KG1 cells were cultured in RPMI-1640 medium (ThermoFisher Scientific) with 10% fetal calf serum (FCS). KG1a cells were cultured in RPMI-1640 medium with 20% FCS. For in vivo experiments, MV4;11 cells were cultured in Iscove's medium (Wisent Bioproducts) with 10% fetal bovine serum and penicillin/streptomycin. All cell lines were cultured in a humidified atmosphere at 37°C and 5% CO_2.

Primary cell culture

Human AML patient material was obtained from patients hospitalized at Amsterdam UMC (location VUmc, Amsterdam, the Netherlands) at time of diagnosis according to HOVON AML protocols. Normal bone marrow samples were obtained from otherwise healthy patients undergoing cardiothoracic surgery. Informed consent was obtained from every used patient, procedures were approved by the ethical committee of Amsterdam UMC, and all experiments were conducted in accordance with the Declaration of Helsinki. Mononuclear cells were isolated from each primary sample using Ficoll-Paque Plus (Amersham Biosciences) separation and cryopreserved in liquid nitrogen. Samples were thawed in IMDM medium (ThermoFisher Scientific) with 20% FCS and incubated with 10 mg/mL DNase I (Roche) and 10 mM magnesium chloride (Sigma Aldrich) for 30 minutes. Samples were cultured in IMDM containing 15% BIT 9500 serum substitute (StemCell Technologies), 50 ng/mL Flt3L (Peprotech), 20 ng/mL IL3 (Peprotech), 100 ng/mL human SCF (Peprotech), and 20 ng/mL G-CSF (Peprotech) in a humidified atmosphere at 37°C and 5% CO_2.

Test compounds and formulations

For in vitro and ex vivo experiments, NEO1132 (Neomed Institute), NEO2734 (Neomed Institute), and CPI-637 (MedChem Express) were dissolved at a concentration of 10 mM in DMSO and preserved at –20°C for future use. Negative controls were treated with DMSO at a concentration equal to the highest concentration of drug in the same experiment.

For in vivo experiments, NEO1132 and NEO2734 were dissolved in 40% polyethylene glycol (PEG400; Sigma Aldrich) in distilled water to yield 1 or 2 mg/mL, providing a 10 mg/kg active dosage. Formulation was vortexed and sonicated for approximately 20–45 minutes at room temperature until the compounds were completely dissolved. Drug formulations were prepared once a week, stored at 4°C, warmed to room temperature before each administration and used for a maximum of 7 consecutive days.

Cell viability assay

AML cell lines and primary AML samples were seeded in a 96-well plate at a density of 8000-10,000 or 20,000-30,000 cells/well, respectively, and incubated with increasing concentrations of CPI-637, NEO1132, or NEO2734 for 96 hours. 10 µL MTT reagent (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma Aldrich) was added and cells were incubated for 4 (AML cell lines) or 6 hours (primary samples). Subsequently, MTT crystals were dissolved using 150 µL isopropanol-HCl and absorbance was measured at 560 nm using a GloMax Discover Microplate reader (Promega). Values are represented as percentages, using the formula optical density (OD) = (treated sample/OD untreated sample) × 100.

Cell cycle assay

AML cell lines were seeded in a 48-well plate at a density of 0.25×10^6 cells/well and incubated with increasing concentrations of CPI-637, NEO1132, or NEO2734 for 24 and 48 hours. Cells were fixed in ice-cold 70% ethanol, incubated for 1 hour at 4°C, and washed twice with phosphate buffered saline (PBS). Subsequently, cells were stained with 50 µg/mL propidium iodide (Sigma Aldrich) in PBS supplemented with 100 µg/

mL RNAse A (Sigma Aldrich) and analysis was performed using a FACS-Fortessa flow cytometer (BD Biosciences). Data analysis was performed with FACS Diva Software (BD Biosciences).

Immunostaining and flow cytometry analysis

AML cell lines and primary cells were treated with CPI-637, NEO1132, or NEO2734 for 96 hours before analysis. Cells were harvested and washed in PBS with 0.1% human serum albumin (PBS/HSA). Samples were incubated for 15-30 minutes at room temperature with monoclonal antibody combinations consisting of antihuman 7AAD-PerCP (1:10), CD45-HV500c (2D1, 1:20), CD33-PE (1:20) or CD33-PC7 (1:20), CD34-APC (8G12, 1:50) or CD34-BV421 (581, 1:20), CD38-APC (HB7, 1:50), CD15-FITC (HI98, 1:100), CD7-APC (M-T701, 1:50) or CD7-FITC (M-T701, 1:20), CD56-PE (MY31, 1:20), HLA-DR-FITC (L243, 1:100), CD3-FITC (SK7, 1:50) or CD3-PE (1:50), CD19-APC-H7 (SJ25C1, 1:10) or CD19-FITC (89B-B4, 1:20), or antimouse CD45-PerCP (30-F11, 1:50), from BD Biosciences, and washed once with PBS/HSA. For detection of apoptosis, cells were stained with 7AAD-PerCP (1:10) for 15-30 minutes, washed with PBS/HSA, and subsequently stained with AnnexinV-FITC 1:1000 (Tau Technologies) in AnnexinV-binding buffer (Invitrogen) for 15 minutes on ice. Flow-count fluorosphere beads (Beckman Coulter, Brea) were added according to manufacturer instruction directly before analysis using a FACS-Fortessa flow cytometer (BD Biosciences). Data analysis was performed with FACS Diva software (BD Biosciences).

RNA isolation and Q-RT-PCR

RNA was isolated using TRIzol (ThermoFisher Scientific) according to manufacturer protocol. Concentration and quality of the RNA was determined with a NanoDrop 1000 (ThermoFisher Scientific). For cDNA synthesis, 1000 ng of RNA was incubated with reverse transcriptase buffer (Invitrogen), 1 mM dithiothreitol (Invitrogen), 1 mM deoxyribonucleotide triphosphates (Roche), 1.89 µg random hexamer primers (Roche), 300 U/µL moloney-murine leukemia virus reverse transcriptase (Invitrogen), 40 U/µL RNasin (Sphaero Q) in nuclease-free water, and incubated for 2 hours at 37°C and 10 minutes at 65°C. Quantitative real-time polymerase chain reactions (Q-RT-PCR) reactions were performed in duplicate with 2 µL cDNA (1 µg), 10 µL TaqMan Gene Expression Master Mix (Applied Biosystems), 1 µL Bcl2 (Hs00608023_m1) or c-Myc (Hs00153408_m1) 20x TaqMan Gene Expression Assay probe mix (Applied Biosystems) and 7 µL nuclease-free water. Expression of the housekeeping gene GUS (0.3 µM Fw primer 5′-GAA AAT ATG TGG TTG TTG GAG AGC TCA TT-3′, 0.3 µM Rv primer 5′-CCG AGT GAA GAT CCC CTT TTT A-3′, and 0.2 µM probe 5′-CCA GCA CTC TCG TCG GTG ACT GTT CA-3′) was used as a control. Q-RT-PCR reactions were performed on an ABI7500 real-time PCR System (Applied Biosystems): 10 minutes at 95°C followed by 45 cycles at 95°C for 15 seconds and 60°C for 1 minute.

Immunoblotting

After 48 hours of treatment, cells were lysed in radioimmunoprecipitation assay lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS) containing protease inhibitor cocktail (Roche). Protein concentrations were determined using Bio-Rad protein assay (#500-00001, Bio-Rad). 2- or 3-units OD of cell lysates were boiled in reduced sample buffer, proteins were separated by 4%-16% precast gels (Bio-Rad) and transferred to polyvinylidene fluoride membranes (Millipore) in Tris/glycine/SDS/20% methanol buffer. Membranes were blocked using 2.5% (w/v) nonfatty dried milk powder (Nutricia) and 2.5% (w/v) bovine serum albumin (Millipore) in PBS/0.1% Tween (ThermoFisher Scientific) and incubated with first antibodies: rabbit anti-Bcl2 (D55G8, 1:1000, #4223, Cell Signaling Technology), rabbit anti-caspase-3 (8G10, 1:1000, #9665, Cell Signaling Technology), rabbit anti-MCL1 (S-19, 1:1000, #sc819, Santa Cruz), mouse anti-c-Myc (C-33, 1:500, #sc42, Santa Cruz), mouse anti-p21 (1:5000, #sc817, Santa Cruz), or mouse anti-actin (C4, 1:5000, #MAB1501R, Millipore). After washing with PBS/0.1% Tween, membranes were incubated with goat anti-mouse-IgG-horse radish peroxidase (HRP) (#p0447, 1:1000, Agilent Dako) or mouse antirabbit-IgG-HRP (1:1000, #sc2004, Santa Cruz). Membranes were developed with enhanced chemiluminescence (GE Healthcare) and imaged using an UVITEC imaging system (Cleaver Scientific).

