Combinatorial molecule screening identified a novel diterpene and the BET inhibitor CPI-203 as differentiation inducers of primary acute myeloid leukemia cells

Simon Hultmark,[1] Aurélie Baudet,[1,2] Ludwig Schmiderer,[1] Pavan Prabhala,[1] Sara Palma-Tortosa,[3] Carl Sandén,[4] Thoas Fioretos,[4] Rajkumar Sasidharan,[5] Christer Larsson,[6] Sören Lehmann,[7,8] Gunnar Juliusson,[2,9] Fredrik Ek[10#] and Mattias Magnusson[1#]

[1]Division of Molecular Medicine and Gene Therapy, Lund Stem Cell Center, Lund University, Lund, Sweden; [2]Division of Molecular Hematology, Lund Stem Cell Center, Lund University, Lund, Sweden; [3]Laboratory of Stem Cells and Restorative Neurology, Lund Stem Cell Center, Lund University, Lund, Sweden; [4]Division of Clinical Genetics, Department of Laboratory Medicine, Lund University, Lund, Sweden; [5]Solvuu, Inc., New York, NY, USA; [6]Division of Translational Cancer Research, Lund University, Lund, Sweden; [7]Department of Medical Sciences, Uppsala University Hospital, Uppsala, Sweden; [8]Department of Medicine, Karolinska Institute, Stockholm, Sweden; [9]Department of Hematology, Skane University Hospital, Lund, Sweden and [10]Chemical Biology & Therapeutics, Lund University, Lund, Sweden.

[#]FE and MM contributed equally as co-senior authors.

 doi:10.3324/haematol.2020.249177

Received: February 17, 2020.

Accepted: August 25, 2020.

Pre-published: August 27, 2020.

Correspondence: *MATTIAS MAGNUSSON* - mattias.magnusson@med.lu.se

Supplemental Methods

Collection of healthy bone marrow, umbilical cord blood and AML samples

Umbilical cord blood (CB) samples were collected from full-term newborns at maternity wards at Lund, Malmö and Helsingborg Hospitals. Bone marrow aspirates from healthy donors were collected at the Department of Hematology at Skåne University Hospital in Lund, Sweden (dnr 2014/596). CD34+ hematopoietic stem and progenitor cells were enriched from filtered bone marrow aspirates and umbilical cord blood by first isolating mononuclear cells using Lymphoprep™ tubes (Axis Shield Cat# 1019818) followed by enrichment of CD34+ cells by magnetic beads (Miltenyl Biotec Cat# 130-046-704) on LS columns (Miltenyl Biotec Cat# 130-042-401). Primary AML samples were obtained from the Skåne University Hospital in Lund and the Karolinska Institute, Stockholm, Sweden (dnr 826/2004 and 2010/1893-31/2 respectively) in accordance with the Declaration of Helsinki. The mononuclear cells were isolated by density gradient, according to manufacturers' instructions (Ficoll-Hypaque, Axis-Shield Cat# 1114547) before freezing. The generation of the AML-12 PDX sample has been previously described (See AML-26BB)[1]. Patient sample characteristics are presented in Table 1 and Supplemental Table 1.

Cell lines

Cell lines (passage# 3-10 after thawing) MV4-11 (ATCC Cat# CRL-9591), MOLM-13 (DSMZ Cat# ACC 554), OCI-AML3 (DSMZ Cat# ACC 582), Kasumi-1 (DSMZ Cat# ACC 220), HL60 (DSMZ Cat# ACC 3), THP1 (DSMZ Cat# ACC 16) and MM6 (DSMZ Cat# ACC124) were authenticated, species verified and tested for mycoplasma by the supplier. All cell lines were maintained in RPMI with L-Glutamine (Gibco® Cat# 21875-034) supplemented with heat-inactivated FCS (HyClone™ Cat# SV30160.03), Kasumi-1 and OCI-AML3 20%, THP1, MOLM-13 and MM6 15%, and for HL60 and MV4-11 10%. OP9M2 cells (passage# 25-30 from collection) were maintained as previously described in Magnusson et al[2].

Small molecule libraries

The natural product library (513 compounds) was obtained from AnalytiCon Discovery, Potsdam, Germany, as a 10 mM stock solution in DMSO. H4 product# NP-000694. The anti-cancer drug library was obtained from Selleckchem (L3000).

Small molecule screening

Small molecule screening has been described earlier[3]. Briefly, primary hematopoietic cells were plated on a layer of irradiated OP9M2 stromal cells in cell culture medium supplemented with the following cytokines (PeproTech): 50 ng/ml stem cell factor (SCF, Cat# 300-07), 25 ng/ml thrombopoietin (TPO Cat# 300-18), 25 ng/ml FLT3 ligand (FLT3L Cat# 300-19), 25 ng/ml Interleukin-6 (IL6 Cat# 200-06), and 25 ng/ml Interleukin-3 (IL3 Cat# 200-03). After 36 to 48 hours, compounds were added by media exchange to reach a final concentration of 0.5 μM and 10 μM. Positive control of 0.5 μM final concentration etoposide (SIGMA-ALDRICH Cat# E1383) and negative control of matching concentration DMSO (SIGMA-ALDRICH Cat# D5879) were added manually. Cells were kept at 37 °C and 5% CO2. AML cell lines were treated with molecules for 3 days and primary AML samples for 4 days before analysis.

Flow cytometry analysis

Cultured cells (primary AML plus stroma or cells from AML cell lines) were carefully resuspended, transferred to 96 well round-bottom plates (Falcon® Cat# 353077), and spun down for 5 minutes at 350 g in 4 °C. Pellets were re-suspended in 30 μl of wash buffer (PBS, 2% FCS and 1 mM EDTA) supplemented with anti-human monoclonal antibodies against CD11b

(BioLegend Cat# 301342, clone ICRF44, RRID:AB_2563395), CD15 (BioLegend Cat# 323030, clone W6D3, RRID:AB_2561670) and CD64 (BioLegend, Cat# 305013, clone 10.1, RRID:AB_1595539). Cells were analyzed using a FACSCanto II analyzer with a high-throughput unit (Becton Dickinson). Data were analyzed with Flow-Jo software (FlowJo, RRID:SCR_008520).

Apoptosis and necrosis assay
Primary AML-3 cells were precultured on irradiated OP9M2 stroma for two days before treatment with H4 10 µM (see sections: small molecule screening and flow cytometry analysis for more details). The apoptotic and necrotic response was measured according to the instructions in the Apoptosis/ Necrosis Assay Kit (Abcam Cat# ab176750).

May-Grünwald Giemsa staining
Cells were cytospun at 550 rpm for 3 min onto microscope glass slides using a Shandon cytospin 3 centrifuge, allowed to air dry, stained with May-Grünwald dye (Merck Cat# 101424) for 5 min, Giemsa dye for 15 min (Merck Cat# 109204), and rinsed twice by immersion in water for 1 min.

Long-term culture
Primary AML-3 cells were precultured on irradiated OP9M2 stroma for two days before treatment (see sections: small molecule screening and flow cytometry analysis for more details). Every four days, cells were harvested, analyzed for immunophenotype, cell numbers by volumetric cell count using flow cytometry and media changed contining with fresh cytokines and H4 10 µM.,Oo day 12 and 24, cells were transferred to new wells with irradiated OP9M2 stroma.