Liquid culture assay and colony forming unit assay

After 7 days of treatment of primary cells in CellGro-SCGM medium (Cellgenix) with 50 ng/mL FLT3L, 20 ng/mL IL3, and 100 ng/mL human SCF, 30,000 AML cells or 15,000 NBM cells were transferred to MethoCult without erythropoietin (StemCell Technologies) and incubated in a humidified atmosphere at 37°C and 5% CO_2. After 8-12 days, colonies were quantified using an Axiovert 25 bright field microscope (Zeiss) and images were acquired using a Nikon D3500 camera (Nikon Corporation).

PDX mouse model

NOD/SCID/IL2r gamma (null) mice (NSG) were purchased from The Jackson Laboratory. All mice were maintained in a specific pathogen-free facility at the Amsterdam Animal Research Center of VU University (the Netherlands) in accordance with a protocol (DEC-HEMA-17-002) approved by the "Centrale Commissie Dierproeven." Female and male mice, 8-10 weeks of age, were irradiated with 200 cGy 1 day prior injection of primary AML cells. Primary AML cells were thawed and incubated with anti-CD3 microbeads (Miltenyi Biotec) and T-cell depleted using MACS columns (Miltenyi Biotec) according to manufacturer protocol. 1.5 to 2.4 × 10^6 AML cells were intravenously injected in the tail vain of the mice. When human CD45 cells were observed in the peripheral blood (PB) of the mice, treatment started.

To evaluate the potential of the NEO drugs to reduce human primary AML8 burden, the mice were orally treated with 10 mg/kg NEO1132, NEO2734, or control (40% PEG400 in distilled water) for 11 times in week 11-12. To evaluate whether the NEO drugs could eliminate residual leukemia cells after treatment with combination chemotherapy, mice were intravenously injected at 10 mL/kg for 2 consecutive days with combination chemotherapy consisting of 50 mg/kg cytarabine and 1.5 mg/kg doxorubicin or PBS in week six or eight after injection of primary T-cell–depleted AML10 or AML9 cells, respectively. Subsequently, 10 mg/kg NEO1132, NEO2734, or control (40% PEG400 in distilled water) was administered 10 or 11 times using oral gavage at 10 mL/kg.

Mice were sacrificed when disease features such as ≥20% weight loss were observed or at the endpoint at 12-15 weeks. Bone marrows were analyzed for the presence of human cells using a FACS-CantoII flow cytometer (BD Biosciences). Data analysis was performed using FACS Diva Software (BD Biosciences).

Tumor xenograft mouse model

SCID (CB17) female mice, 5-6 weeks old, were purchased from Charles River Canada Inc. Mice were housed in a controlled

animal room environment at the AAALAC accredited animal facility of Amplia PharmaTek Inc. (Montreal, Canada) in accordance with procedures (including protocol PRN008) approved by the Institutional Animal Care and Use Committee and conducted in accordance with the principles outlined in the current Guide to the Care and Use of experimental Animals as published by the Canadian Council. Mice were injected subcutaneously with 3×10^6 MV4;11 cells on the right side of the lower flanks (1 graft per mouse). Tumor growth was monitored once weekly starting 14 days after cell inoculation. About 3 weeks postinjection, mice were treated once every day (ED) for 11 consecutive days during treatment period 1 (day 1-11) or for 11 times every other day (EOD) during treatment period 1 (day 1-11) and treatment period 2 (day 19-27) with 10 mg/kg NEO1132 (Neomed Institute), NEO2734 (Neomed Institute), or control (40% PEG400 in distilled water), administered by oral gavage at 5 mL/kg. During the first treatment period, tumor volume and body weight were monitored EOD. Thereafter, monitoring was done 3 times per week until study termination. PB was collected from each animal by tail snip on day 12, and whole blood count with white cell differential was analyzed using a scil Vet ABC Automated Blood Counter (ABX Diagnostics). Mice were sacrificed when disease features, such as ≥20% weight loss, tumor volume >3000 mm^3 or with ulcer were observed or at the endpoint at day 27. Blood was collected, centrifuged at 6010 rcf for 10 minutes at 4°C, and serum was collected. Serum was sent to Biovet Laboratory for Blood Chemistry analysis.

Data quantification and statistical analysis

Statistical analyses were performed using GraphPad Prism Software (version 7.0 and 8.0). Results were reported as mean ± SEM or SD. The concentration whereby a drug reduces cell survival by half (IC50) values were calculated using nonlinear regression curve fitting in GraphPad Prism Software 8.0. Statistical significance between 2 measurements was determined using 2-tailed (un)paired Student's *t* tests. To compare multiple groups, 1- or 2-way (repeated measure) ANOVA tests with post-hoc Dunnett's or Tukey's multiple comparison tests were used. In all figures and Supplemental Digital Content, Tables S1 and S2, http://links.lww.com/HS/A176, $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $****P < 0.0001$.

Results

NEO2734 and NEO1132 reduce AML cell growth by inducing apoptosis, and efficiently downregulate c-Myc and Bcl2 expression

Given that the dual BET-CBP/p300 inhibitor NEO2734 has demonstrated a stronger inhibition of cell growth than either targeting BET or CBP/p300 in DLBLC and prostate cancer,[38,39] we sought to assess the biological activity of NEO2734 in AML. First, we treated a panel of AML cell lines with NEO2734, NEO1132, and the CBP/p300 inhibitor CPI-637. After treatment with nanomolar concentrations of NEO2734, reduced cell viability was observed in all tested AML cell lines, with IC50 values ranging from 27 to 125 nM (Figure 1A, Supplemental Digital Content, Table S1, http://links.lww.com/HS/A176). In all cell lines, the reduction of cell viability after NEO2734 treatment was significantly better than after the mono CBP/p300 inhibitor CPI-637, and NEO2734 was significantly better in decreasing the viability of HL60, KG1, KG1a and MV4;11 cells than NEO1132 (Figure 1A, Supplemental Digital Content, Table S1, http://links.lww.com/HS/A176).

Next, we investigated whether NEO2734 treatment of AML cells could reduce *c-Myc*, a target of both BETi and CBPi,[40] and *Bcl2*, the top gene upregulated in NEO2734-resistant lymphoma cells.[39] The AML cell lines HL60, THP1, and MV4;11 were treated with increasing concentrations of NEO2734 and mRNA levels of *c-Myc* and *Bcl2* were measured after 2 and 5 hours by Q-RT-PCR. In all 3 cell lines, *c-Myc* and *Bcl2* were already significantly reduced on treatment with 100 nM NEO2734 (Supplemental Digital Content, Figure 1A, B, http://links.lww.com/HS/A176). Immunoblotting confirmed that treatment with NEO2734 reduced protein expression of c-Myc and Bcl2 in HL60, THP1, and KG1 cells (Figure 1B). In MV4;11 cells, treatment with NEO2734 reduced c-Myc protein; however, the Bcl2 levels were hardly affected (Figure 1B). Since Bcl2 protein did not change on NEO2734 treatment in MV4;11, we measured protein levels of the Bcl2 family member MCL1 and showed that NEO2734 reduced MCL1 in MV4;11 cells (Figure 1C). To investigate whether the reduction in cell viability after treatment with the NEO drugs was due to induction of apoptosis, the AML cell lines were tested in a flow cytometry-based apoptosis assay. Indeed, NEO2734 significantly enhanced binding of Annexin-V and 7AAD as compared with CPI-637 in all tested cell lines (Figure 1D, E, Supplemental Digital Content, Figure 1C, D, http://links.lww.com/HS/A176). Moreover, NEO2734 was in general better in inducing apoptosis than NEO1132 (Figure 1D, E, Supplemental Digital Content, Figure 1C, D, http://links.lww.com/HS/A176). We also measured expression of cleaved caspase 3 and cyclin-dependent kinase inhibitor 1 protein p21 (Figure 1E), 2 known downstream targets of c-Myc,[41,42] after treatment of THP1 cells with NEO2734. Treatment with NEO2734 induced cleavage of caspase-3 and upregulation of p21, again indicating that NEO2734 induces apoptosis in AML cells.

NEO2734 and NEO1132 induce a G1-phase cell cycle arrest in AML cells

Inhibition of both BET and CBP/p300 demonstrated to induce a G1-phase cell cycle arrest,[43,44] which, in case of CBP/p300 inhibition in multiple myeloma cell lines, also induced downregulation of c-Myc.[44] Similarly, NEO2734 and NEO1132 were recently identified to induce a G1-phase cell cycle arrest in NUT midline carcinoma cells and multiple myeloma cells,[31,45] and we therefore investigated the effect of the NEO drugs on cell cycle progression of AML cells. Indeed, after 24 or 48 hours of treatment, NEO2734 could induce a significant G1-phase cell cycle arrest in all 4 tested AML cell lines (Figure 2A, Supplemental Digital Content, Figure 2A, http://links.lww.com/HS/A176). Treatment with NEO1132 only resulted in a significant induction of a G1 block in HL60, THP1, and MV4;11 cells (Figure 2A, Supplemental Digital Content, Figure 2A, http://links.lww.com/HS/A176). Together these results suggest that NEO2734 is more effective in inducing a G1-phase cell cycle arrest than NEO1132 and CPI-637.

Every day dosing of NEO2734 and NEO1132 efficiently inhibits tumor growth of MV4;11 cells in a tumor xenograft mouse model

To evaluate whether 10 mg/kg NEO1132 or NEO2734 dosed orally either once a day over 1 treatment period (ED) of 11 days or EOD in 2 treatment periods with a rest period of a week was efficacious to inhibit the growth of the human leukemia MV4;11 cell line in vivo, we subcutaneously injected MV4;11 cells in the lower flank of SCID (CB17) mice (treatment schedule in Figure 2B, left). ED dosing was shown to be significantly more effective in inhibition of tumor growth than EOD treatment ($**P < 0.01$ for NEO1132 ED versus NEO1132 EOD between day 11 and 18, and $*P < 0.05$ for NEO2734 ED versus NEO2734 EOD between day 13 and 27) (Figure 2B, right).

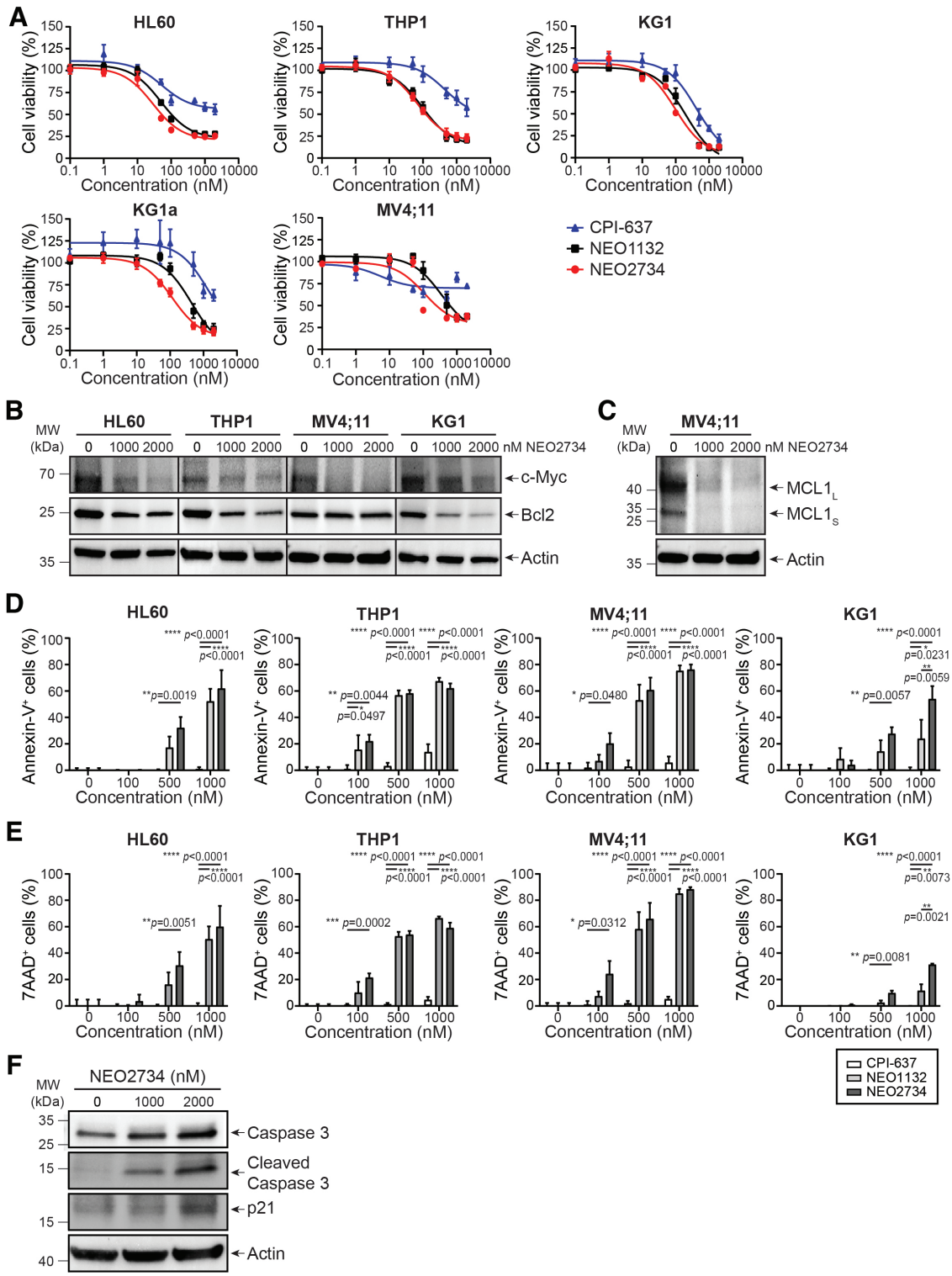

Figure 1. NEO2734 and NEO1132 reduce AML cell growth by inducing apoptosis, and efficiently downregulate c-Myc and Bcl2 expression. For all in vitro experiments, AML cell lines were incubated with increasing concentrations of CPI-637, NEO1132, or NEO2734 for 96 h, unless stated otherwise. Data are plotted as mean ± SEM and *P* values were calculated using a 2-way ANOVA with post-hoc Tuckey's multiple comparison test. (A), Cell viability of AML cell lines was measured using an MTT assay, quantified as relative absorbance and normalized against untreated controls. Graphs are representative of 3 independent experiments (in triplicate). IC50 values and statistics are shown in Supplemental Digital Content, Table S1, http://links.lww.com/HS/A176. (B and C), Immunoblot analysis of c-Myc and Bcl2 expression in AML cell lines treated with NEO2734. (D–E), Percentage of (D) Annexin-V+ and (E) 7AAD+ cells in AML cell lines after treatment with increasing concentrations of indicated drugs. Untreated control samples were set to 0%. (F), Immunoblot analysis of caspase 3, cleaved caspase 3, and p21 in THP1 cells treated with NEO2734 for 48 h. AML = acute myeloid leukemia; IC50 = the concentration whereby a drug reduces cell survival by half; MTT = 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide.

Also, from day 9 to 18, the average tumor volume after ED treatment with either NEO1132 or NEO2734 was significantly lower than the average tumor volume in the control group (**$P < 0.01$). For EOD treatment, only in the period between treatment period 1 and 2, the tumor growth was significantly inhibited by NEO2734 (*$P < 0.05$), while ED dosing of both