Cell cycle inhibition
Primary AML-3 cells were precultured on irradiated OP9M2 stroma for five days with Palbociclib (Selleckchem Cat# S1116) 5 µM or DMSO (see sections: small molecule screening and flow cytometry analysis for more details). On day 5, immunostaining, volumetric cell count, and cell cycle analysis (using DAPI and PE Mouse Anti-Ki-67 Set (BioLegend Cat# 556027)) was performed according to manufactures instructions using flow cytometry. Media was changed by aspirating the old media (complete media change) and adding new media containing DMSO, H4 10 µM, Palbociclib 5 µM, or combination of H4 10 µM and Palbociclib 5 µM. On day 9, AML-3 cells were analyzed by immunostaining and volumetric cell count.

***In vitro* treatment, transplantation to NRGS mice and analysis of engraftment**
Primary AML-3 cells were precultured on irradiated OP9M2 stroma for two days followed by four days treatment with DMSO or H4 µM (see section: small molecule screening for more details). Cultured cells from an equal number of wells per treatment group were harvested and pooled, counted with trypan blue, and transplanted to pre-conditioned (600 cGy radiation) NRGS (NRG-SGM3) mice, obtain from Jackson laboratory stock nr: 024099, intravenously through the tail vein. When mice from the DMSO group showed signs of sickness, mice from both groups were sacrificed, hip, femur, tibia, and spleen collected, bones crushed by mortar and pestle, and bone marrow and spleen filtered through 40 µM cell strainers (Fisher Scientific Cat# 22-363-547). Aliquots of cells were spun down, treated with ammonium chloride to lyse red blood cells (2x10 min with PBS wash steps), and spun down pellets were re-suspended in 30 µl of wash buffer (PBS, 2% FCS) supplemented with anti-human monoclonal antibodies against CD45 (BioLegend Cat# 304037, clone HI30, RRID:AB_2562049) and CD33 (BioLegend Cat# 303416, clone WM53, RRID:AB_2561690). Cells were analyzed using a

FACSCanto II analyzer with a high-throughput unit (Becton Dickinson). Data were analyzed with Flow-Jo software (FlowJo, RRID:SCR_008520).

Gene-expression analysis
AML-3 cells were treated with either DMSO or H4 (10 µM) for 16 hours and sorted into RLT cell lysis buffer (QIAGEN Cat# 79216). Gene expression analysis on Human Gene 2.0 ST arrays was performed at the KFB UR Center of Excellence for Fluorescent Bioanalysis at the University of Regensburg. Bioconductor was used for computational analyses and the Limma package was used to obtain differentially expressed genes. Modulated genes were visualized using CellRadar (https://karlssong.github.io/cellradar/) developed by G. Karlsson lab Lund University, Dhapola et al. in preparation, based on gene expression data from normal human hematopoiesis (HemaExplorer, http://servers.binf.ku.dk/bloodspot/) [4]. Gene Ontology (GO) analysis was performed using the DAVID bioinformatics suite (DAVID, RRID:SCR_001881). Ranked lists of genes with a fold change of greater than 1.5 were used as input for GSEA pre-rank. Pre-ranked; all p and FDR q-values were calculated as described previously[5]. Data were deposited in GEO under accession GEO: GSE126439.

Sequencing of mutations in primary AML cells
Primary AML samples were sequenced for mutations by their respective hospitals except for AML-9 and AML-15. DNA was extracted from them using the QIAmp DNA Mini kit (QIAGEN Cat# 51204), and the Center for Translational Genomic in Lund sequenced them for mutations using an illumine myeloid panel with a FLT3-ITD spike-in.

Protein synthesis measurement assay
AML-3 cells were treated for 19 hours with H4 10 µM or DMSO. Protein synthesis rate was measured with the Click-iT™ Plus OPP Alexa Fluor™ 488 Protein Synthesis Assay Kit (Invitrogen Cat# C10428). In brief, cell culture media was replaced by a working solution of cell culture media containing Click-iT® OPP and incubated for 30 min at 37 °C. Cells were washed with PBS and fixed with 3.7% formaldehyde solution for 15 min at room temperature followed by 0.5% Triton® X-100 (SIGMA-ALDRICH Cat# X100) solution for 15 min. Cells were then washed twice with PBS and treated with Click-iT® Plus OPP reaction cocktail for 30 min and kept in the dark in accordance with the kit instructions. Cells were then rinsed with Click-iT® Reaction Rinse Buffer and resuspended in PBS for the detection of Alexa Fluor™ 488 by flow cytometry.

Inhibitor verification screen
THP1 and MM6 were seeded out in 96 half area wells and treated (in duplicates) the same day by 30 minutes pre-treatment with the inhibitors (5 µM, 0.5 µM, 0.05 µM: GF 109203X (TOCRIS Cat# 0741), Quizartinib (TOCRIS Cat# 6788), Trametinib (Selleckchem Cat# S2673)), Ravoxertinib (Selleckchem Cat# S7554) before H4 10 µM was added. Primary AML-3 cells were pre-cultured on OP9M2 stroma for two days before they were treated with 4 µM of GF 109203X (TOCRIS Cat# 0741) with and without H4 10 µM. AML cell lines were treated for three days and AML-3 for four days before analysis with flow cytometry. See sections: small molecule screening and flow cytometry analysis for more details on primary AML screening and flow cytometry analysis.

PKC-GFP translocation assay
Plasmids used for the PKC translocation assay encoded full-length EGFP-tagged classical isoforms PKCα and βII, and novel isoforms δ, and ε, described previously[6]. Transfection was performed on HEK-293T cells using the calcium phosphate method. The day after transfection,

25 000 cells were transferred to ibiTreated µ-Plate 96 Well Black plates (ibidi cat# 89626) and allowed to attach overnight. Cells were treated with H4 at a concentration of 40 µM. Translocation of PKC-GFP from the cytoplasm to the plasma membrane was measured using live imaging (1 picture every 5s for 20 min or 30 min) in a CO2 (5%)/humidity/temperature (37 °C) controlled incubator added onto a Zeiss 780 Confocal Laser Scanning Microscope.

Immunostaining of PKC phosphorylation

Primary AML-3 cells and MOLM-13 were treated for 30 min with H4 10 µM and 40 µM concentration. Cells were cytospun onto polysine adhesion slides (Thermo Scientific Cat# J2800AMNZ), dried, and fixated with ice-cold 100% methanol. Cells were stained with primary anti-PKCα phospho S657 + Y658 (Abcam Cat# ab23513, RRID:AB_2237450) and anti-PKCε phospho S729 (Abcam Cat# ab63387, RRID:AB_1142277) antibodies for 1hr at room temperature followed by the secondary antibody Donkey Anti-Rabbit IgG H&L (Abcam Cat# ab150075, RRID:AB_2752244). Nuclei were stained with Hoechst (Thermo Fisher Scientific) for 5 min and sections mounted with Dabco mounting medium. Confocal images were taken with a maximum intensity projection of 5 µm using a 63X objective (LSM780, Zeiss, Germany).

Real-time semi-quantitative PCR

Total RNA was isolated using RNeasy Micro Kit (QIAGEN Cat# 74004), including on-column DNase treatment. cDNA was generated using random hexamers (Thermo Scientific Cat# SO142) and Superscript III following the manufacturer instructions (Invitrogen Cat# 18080093). *MYC* expression in primary AML cells and leukemic cell lines was measured by Taqman probe Hs00153408_m1 (Thermo Fisher Scientific Cat# 4331182) on a 7900HT Fast Real-Time PCR system (Applied Biosystems). For THP1, cDNA input was normalized between each treatment and time point by measuring cDNA concentration on a NanoDrop (Thermo ScientificTM).