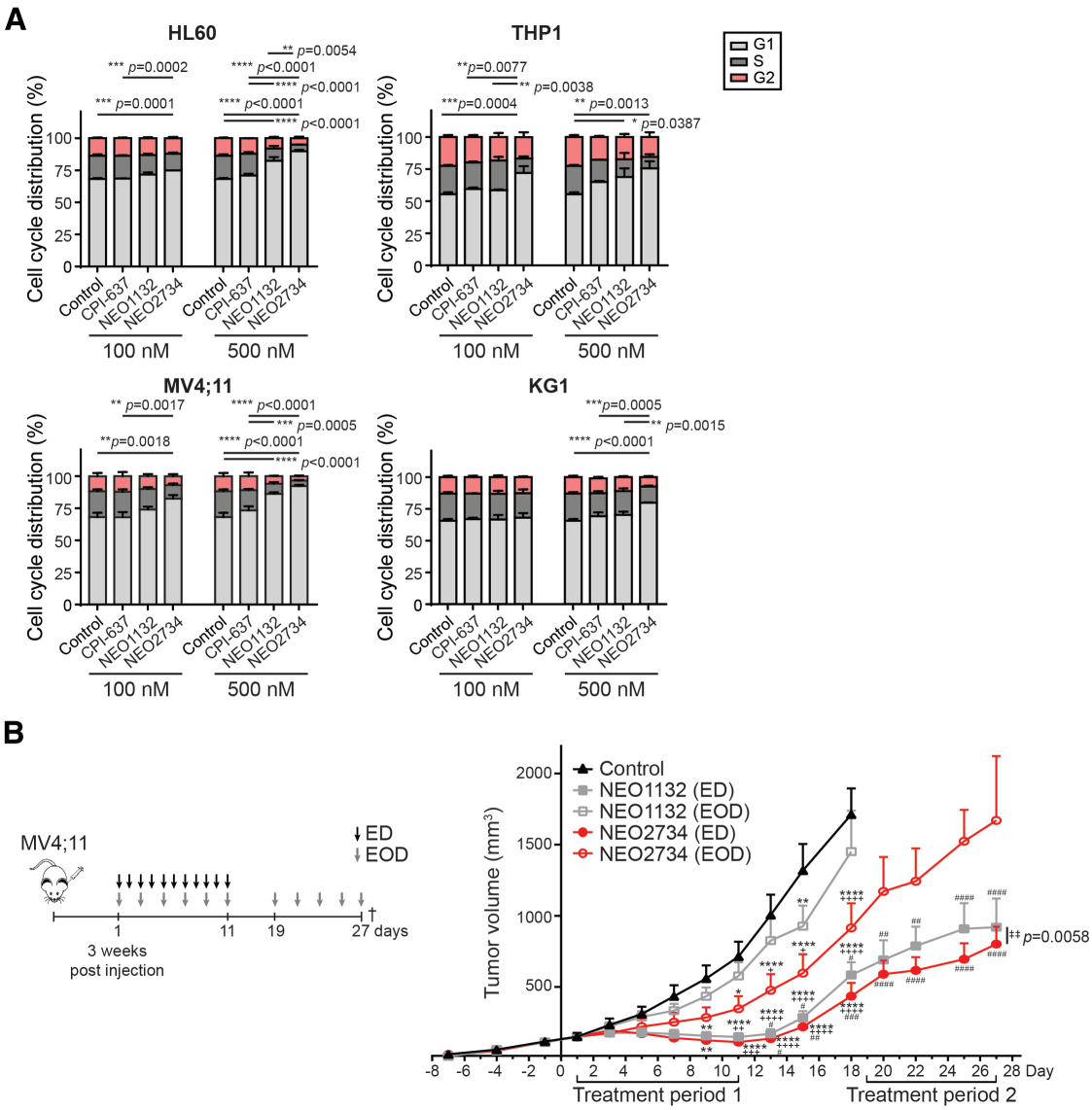

Figure 2. NEO2734 and NEO1132 induce a G1-phase cell cycle arrest, and efficiently inhibit tumor growth of MV4;11 cells in a tumor xenograft mouse model. (A), Cell cycle analysis of AML cell lines incubated with 100 and 500 nM of CPI-637, NEO1132, or NEO2734 for 24 h (THP1 and MV4;11) or 48 h (HL60 and KG1). The percentage of cells in G1, S, and G2 phase was measured using flow cytometry in 3 independent experiments and plotted as mean ± SEM. *P* values were calculated using a 2-way ANOVA with post-hoc Tuckey's multiple comparison test for statistical comparison of the G1 phase. (B), Schematic overview of the experiment (left). 3 × 10^6 MV4;11 cells were subcutaneously injected on the right side of the lower flanks of SCID (CB17) mice. 3 wk postinjection, mice were treated once daily (ED) for 11 consecutive days during treatment period 1 (day 1-11) or for 11 times EOD during treatment period 1 (day 1-11) and treatment period 2 (day 19-27) with 10 mg/kg NEO1132, NEO2734, or control (40% PEG400 in distilled water), administered by oral gavage at 5 mL/kg. Tumor volume from 6 mice each group was monitored until study termination (right). *P* values were calculated using a 2-way repeated measure ANOVA with post-hoc Tukey's multiple comparison test for comparison of 3 or more groups, * vs control group, ⁺ vs NEO1132 EOD, # vs NEO2734 EOD. For comparison of 2 groups, *P* values were calculated using a paired Student's *t* test, ‡NEO1132 vs NEO2734. AML = acute myeloid leukemia; ED = every day; EOD = every other day.

NEO1132 and NEO2734 completely blocked the tumor growth from the start of the treatment till day 11. Notably, the tumor started to regrow after stopping the treatment, suggesting that tumor growth inhibition requires daily treatment. Comparing ED treatment of both drugs revealed that NEO2734 was more efficient in inhibition of tumor growth than NEO1132 (**P* = 0.006; Figure 2B, right).

NEO2734 and NEO1132 eliminate primary AML blasts by inducing apoptosis

To investigate whether NEO2734 and NEO1132 can efficiently eradicate primary AML patient cells, we incubated 6 primary AML samples with the NEO drugs or CPI-637 for 4 days. Both NEO drugs efficiently eliminated the total AML cell population, as measured by an MTT assay, with IC50 values ranging from 6 to 175 nM for NEO2734 (mean IC50 of 86.5 ± 26.9 nM) and 26 to 412 nM for NEO1132 (mean IC50 of 215.2 ± 56.2 nM) (Figure 3A, B, Supplemental Digital Content, Figure 2B, Table S2, http://links.lww.com/HS/A176). The potency of NEO2734 to eliminate primary AML cells was significantly higher than that of NEO1132 (**P* = 0.0236) and CPI-637 (mean IC50 of 4.0 ± 1.2 μM, **P* = 0.0461) (Figure 3B). As an MTT assay could not detect the identity of the cell populations that are eliminated after treatment, we also measured the potential of the drugs to eradicate primary AML cells using flow cytometry. Survival of AML $CD45^{dim}$ blasts (example in Supplemental

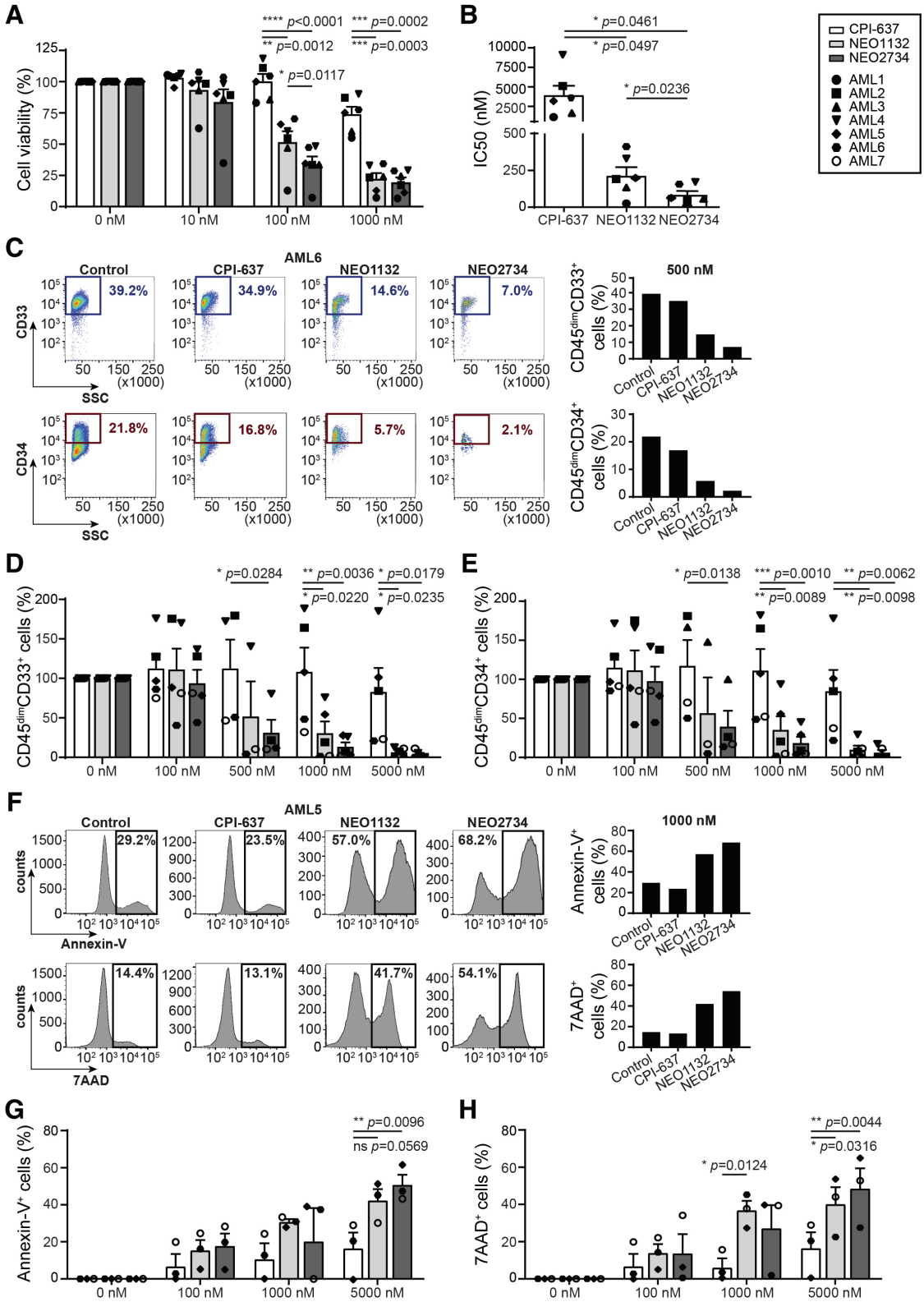

Figure 3. NEO2734 and NEO1132 eliminate primary AML blasts by inducing apoptosis. Primary AML samples were incubated for 4 d with increasing concentrations of CPI-637, NEO1132, or NEO2734. Percentages of AML cell populations were measured using flow cytometry, quantified relative to flow count beads, normalized against untreated controls and plotted as mean ± SEM. *P* values were calculated using a repeated measure 1- or 2-way ANOVA with post-hoc Tukey's multiple comparison test. Patient characteristics and IC50 values are shown in Supplemental Digital Content, Table S2, http://links.lww.com/HS/A176. (A and B), In 6 primary AML samples, cell viability was quantified using an MTT assay, (A) measured as relative absorbance (in triplicate) and normalized against untreated controls. (B), IC50 values in nM were calculated. (C), Viable $CD45^{dim}CD33^{+}$ (top, blue) and $CD45^{dim}CD34^{+}$ (bottom, red) cells in AML6 after treatment with 500 nM of indicated drugs. (D), Percentage of viable $CD45^{dim}CD33^{+}$ and (E) $CD45^{dim}CD34^{+}$ cells in 5 primary AML samples. (F), Annexin-V^{+} (top) and $7AAD^{+}$ (bottom) cells in AML5 after treatment with 1000 nM of indicated drugs. (G), Percentage of annexin-V^{+} and (H) $7AAD^{+}$ cells in 3 primary AML samples. Untreated control samples were set to 0%. AML = acute myeloid leukemia; IC50 = the concentration whereby a drug reduces cell viability by half.

Digital Content, Figure 2C, D, http://links.lww.com/HS/A176), myeloid CD45^{dim}CD33^{+} cells (example in Figure 3C, top, Figure 3D), and CD45^{dim}CD34^{+} immature blasts (example in Figure 3C, bottom, Figure 3E) was significantly reduced after incubation with 500 nM NEO2734 and 1 μM NEO1132, while the lymphocytes were unaffected after incubation with concentrations up to 5 μM (example in Supplemental Digital Content, Figure 2C, E, http://links.lww.com/HS/A176). NEO2734 and NEO1132 induced apoptosis in the primary CD45^{dim} leukemic blasts as shown by enhanced binding of annexin-V (example in Figure 3F, top, Figure 3G) and 7AAD (example in Figure 3F, bottom, Figure 3H) after treatment of the cells with both NEO drugs.

NEO2734 significantly impairs leukemic stem/progenitor cell survival

In most AML cases, LSC reside within the CD34^{+}CD38^{-} leukemic cell compartment,[46] which can be identified by flow cytometry using leukemia-associated immunophenotypic (LAIP) markers expressed on the membrane of the leukemic CD34^{+}CD38^{-} cells.[47] The presence of LSC is a major risk factor for AML relapse,[48] implicating that elimination of these leukemic (stem/progenitor) cells will be crucial for relapse prevention. To investigate the potential of the NEO drugs to eliminate CD45^{dim}CD33^{+}LAIP^{+} and immature CD34^{+}CD38^{-}LAIP^{+} AML cells, we incubated 2 AML samples with the NEO drugs and determined the presence of both cell compartments by flow cytometry. Treatment with NEO1132 and NEO2734 resulted in a significant reduction in both AML CD45^{dim}CD33^{+}LAIP^{+} blasts (Figure 4A, B) and immature CD45^{dim}CD34^{+}CD38^{-}LAIP^{+} leukemic cells (Figure 4C, D).

To evaluate whether NEO1132 and NEO2734 treatment could inhibit clonogenic capacity of leukemic stem/progenitor cells, 5 primary AML samples were incubated with the NEO drugs for 1 week. Already after treatment with 10 nM of either NEO1132 or NEO2734, a significant reduction in the number of colonies was observed in all primary AML cases (Figure 4E, Supplemental Digital Content, Figure 2F, http://links.lww.com/HS/A176). Interestingly, in 2 AML cases (AML4 and AML7), NEO2734 was capable of complete attenuation of colony forming leukemia cells at 100 nM, while it could completely eliminate the leukemic stem/progenitors at 500 nM in all the cases (Supplemental Digital Content, Figure 2F, http://links.lww.com/HS/A176). The colonies consisted of clusters of cells, which looked like small colonies derived from granulocyte-macrophage progenitor cells (Supplemental Digital Content, Figure 2G, http://links.lww.com/HS/A176).

NEO2734 reduces leukemic engraftment and adds to chemotherapy treatment in a PDX AML mouse model

To evaluate the potential of the NEO drugs to reduce human primary AML burden in vivo, patient AML cells (containing a FLT3-ITD and NPM1 mutation) were intravenously injected into NSG mice. When human CD45 (hCD45) leukemia cells were detected in the PB of the mice, the mice were orally treated with 10 mg/kg NEO1132 or NEO2734 for 11 times (treatment schedule in Figure 5A, left). A significant reduction in leukemic engraftment of hCD45^{+}CD33^{+}LAIP^{+} myeloid leukemia cells (Figure 5A, middle), and immature hCD45^{+}CD34^{+} cells (Figure 5A, right) in the bone marrows of the mice was observed after treatment with NEO2734, indicating that NEO2734 monotherapy efficiently reduced leukemic burden in vivo. In contrast, after treatment with NEO1132, no significant reduction in leukemic burden was observed.

Since persistence of residual leukemia cells (MRD) is the major cause of relapse in AML patients, we investigated whether the NEO drugs could deplete residual leukemia cells after treatment with combination chemotherapy in vivo. We transplanted mice with primary AML cells (AML9; EVI-1+ and AML10; FLT3-ITD and a NPM1 mutation). AML9 was derived from a patient that quickly reached CR after chemotherapy, while the AML10 patient was initially refractory and reached CR after chemotherapy at a later time point than AML9. When human CD45 (hCD45) leukemia cells were detected in the PB, we treated the mice first with a combination of cytarabine (50 mg/kg) and doxorubicin (1.5 mg/kg) for 2 consecutive days, and subsequently with 10 mg/kg NEO1132 or NEO2734 for 10 times (treatment schedules in Figure 5B, top left [AML9] and Figure 5C, top [AML10]). For AML9 and AML10, 2 times treatment with combination chemotherapy hardly reduced leukemic burden, likely due to the low frequency (2 times) of the treatment. Moreover, also 10 times monotherapy with NEO1132 was not effective in reducing engraftment. Interestingly, treatment with NEO2734 after chemotherapy could reduce the leukemic engraftment of hCD45^{+} cells (Figure 5B; AML9, Figure 5C; AML10, example in Supplementary Digital Content, Figure 3A, http://links.lww.com/HS/A176), hCD45^{+}CD33^{+}LAIP^{+} myeloid leukemia cells (Figure 5B; AML9, and Figure 5C; AML10) and immature hCD45^{+}CD34^{+} cells (Figure 5B; AML9) in the bone marrows of the mice, suggesting that NEO2734 could enhance the effect of chemotherapy or could eliminate chemotherapy-resistant primary AML cells.