Quantification and statistical analysis

GraphPad Prism 8 software was used to perform statistical analysis (GraphPad Prism, RRID:SCR_002798). Unpaired two-tailed t tests and Welch's t test were used for pairwise comparisons and ordinary one-way ANOVA and multiple comparisons for three or more comparisons.

Data and software availability

The accession number for the gene expression of primary AML-3 cells treated with DMSO and H4 10 µM is GEO: GSE126439.

References

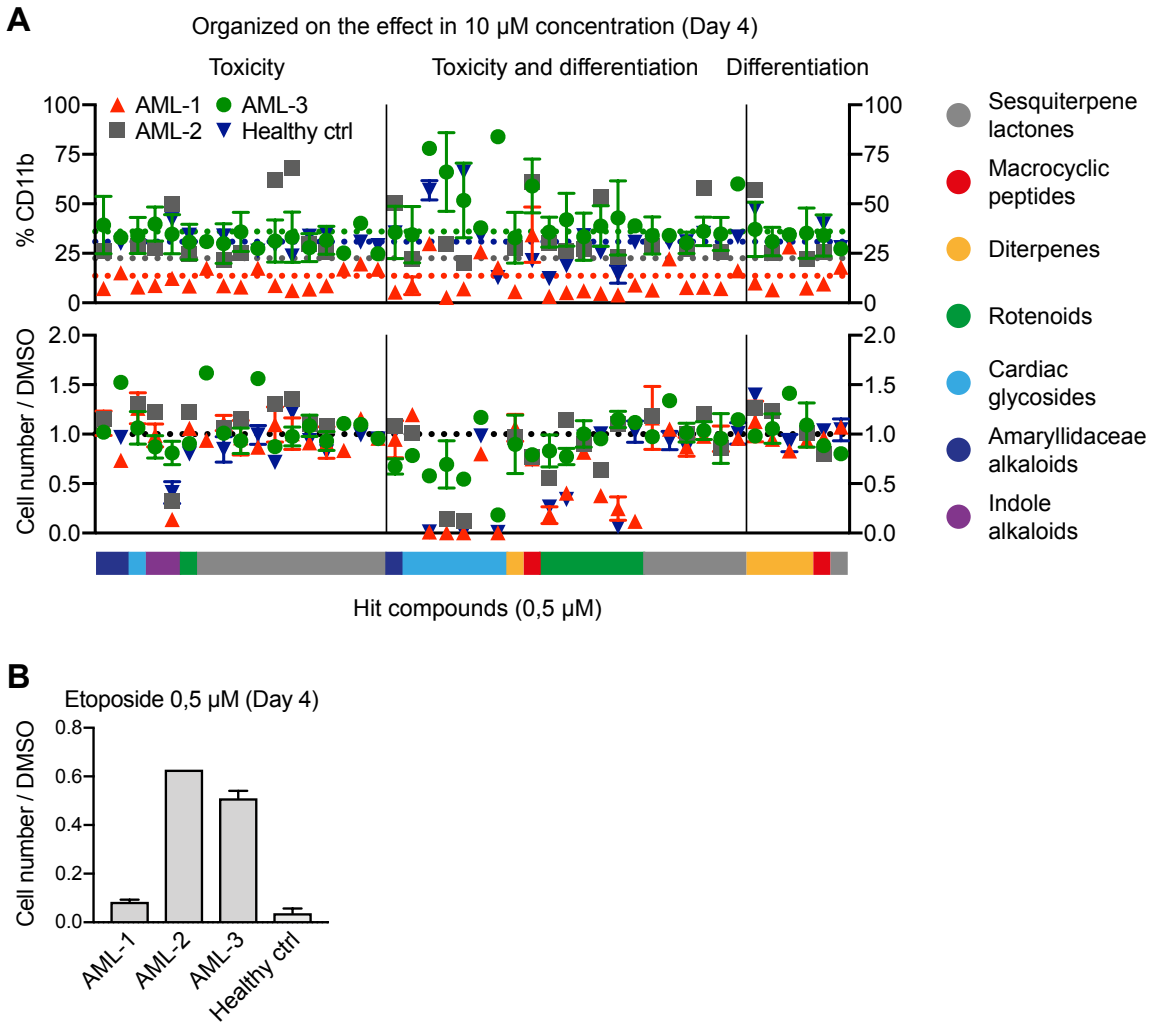
1. Sanden C, Lilljebjorn H, Orsmark Pietras C, et al. Clonal competition within complex evolutionary hierarchies shapes AML over time. Nat Commun. 2020;11(1):579.
2. Magnusson M, Sierra MI, Sasidharan R, et al. Expansion on stromal cells preserves the undifferentiated state of human hematopoietic stem cells despite compromised reconstitution ability. PLoS One. 2013;8(1):e53912.
3. Baudet A, Ek F, Davidsson J, et al. Small molecule screen identifies differentiation-promoting compounds targeting genetically diverse acute myeloid leukaemia. Br J Haematol. 2016;175(2):342-346.
4. Bagger FO, Kinalis S, Rapin N. BloodSpot: a database of healthy and malignant haematopoiesis updated with purified and single cell mRNA sequencing profiles. Nucleic Acids Res. 2019;47(D1):D881-D885.

5. Mootha VK, Lindgren CM, Eriksson KF, et al. PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. Nat Genet. 2003;34(3):267-273.
6. Zeidman R, Lofgren B, Pahlman S, Larsson C. PKCepsilon, via its regulatory domain and independently of its catalytic domain, induces neurite-like processes in neuroblastoma cells. J Cell Biol. 1999;145(4):713-726.