NEO2734 shows higher activity in primary AML cells than in normal hematopoietic bone marrow cells, providing a therapeutic window

To restore normal hematopoiesis after therapy, therapeutic approaches targeting leukemia cells should spare normal hematopoietic cells. Hence, we tested the ex vivo effect of the NEO drugs on normal bone marrows cells derived from 2 healthy donors (Figure 6A, Supplemental Digital Content, Table S2, http://links.lww.com/HS/A176). After 4 days of treatment, both NEO2734 and NEO1132 reduced the survival of normal bone marrow cells; however, only at high concentrations (1 μM) and with 3.4- and 2.0-fold lower efficiency than the drugs affected primary AML cells (mean IC50 of 292.9 ± 65.7 nM and 427.2 ± 125.7 nM for NEO2734 and NEO1132, respectively; Figure 6A, B, Supplemental Digital Content, Figure 3B, Table S2, http://links.lww.com/HS/A176). Although the single CBP/p300 inhibitor CPI-637 was significantly less efficient in eliminating normal bone marrow cells than the NEO drugs (mean IC50 of 4.7 ± 2.4 μM; Figure 6A, B), its IC50 value for AML cells was only 1.2-fold lower than for normal bone marrow cells (Figure 6B, Supplemental Digital Content, Table S2, http://links.lww.com/HS/A176). Flow cytometric analysis revealed that only high concentration (1 μM) of NEO2734 reduced the number of normal CD45^{dim} (Supplemental Digital Content, Figure 3C, http://links.lww.com/HS/A176) and myeloid CD45^{dim}CD33^{+} (Figure 3C) cells. Also, the number of CD3^{+} T- and CD19^{+} B-cells were not affected by treatment with NEO2734 or NEO1132 (example in Supplemental Digital Content, Figure 3D, http://links.lww.com/HS/A176, Figure 6D, E). Moreover, only at high concentrations of NEO2734 (0.5 μM), the colony-forming capacity of progenitor cells was significantly affected (Figure 6F, Supplemental Digital Content, Figure 3E, http://links.lww.com/HS/A176).

To evaluate the toxicity of the NEO drugs in vivo, the weight of SCID (CB17) mice orally treated with NEO2734 or NEO1132 either once a day or EOD was monitored (treatment schedule in Figure 2B, left). NEO1132 and NEO2734 did not significantly affect body weight loss during the experiment (Figure 6G). Although there was a slight decrease in white blood cells (WBC) (Figure 6H) and granulocytes (Figure 6I) after EOD

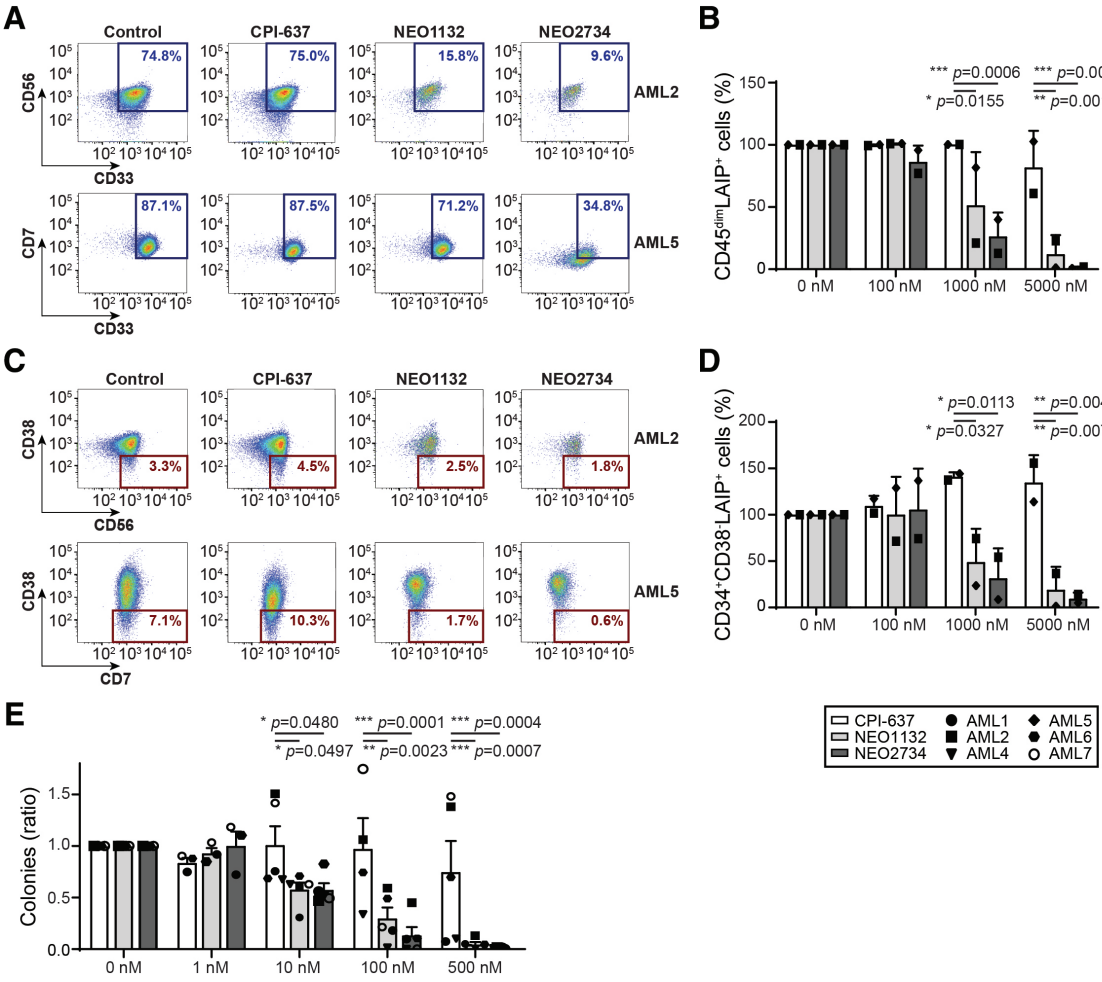

Figure 4. NEO2734 significantly impairs leukemic stem/progenitor cell survival. Primary AML samples were incubated with increasing concentrations of CPI-637, NEO1132, or NEO2734. Data were normalized against untreated controls and depicted as mean ± SD, unless stated otherwise. *P* values were calculated using a repeated measure 2-way ANOVA with post-hoc Tukey's multiple comparison test. Patient characteristics are shown in Supplemental Digital Content, Table S2, http://links.lww.com/HS/A176. (A–D), After 4 d of treatment, $CD45^{dim}CD33^{+}LAIP^{+}$ blasts and $CD45^{dim}CD34^{+}CD38^{-}LAIP^{+}$ cells in AML2 (LAIP = CD56) and AML5 (LAIP = CD7) were measured using flow cytometry and quantified relative to flow count beads. (A), Flow cytometric analysis after treatment with 1000 nM of indicated drugs and (B) percentages of $CD45^{dim}CD33^{+}LAIP^{+}$ cells. (C), Flow cytometry analysis after treatment with 1000 nM of indicated drugs and (D) percentages of $CD45^{dim}CD34^{+}CD38^{-}LAIP^{+}$ cells. (E), CFU progenitor assays (in duplicate) of primary AML samples. After treatment of the primary cells for 1 wk, cells were incubated in MethoCult without erythropoietin for 8-12 d, and colonies were quantified using a microscope. Data were normalized against untreated controls and plotted as mean ± SEM. AML = acute myeloid leukemia; CFU = colony-forming unit; LAIP = leukemia-associated immunophenotypic.

treatment with NEO2734, there was no decrease in the total numbers of WBC (Figure 6H), granulocytes (Figure 6I), red blood cells (Figure 6J), monocytes (Figure 6K), and lymphocytes (Figure 6L) after treatment with NEO1132, and importantly not with NEO2734 ED treatment, the treatment schedule most efficiently reducing tumor growth. Overall, ED treatment with NEO1132 and NEO2734 did not affect the survival of normal hematopoietic cells and was well tolerated in mice.

Discussion

The 5-year survival of AML patients is relatively low, with high relapse rates after standard chemotherapy, especially for older patients. Therefore, novel alternative therapeutic strategies are warranted. AML cells are characterized by aberrant gene transcription that is, at least in part, due to convergence of developmental and oncogenic gene expression regulated by epigenetic modifiers and transcription factors. The recent discovery that epigenetic readers of the BRD and BET protein family are crucial for AML maintenance by regulating the transcription of *c-Myc* has led to the rapid development of many BETi. BETi JQ1 and OTX015 demonstrated to induce apoptosis in AML cells, to reduce in vivo AML engraftment and to increase the survival of leukemic mice.[9,20,49] Nevertheless, single activity of BETi appeared to be limited and several resistance mechanisms to BETi have been identified, especially in LSC.[4,28,29]

The basal transcription factor machinery in AML cells involves aberrant activity of hematopoietic transcription factors and their coactivators, such as Myb and CBP/p300. CBP/p300 acetylates histone 3 lysine 27 (H3K27) at enhancers, thereby recruiting the BRD proteins to active promoters. BET proteins regulate gene expression through binding to acetylated histones, controlling enhancer-dependent transcriptional elongation. Both CPB/p300 and BET proteins play an important role in the expression of *c-Myc*.[32,35,50] We demonstrated that NEO1132 and NEO2734 have activity against AML cell lines and primary AML by inducing apoptosis in the leukemic blasts, and that this effect is accompanied by a rapid downregulation of *c-Myc* and *Bcl2*. Previously, it was demonstrated that NEO2734-resistant lymphoma cells have upregulated *Bcl2*.[39] Downregulation of *c-Myc* and *Bcl2* can be established by blocking the interaction of CBP/p300 with Myb, resulting in displacement of Myb-CBP/

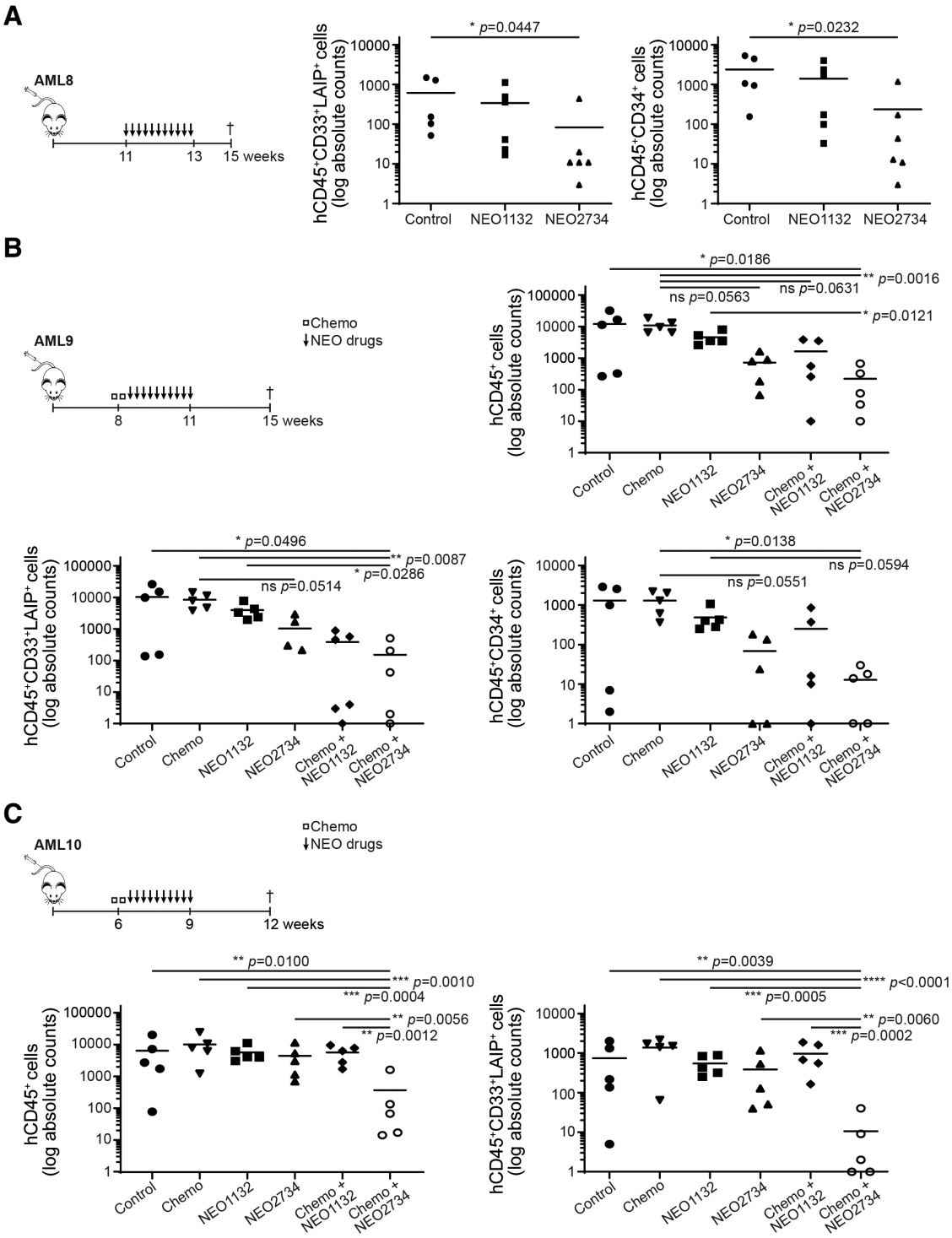

Figure 5. NEO2734 reduces leukemic engraftment and adds to chemotherapy treatment in a PDX AML mouse model. After IV injection of T-cell–depleted primary AML cells, NSG mice were IV treated at 10 mL/kg with combination chemotherapy (50 mg/kg cytarabine and 1.5 mg/kg doxorubicin) or control (PBS), and/or 10 mg/kg NEO1132, NEO2734, or control (40% PEG400 in distilled water) administered by oral gavage at 10 mL/kg. At study termination, bone marrows were collected and analyzed using flow cytometry. *P* values were calculated using a 1-way ANOVA with post-hoc Tukey's multiple comparison. Patient characteristics are shown in Supplemental Digital Content, Table S2, http://links.lww.com/HS/A176. (A), Schematic overview of the experiment (left). After injection of 1.5×10^6 AML8 cells, NSG mice were treated with NEO1132, NEO2734, or control for 11 times (week 11-12). At week 15, the bone marrows of the mice were analyzed for the presence of myeloid hCD45$^+$CD33$^+$LAIP$^+$ leukemia cells (LAIP = CD7, middle) and immature hCD45$^+$CD34$^+$ cells (right). (B), Schematic overview of the experiment (top left). After injection of 2.4×10^6 AML9 cells, NSG mice were treated with combination chemotherapy for 2 consecutive days in week 8 and subsequently 10 times with NEO1132 or NEO2734 in week 8-11. At week 15, the bone marrows of the mice were analyzed for the presence of human CD45$^+$ cells (top right), myeloid leukemia hCD45$^+$CD33$^+$LAIP$^+$ cells (LAIP = CD15$^-$/HLA-DR$^-$, bottom left), and immature hCD45$^+$CD34$^+$ cells (bottom right). (C), Schematic overview of the experiment (top). After injection of 1.6×10^6 AML10 cells, NSG mice were treated with combination chemotherapy for 2 consecutive days in week 6 and subsequently 10 times with NEO1132 or NEO2734 in week 6-9. At week 12, the bone marrows of the mice were analyzed for the presence of human CD45$^+$ cells (left) and myeloid leukemia hCD45$^+$CD33$^+$LAIP$^+$ cells (LAIP = CD7, right). AML = acute myeloid leukemia; IV = intravenously; LAIP = leukemia-associated immunophenotypic; NSG = NOD/SCID/IL2r gamma; PBS = phosphate buffered saline; PDX = patient-derived xenograft; PEG400 = 40% polyethylene glycol.