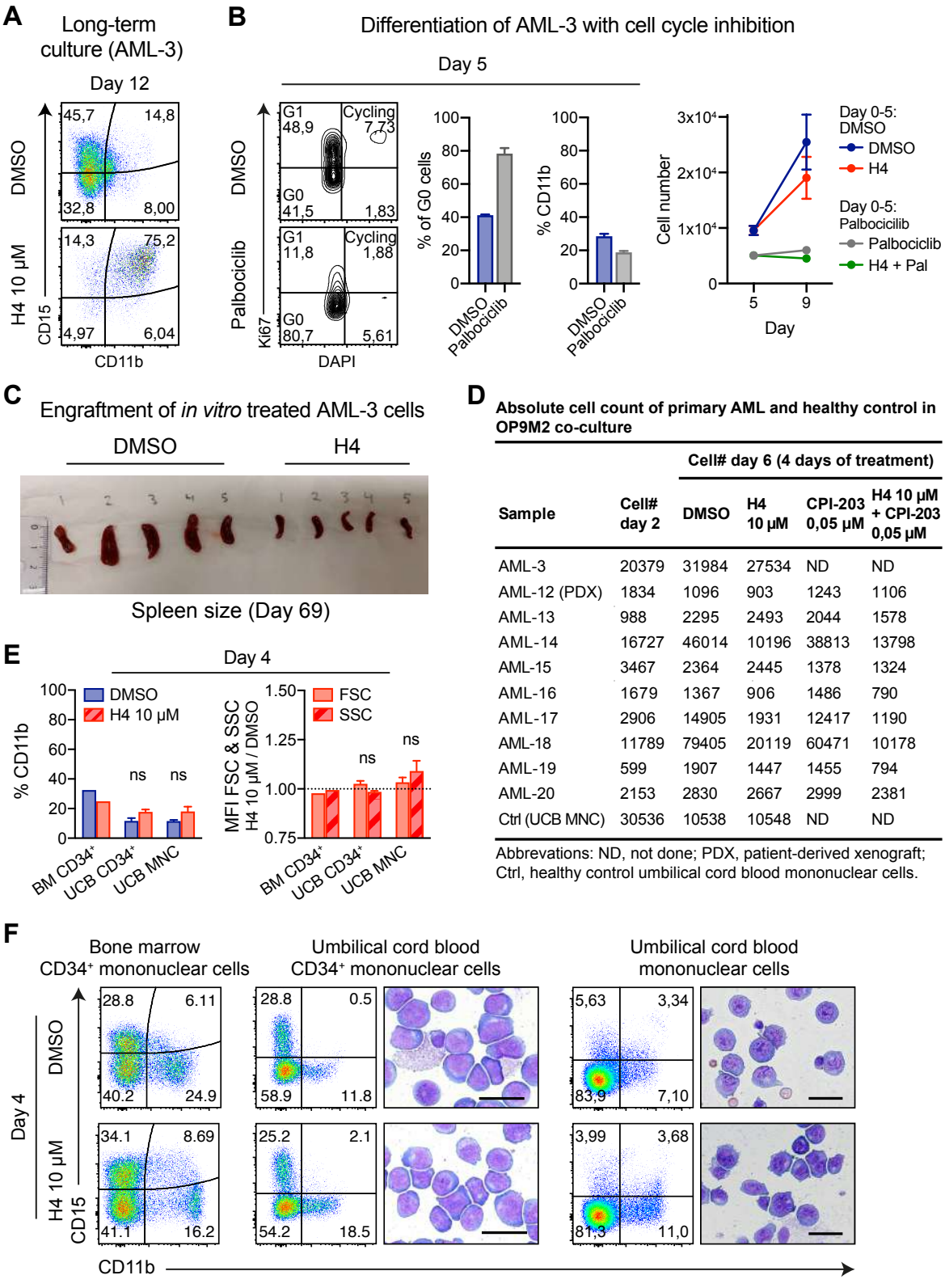
Supplemental Table 1: Cytogenetics of AML samples and cell lines used in the study

Sample	Cytogenetics
AML-1	46,XX
AML-2	45,X,-Y
AML-3	49,XY,+4,?der(7)ins(7;8)(p?;q22q22)ins(7;8)(q?;q22q22),ider(8)(q10)del(8)(q23),?-17,+20,del(21)(q22),+2mar[5]/46,XY[8].
AML-4	46,XX
AML-5	46,XY
AML-6	46,XX,del(3)(q2),add(5)(q3),del(5)(q13q33),del(7)(q22)[6]/45,idem,der(13;17)(q10;q10)[18]/46,XX[1]
AML-7	46,XX,der(7)t(1;7)(q31;q22)[11]/47,XX,idem,+,mar[5](46,XX,idem,add(10)(q13][4]/46,XX[4]
AML-8	46,XX
AML-9	ND
AML-10	46,XX
AML-11	ND
AML-12 (PDX)	del19p
AML-13	Normal
AML-14	del5q, complex
AML-15	t(15;17)
AML-16	47, XX, +11
AML-17	Normal
AML-18	del9
AML-19	Normal
AML-20	ND
MV4-11 [a]	48, XY, t(4;11)(q21;q23), +8, +19
MOLM-13 [b]	Human hyperdiploid karyotype with 4% polyploidy - 51(48-52)<2n>XY, +8, +8, +8, +13, del(8)(p1?p2?), ins(11;9)(q23;p22p23) - resembles published karyotype - sideline with idem, +19 - carries occult insertion effecting KMT2A-MLLT3 (MLL-MLLT3; MLL-AF9) fusion
MM6 [a]	Human flat-moded hypotetraploid karyotype with near-diploid (8%) and polyploid (17%) sidelines - 84-90<4n>XX/XXX, -Y, +6, +7, -12, -13, -13, -16, -16, +2mar, t(9;11)(p22;q23)x2, add(10)(p11)x2, add(12)(q21), del(13)(q13q14)der(13)t(13;14)(p11;q12)x2, der(17)t(13;17)(q21;p11)x2 - first cell line shown to carry t(9;11) characteristic of AML M5
Kasumi-1 [b]	Human hypodiploid karyotype - 45<2n>X, -Y, -9, -13, -16, +3mar, t(8;21)(q22;q22), der(9)t(9;?)(p22;?), der(15)t(?9;15)((?q11;?p11) - carries both partners of 8;21 translocation associated with AML (mainly FAB M2)
OCI-AML3 [b]	Human hyperdiploid karyotype - 48(45-50)<2n>X/XY, +1, +5, +8, der(1)t(1;18)(p11;q11), i(5p), del(13)(q13q21), dup(17)(q21q25) - sideline with r(Y)x1-2 - hemizygous for RB1
HL60 [b]	Human flat-moded hypotetraploid karyotype with hypodiploid sideline and 1.5% polyploidy - 82-88<4n>XX, -X, -X, -8, -8, -16, -17, -17, +18, +22, +2mar, ins(1;8)(p?31;q24hsr)x2, der(5)t(5;17)(q11;q11)x2, add(6)(q27)x2, der(9)del(9)(p13)t(9;14)(q?22;q?22)x2, der(14)t(9;14)(q?22;q?22)x2, der(16)t(16;17(q22;q22)x1-2, add(18)(q21) - sideline with: -2, -5, -15, del(11)(q23.1q23.2) - c-myc amplicons present in der(1) and in both markers
THP1 [b]	Human near-tetraploid karyotype - 94(88-96)<4n>XY/XXY, -Y, +1, +3, +6, +6, -8, -13, -19, -22, -22, +2mar, add(1)(p11), del(1)(q42.2), i(2q), del(6)(p21)x2-4, i(7p), der(9)t(9;11)(p22;q23)i(9)(p10)x2, der(11)t(9;11)(p22;q23)x2, add(12)(q24)x1-2, der(13)t(8;13)(p11;p12), add(?18)(q21) - carries t(9;11) associated with AML M5

Abbrevation: ND, not done; PDX, patient-derived xenograft; [a] Data from ATCC; [b] Data from DSMZ.



Supplemental Figure 1. The natural compound screen on primary patient cells resulted in 45 hits categorized in groups based on molecular structure and biological activity
(A) Hit compounds were categorized into biological response groups and then on molecular structure based on the effect in 10 µM concentration. The baseline expression of CD11b for each sample is indicated with a dotted line in a designated color. Data are shown as mean ± SEM from independent experiments, healthy control (CD34+ bone marrow) n=2, AML-1 n=2-3, AML-2=1, AML-3 n=2-3. (B) Resistance to chemotherapy drug etoposide. The data are shown as mean ± SEM of independent experiments, AML-1, AML-3, and healthy control (CD34+ bone marrow) n=3, and AML-2 n=1.



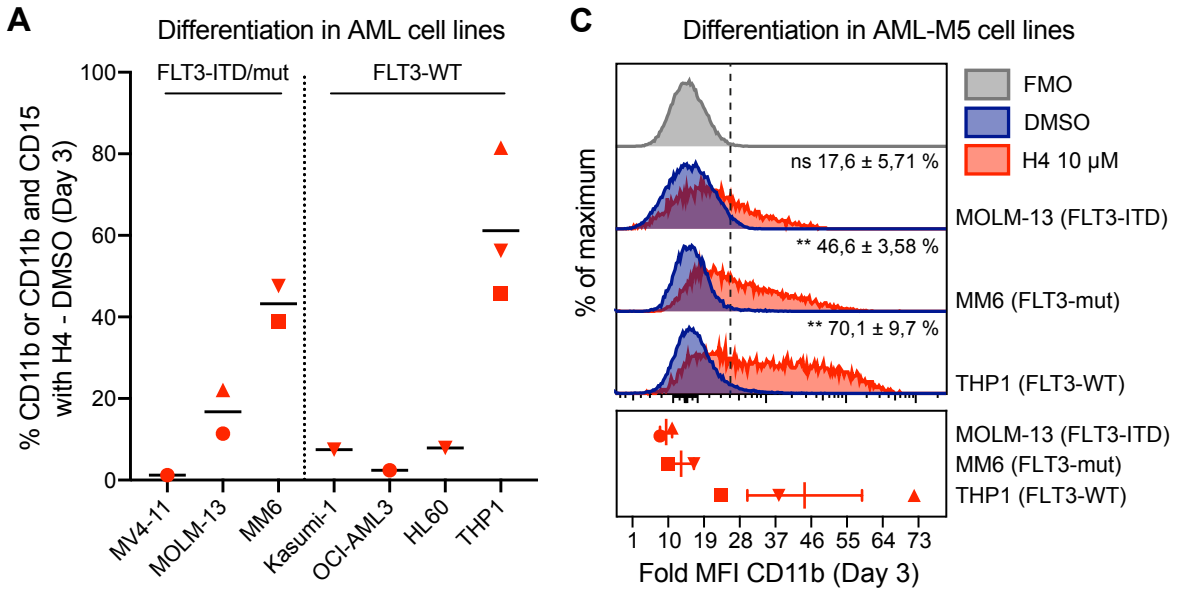
Sample	Cell# day 2	Cell# day 6 (4 days of treatment) DMSO	H4 10 µM	CPI-203 0,05 µM	H4 10 µM + CPI-203 0,05 µM
AML-3	20379	31984	27534	ND	ND
AML-12 (PDX)	1834	1096	903	1243	1106
AML-13	988	2295	2493	2044	1578
AML-14	16727	46014	10196	38813	13798
AML-15	3467	2364	2445	1378	1324
AML-16	1679	1367	906	1486	790
AML-17	2906	14905	1931	12417	1190
AML-18	11789	79405	20119	60471	10178
AML-19	599	1907	1447	1455	794
AML-20	2153	2830	2667	2999	2381
Ctrl (UCB MNC)	30536	10538	10548	ND	ND

Abbrevations: ND, not done; PDX, patient-derived xenograft; Ctrl, healthy control umbilical cord blood mononuclear cells.

Supplemental Figure 2. Response to H4 treatment in primary AML and healthy control cells

(A) Dot-Plots from AML-3 cells cultured for 12 days with H4 10µM and DMSO. (B) Shown is percentage of G0 cells and CD11b in AML-3 after five days pre-treatment with Palbociclib at 5 µM as well as change in cell numbers (graph to the right) after pre-treatment with Palbo-

ciclib (Day 5) or DMSO and after additional four days of treatment (Day 9) with H4 10 μM and/or Palbociclib 5 μM. The data are shown as mean ± SD from technical replicates. (C) Spleens from the NRGS mice 10 weeks post transplantation with primary AML-3 cells treated in vitro for four days with DMSO or H4 (10 μM). (D) Absolut cell count (mean of one experiment) of primary samples on day 2 (pre-cultured for two days) and day 6 (treated for four days).



B

Differentiation response to H4 in AML cell lines

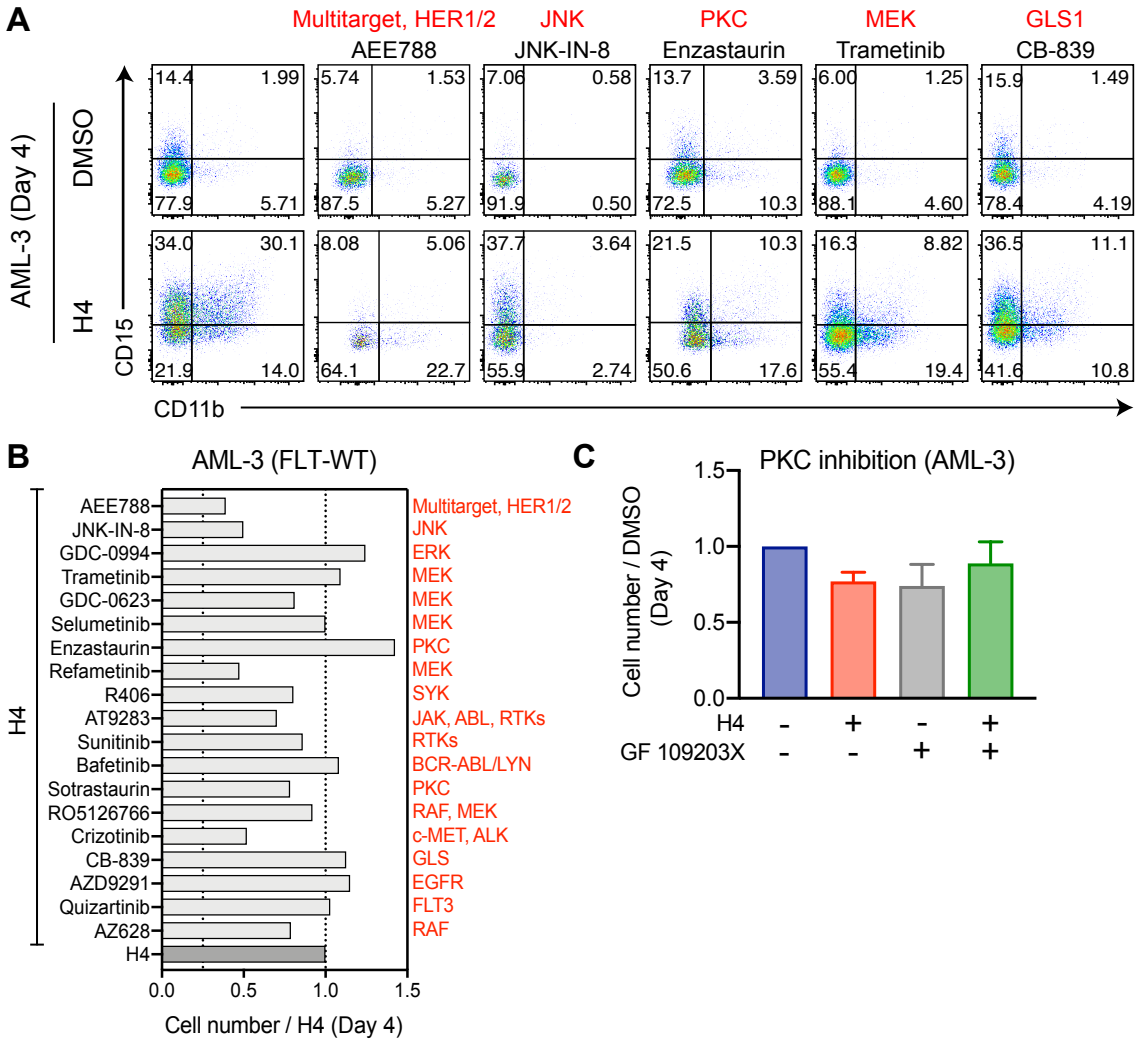
Sample	Subtype	Karyotype	FLT3	Additional Mutations	% CD11b or CD11b and CD15* DMSO	% CD11b or CD11b and CD15* H4 10 µM	Cell# / DMSO	MFI FSC-A & SSC-A / DMSO
MV4-11	MPAL	Complex	ITD	MLL-AF4 [a]	0,5%	1,8%	3,16	1,01 / 0,96
MOLM-13	M5a	Complex	ITD	MLL-AF9 [a]	1,0%	17,6%	0,73	0,96 / 1,30
MM6	M5	Complex	Mut	MLL-AF9 [a]	3,3%	46,6%	0,47	1,01 / 1,11
Kasumi-1	M2	Complex	WT	TP53, Kit, RUNX1-ETO [a]	25,8%*	33,2%*	1,58	0,95 / 0,92
OCI-AML3	M4	Complex	WT	NPM1, DNMT3A	0,6%	3,0%	0,42	0,89 / 1,21
HL60	M2	Complex	WT	TP53, CDKN2A, NRAS [a]	0,2%	8,1%	1,63	0,93 / 0,94
THP1	M5	Complex	WT	CDKN2A, KDM6A, NRAS, MLL-AF9 [a]	6,4%	70,1%	0,18	1,40 / 1,58