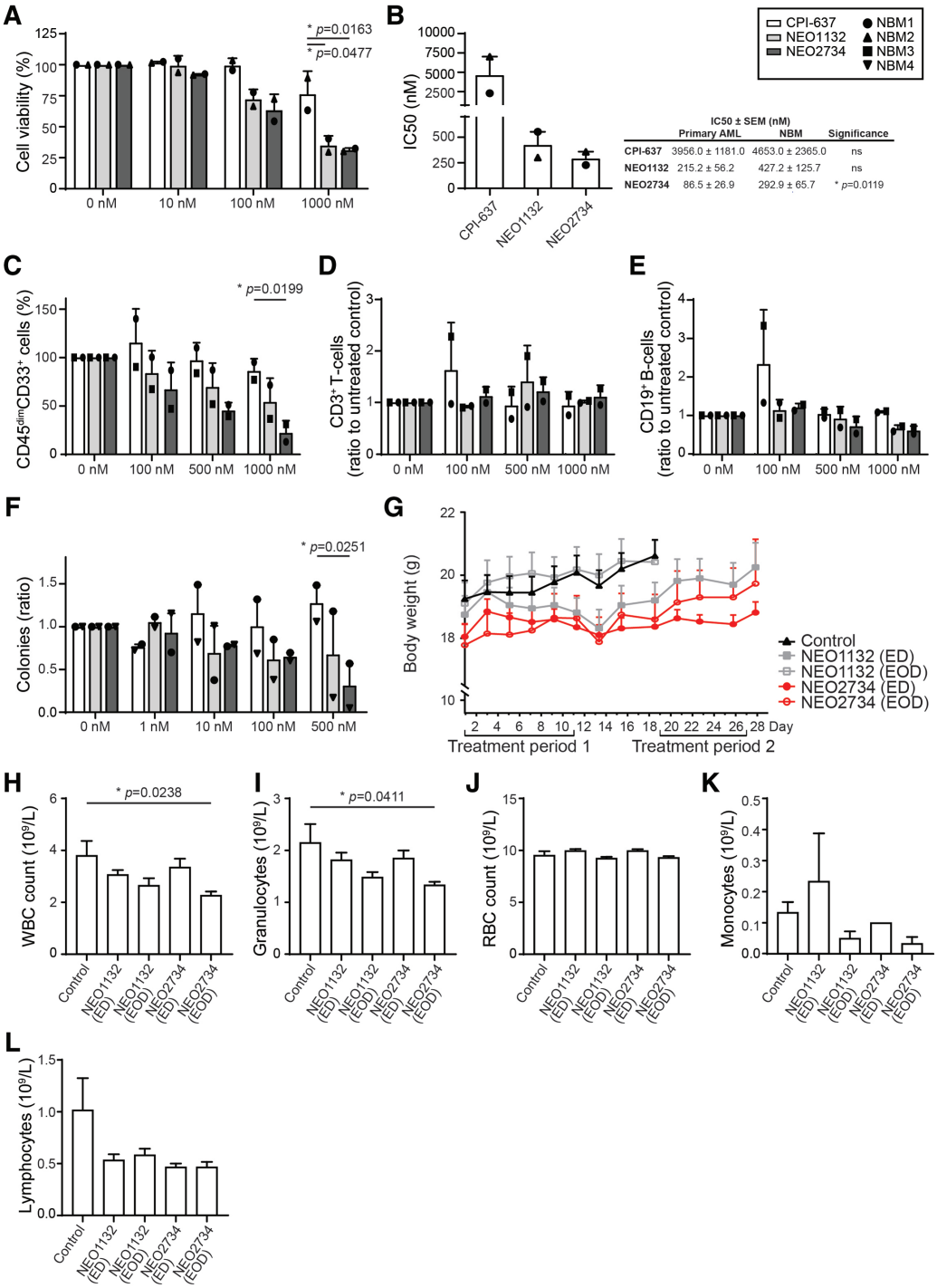

Figure 6. NEO2734 shows higher activity in primary AML cells than in normal hematopoietic bone marrow cells, providing a therapeutic window. For all ex vivo experiments, NBM cells from healthy donors were incubated for 4 d with increasing concentrations of CPI-637, NEO1132, or NEO2734, and *P* values were calculated using a 1 or 2-way ANOVA with post-hoc Tukey's multiple comparison test unless stated otherwise. NBM sample characteristics and IC50 values are shown in Supplemental Digital Content, Table S2, http://links.lww.com/HS/A176. (A and B), Using an MTT assay, cell viability (in triplicate) was quantified in 2 NBM samples, (A) measured as relative absorbance and normalized against untreated controls, and (B) IC50 values (in nM) were calculated. Data are plotted as mean ± SD and *P* values were calculated using a repeated measure 1-way ANOVA with Dunnett's multiple comparison test. For each drug, the mean IC50 ± SEM in primary AML and NBM samples (right table) was compared and *P* values were determined using a Student's *t* test. (C–E), Percentage of (C) viable $CD45^{dim}CD33^{+}$, (D) $CD3^{+}$ T-cells, and (E) $CD19^{+}$ B-cells in 2 NBM samples, measured using flow cytometry, quantified against untreated controls and plotted as mean ± SD. (F), CFU progenitor assays (in duplicate) of 2 NBM samples. After treatment of the NBM cells for 1 wk, cells were incubated in MethoCult without erythropoietin for 8-12 d, and colonies were quantified using a microscope. Data were normalized against untreated controls and depicted as mean ± SEM. *P* values were calculated using a 2-way ANOVA with post-hoc Tukey's multiple comparison test. (G–L), 3×10^6 MV4;11 cells were subcutaneously injected on the right side of the lower flanks of SCID (CB17) mice. 3 wk postinjection, mice were treated once daily (ED) for 11 consecutive days during treatment period 1 (day 1-11) or for 11 times EOD during treatment period 1 (day 1-11) and treatment period 2 (day 19-27) with 10 mg/kg NEO1132, NEO2734, or control (40% PEG400 in distilled water), administered by oral gavage at 5 mL/kg. Schematic overview of the experiment is shown in Figure 2B, left. Data are depicted as mean ± SEM, derived from 6 mice each group. (G), Body weight (in g) of the mice was monitored until study termination. The numbers of (H) WBC, (I) granulocytes, (J) RBC, (K) monocytes, and (L) lymphocytes were measured after treatment period 1 at day 12. AML = acute myeloid leukemia; CFU = colony-forming unit; ED = every day; EOD = every other day; IC50 = the concentration whereby a drug reduces cell viability by half; MTT = 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; NBM = normal bone marrow; PEG400 = 40% polyethylene glycol; RBC = red blood cells; SCID = severe combined immunodeficiency; WBC = white blood cells.

p300 complexes from oncogenic enhancers and downregulation of Myb-dependent gene expression.[35,40] Inhibition of CBP/p300 was found to transcriptionally silence *Myc* in numerous myeloma and leukemia derived cell lines in a manner comparable to downregulation of *Myc* by inhibition of BET.[15,23,50] However, while both BET and CBP/p300 bromodomains regulate *c-Myc*, their transcriptional and phenotypical effects are distinct, suggesting that CBP/p300 inhibition may represent an alternative or complementary therapeutic option to BET bromodomain inhibition and that targeting both may reduce appearance of resistance.

The selective Bcl2 inhibitor venetoclax has demonstrated clinical benefits in AML patients,[51–53] leading to its recent U.S. Food and Drug Administration approval in combination with hypomethylating agents or low-doses cytarabine in unfit adults with newly diagnosed AML.[54] Unfortunately, nonrecurrent changes in oncogenic pathways and gene expression signatures, including overexpression of Bcl2 and MCL1, are associated with resistance to venetoclax.[51] Since we here showed that NEO2734 is able to rapidly modulate *Bcl2* expression in AML cells, combination treatment of NEO2734 might enhance the clinical efficacy of Bcl2 inhibition, preventing or overruling venetoclax resistance in AML.

AML relapse is thought to be caused by leukemia cells contained with self-renewal capacity, referred to as LSC. In the major part of AML cases, these LSC are residing within the $CD34^+CD38^-$ AML cell compartment. We showed that $CD34^+CD38^-$ immunophenotypically defined LSC within AML are sensitive to the NEO drugs, but also that the drugs efficiently eliminated clonogenic leukemic stem/progenitor cells ex vivo. LSC demonstrated to be initially sensitive to BETi.[55] However, in an MLL-AF9–driven AML mouse model long time inhibition of only BET selected for survival of LSC,[4] suggesting that during BETi mono treatment immature leukemia cells will survive.

We showed that every day treatment with NEO1132 and NEO2734 could almost completely prevent growth of MV4;11 AML cells in vivo in a subcutaneous tumor xenograft mouse model. Moreover, using a PDX AML mouse model, we showed that oral treatment with NEO2734 significantly reduced the leukemic burden in mice. Since most AML patients are still treated with a combination of cytarabine and an anthracycline, and persistence of residual leukemia cells (MRD) is the major cause of relapse, we also determined whether the NEO drugs could eliminate residual leukemia cells after treatment with combination chemotherapy. We observed that only NEO2734 could add to in vivo treatment of AML PDX mice with low doses combination chemotherapy consisting of cytarabine and doxorubicin. These results suggest that a combination treatment of chemotherapy and NEO2734 may reduce MRD load, and these results are in concordance with data showing that inhibition of CPB/p300 by I-CBP112 could sensitize leukemic cells to BET inhibition but also to the anthracycline doxorubicin.[13]

NEO2734 reduced the numbers of WBC and granulocytes in the blood of healthy mice when used EOD; however, no effect on healthy blood cells was observed when treatment was applied every day over a shorter time period. The cell viability and reduction in clonogenic capacity after treatment with NEO2734 significantly differed for AML cells and healthy normal bone marrow cells, leaving a therapeutic window allowing efficient elimination of patient AML cells while sparing healthy bone marrow cells. Together, our findings highlight the efficacy of NEO2734 in elimination of primary AML cells, leukemic progenitors and LSC, and in reducing leukemic cell load in combination with chemotherapy treatment in AML patients.

Disclosures

FG has served as consultant to Epigene Therapeutics Inc. All the other authors have no conflicts of interest to disclose.

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