[a] From ATCC and DSMZ.

Supplemental Figure 3. Differentiation response to H4 in AML cell lines
(A) H4 triggers the strongest differentiation response in monocytic AML cell lines THP1, MM6 and MOLM-13. The average increase in CD11b is shown with a line and individual experiments with symbols. (B) Representative histogram plots showing the differentiation effect of H4 (10 µM) in M5-AML cell lines. Lower graphs show fold change of mean fluorescent intensity (MFI) of CD11b relative to DMSO. The data are shown as mean ± SEM from independent experiments designated with individual symbols. (C) Differentiation response to H4 in AML cell lines.

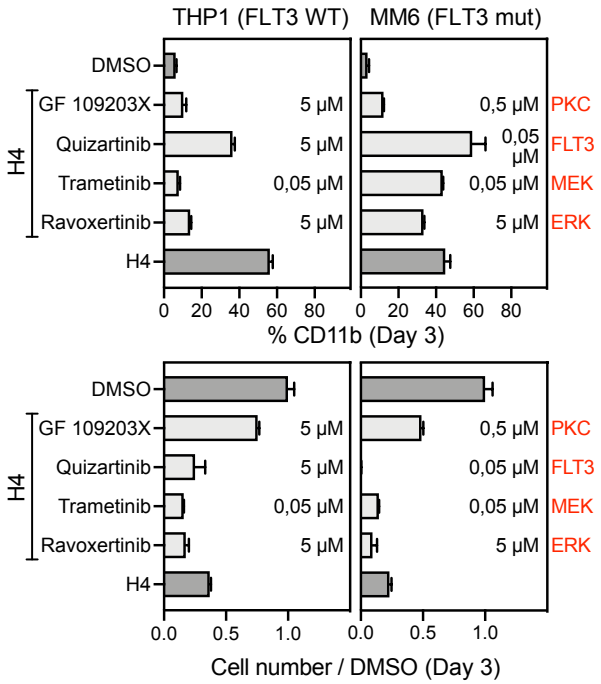
Supplemental Table 2: The most significantly changed GO GESA processes

Gene Ontology GSEA	NES	FDR q-val
Ribosome biogenesis	2.56	<0.0001
Mitochondrial translation	2.28	<0.0001
Translational elongation	2.25	<0.0001
Response to type 1 interferon	2.02	0.0063
Mitochondrial respiratory chain complex assembly	1.99	0.0085
Positive regulation of NF-KB signaling	1.87	0.0426
Regulation of P38MAPK cascade	1.84	0.0523
Leukocyte proliferation	1.77	0.0972



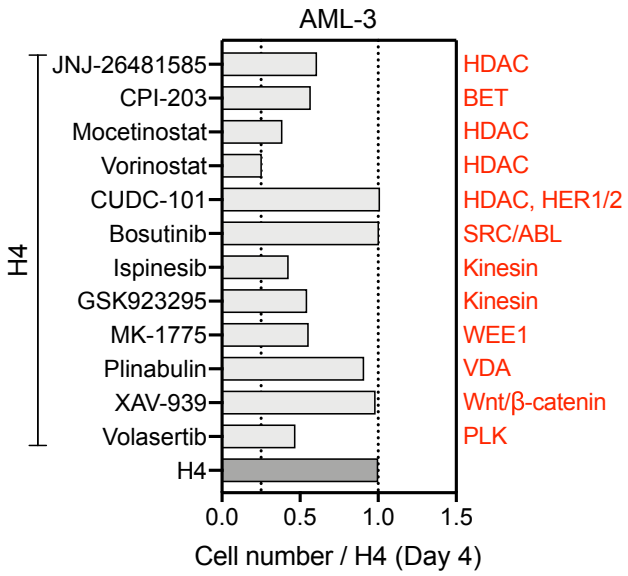
Supplemental Figure 4. Dot-plots and cell number from compound combinations that inhibit H4 induced differentiation in AML-3 cells

(A) Dot-plots from kinases and their targets that inhibit H4 differentiation in AML-3 cells. (B) Cell number compared to H4 10 µM from the most potent inhibitory combinations of H4 differentiation. Cut-off at 0.25 (dotted line) indicates the threshold for which combinations were excluded due to cytotoxicity. (C) Cell number in AML-3 cells treated with PKC inhibitor GF 109203X (4 µM).



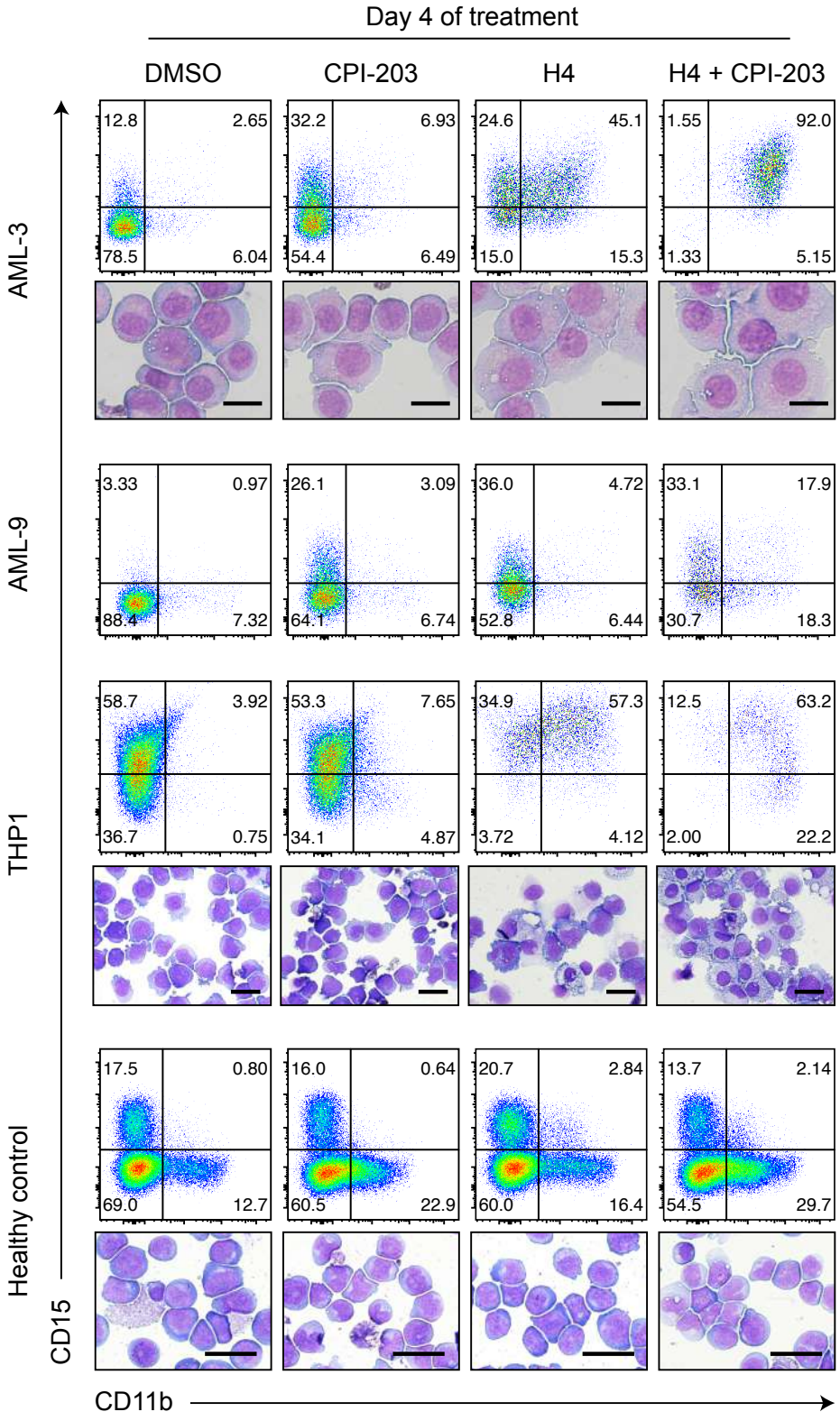
Supplemental Figure 5. Selected combinations that inhibit H4 induced differentiation in AML cell lines

Inhibition of H4 induced differentiation in AML cell lines from selected kinases/targets identified in the AML-3 screen.



Supplemental Figure 6. Cell number from compound combinations that enhance H4 induced differentiation

Cell number from the most potent compound combinations that enhance H4 myeloid differentiation response by upregulation of CD11b. Cut-off at 0.25 (dotted line) indicates the threshold for which combinations were excluded due to cytotoxicity.



Supplemental Figure 7. Representative dot plots and changes in cell morphology after treatment with H4 and CPI-203

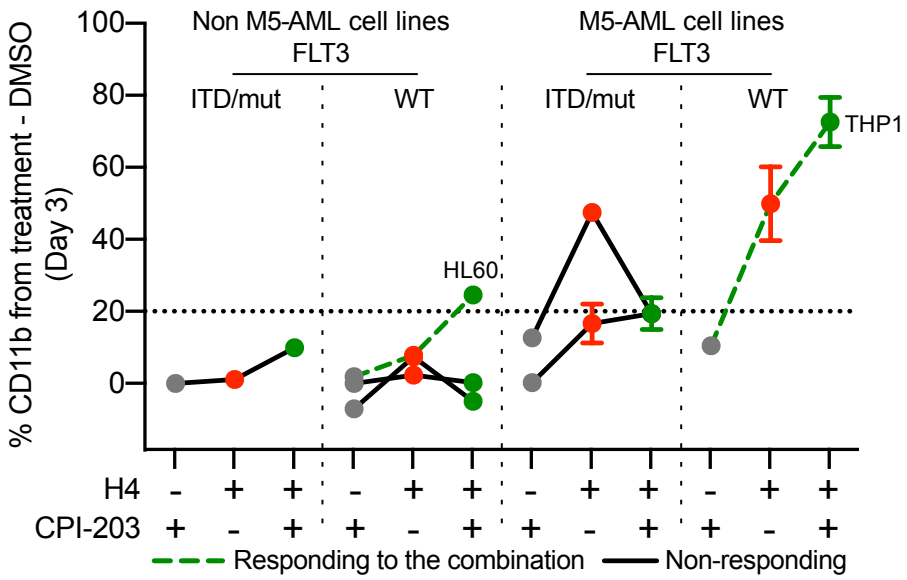
Representative dot-plots and May-Grünwald Giemsa staining (scale bars 10 µm) are shown after treatment with H4 10 µM and CPI-203 0.5 µM as a single treatment and in combination. Healthy control (CD34+ mononuclear cells from umbilical cord blood).

Supplemental Table 3: Response to H4 and CPI-203 combination

Sample	Subtype	FLT3	Parameter	DMSO	H4 10 µM	CPI-203 0,05 µM	H4 10 µM + CPI-203 0,05 µM
AML-1	M4	ITD	% CD11b and CD15	23,2%	23,8%	11,7%	12,9%
			Cell# / DMSO	NA	1,39	1,33	1,50
			MFI FSC-A & SSC-A / DMSO	NA	1,02 / 0,98	0,94 / 0,88	0,93 / 0,88
AML-2	M2	WT	% CD11b and CD15	31,4%	47,4%	23,7%	36,3%
			Cell# / DMSO	NA	0,88	0,66	0,35
			MFI FSC-A & SSC-A / DMSO	NA	0,95 / 1,00	0,82 / 0,94	0,82 / 1,02
AML-3	M5	WT	% CD11b	20,8%	62,9%	19,8%	77,6%
			Cell# / DMSO	NA	0,73	0,94	0,64
			MFI FSC-A & SSC-A / DMSO	NA	1,09 / 1,27	0,93 / 0,84	1,05 / 1,13
AML-4	M1	ITD	% CD11b and CD15	14,6%	19,0%	23,2%	25,4%
			Cell# / DMSO	NA	1,02	1,16	0,85
			MFI FSC-A & SSC-A / DMSO	NA	0,89 / 0,78	0,89 / 0,84	0,87 / 0,81
AML-5	M0	WT	% CD11b and CD15	14,8%	7,6%	17,2%	11,7%
			Cell# / DMSO	NA	0,62	0,97	0,40
			MFI FSC-A & SSC-A / DMSO	NA	0,82 / 0,89	0,93 / 0,96	0,86 / 0,95
AML-6	Unspecified	WT	% CD11b	65,2%	73,1%	82,1%	60,4%
			Cell# / DMSO	NA	0,04	0,34	0,04
			MFI FSC-A & SSC-A / DMSO	NA	0,86 / 1,15	0,84 / 0,89	0,85 / 1,14
AML-7	Unspecified	WT	% CD11b and CD15	28,3%	57,7%	37,2%	56,0%
			Cell# / DMSO	NA	0,64	0,77	0,19
			MFI FSC-A & SSC-A / DMSO	NA	0,91 / 0,77	0,85 / 0,79	0,85 / 0,84
AML-8	M5	ITD	% CD11b	23,1%	34,9%	23,5%	48,0%
			Cell# / DMSO	NA	1,18	0,75	0,79
			MFI FSC-A & SSC-A / DMSO	NA	1,02 / 1,01	0,93 / 0,86	0,94 / 1,00
AML-9	M5	WT	% CD11b and CD15	15,9%	45,0%	39,4%	67,5%
			Cell# / DMSO	NA	0,73	0,93	0,49
			MFI FSC-A & SSC-A / DMSO	NA	0,97 / 1,05	1,00 / 1,00	0,92 / 1,06
AML-10	M5	Mut	% CD11b	28,8%	29,6%	30,9%	34,3%
			Cell# / DMSO	NA	0,69	1,09	0,75
			MFI FSC-A & SSC-A / DMSO	NA	0,96 / 0,90	0,98 / 0,88	0,93 / 0,92
AML-11	M5	Mut	% CD11b	25,4%	35,2%	22,3%	22,4%
			Cell# / DMSO	NA	0,49	0,79	0,41
			MFI FSC-A & SSC-A / DMSO	NA	0,98 / 1,30	1,00 / 1,15	0,92 / 1,36
AML-12 (PDX)	M4	WT	% CD11b	64,3%	75,5%	67,1%	63,6%
			Cell# / DMSO	NA	0,82	1,13	1,01
			MFI FSC-A & SSC-A / DMSO	NA	0,97 / 0,98	0,92 / 0,92	0,91 / 0,91
AML-13	M1	WT	% CD11b	23,3%	25,0%	20,0%	16,7%
			Cell# / DMSO	NA	1,09	0,89	0,69
			MFI FSC-A & SSC-A / DMSO	NA	1,02 / 1,00	0,93 / 0,88	0,96 / 0,95
AML-14	M1	WT	% CD11b	38,8%	64,4%	30,6%	50,2%
			Cell# / DMSO	NA	0,22	0,84	0,30
			MFI FSC-A & SSC-A / DMSO	NA	0,93 / 1,06	0,94 / 0,89	0,87 / 0,93
AML-15	M3	Mut	% CD11b	21,5%	29,3%	27,6%	36,3%
			Cell# / DMSO	NA	1,03	0,58	0,56
			MFI FSC-A & SSC-A / DMSO	NA	1,01 / 1,11	0,96 / 0,95	0,97 / 1,06
AML-16	M0	WT	% CD11b	69,8%	72,4%	70,9%	65,1%
			Cell# / DMSO	NA	0,66	1,09	0,58
			MFI FSC-A & SSC-A / DMSO	NA	0,97 / 1,03	0,93 / 0,88	0,91 / 1,02
AML-17	NOS	ITD	% CD11b	78,5%	78,3%	85,3%	72,4%
			Cell# / DMSO	NA	0,13	0,83	0,08
			MFI FSC-A & SSC-A / DMSO	NA	0,80 / 0,81	0,91 / 0,93	0,78 / 0,77
AML-18	MD	WT	% CD11b	76,3%	86,9%	67,3%	72,4%
			Cell# / DMSO	NA	0,25	0,76	0,13
			MFI FSC-A & SSC-A / DMSO	NA	0,93 / 0,88	0,92 / 0,99	0,82 / 0,84
AML-19	M2	ITD	% CD11b	44,9%	47,6%	51,3%	54,9%
			Cell# / DMSO	NA	0,49	1,06	0,42
			MFI FSC-A & SSC-A / DMSO	NA	1,00 / 0,94	0,94 / 0,88	0,82 / 0,96
AML-20	M1	ITD	% CD11b and CD15	64,5%	72,5%	46,8%	68,0%
			Cell# / DMSO	NA	0,94	1,06	0,84
			MFI FSC-A & SSC-A / DMSO	NA	0,91 / 0,94	0,91 / 0,86	0,89 / 0,99

Healthy control[a]	NA	NA	% CD11b	12,7%	18,3%	19,0%	28,6%
			Cell# / DMSO	NA	0,32	0,64	0,69
			MFI FSC-A & SSC-A / DMSO	NA	1,01 / 0,99	0,90 / 0,81	0,91 / 0,83
MV4-11	MPAL	ITD	% CD11b	0,5%	1,8%	0,6%	10,5%
			Cell# / DMSO	NA	3,16	3,24	1,45
			MFI FSC-A & SSC-A / DMSO	NA	1,01 / 0,96	0,95 / 0,81	0,94 / 0,81
MOLM-13	M5a	ITD	% CD11b	1,0%	17,6%	1,2%	20,4%
			Cell# / DMSO	NA	0,73	0,96	0,36
			MFI FSC-A & SSC-A / DMSO	NA	0,96 / 1,30	0,98 / 0,92	0,92 / 1,44
MM6	M5	Mut	% CD11b	3,3%	46,6%	15,71%	22,45%
			Cell# / DMSO	NA	0,47	1,01	0,20
			MFI FSC-A & SSC-A / DMSO	NA	1,01 / 1,11	0,97 / 0,78	0,93 / 1,08
Kasumi-1	M2	WT	% CD11b and CD15	25,8%	33,2%	18,9%	21,0%
			Cell# / DMSO	NA	1,58	0,27	0,2
			MFI FSC-A & SSC-A / DMSO	NA	0,95 / 0,92	0,87 / 0,95	0,93 / 1,08
OCI-AML3	M4	WT	% CD11b	0,6%	3,0%	0,7%	0,9%
			Cell# / DMSO	NA	0,42	0,40	0,04
			MFI FSC-A & SSC-A / DMSO	NA	0,89 / 1,21	0,76 / 0,77	0,80 / 1,17
HL60	M2	WT	% CD11b	0,2%	8,1%	2,4%	25,1%
			Cell# / DMSO	NA	1,63	1,37	1,23
			MFI FSC-A & SSC-A / DMSO	NA	0,93 / 0,94	0,87 / 0,79	0,80 / 0,80
THP1	M5	WT	% CD11b	6,4%	58,4%	16,2%	78,3%
			Cell# / DMSO	NA	0,30	0,71	0,20
			MFI FSC-A & SSC-A / DMSO	NA	1,31 / 1,45	1,14 / 0,88	1,32 / 1,37

Abbrevation: NA, not applicable; PDX, patient-derived xenograft; [a] $CD34^+$ mononuclear cells from umbilical cord blood.



Supplemental Figure 8. Combination treatment with H4 and CPI-203 in AML cell lines
Combining H4 (10 µM) and the BET inhibitor CPI-203 (0,05 µM) enhances differentiation in THP1 and HL60 cell lines with FLT3 wild-type alleles. None of FLT3-ITD/mutated cell lines responded to the combination. Responders to the combination (green dashed line) had to exceed the 20% cut off (dotted line) in increased CD11b expression to be categorized as responding.

Supplemental Video 1: H4 translocate PKCα-GFP to the plasma membrane in HEK-293T cells.
Cell were treated for 20 min with H4 40 µM. GFP is shown in the left field and brightfield in the right.

Supplemental Video 2: H4 translocate PKCβII-GFP to the plasma membrane in HEK-293T cells.
Cell were treated for 20 min with H4 40 µM. GFP is shown in the left field and brightfield in the right.

Supplemental Video 3: H4 translocate PKCδ-GFP to the plasma membrane in HEK-293T cells.
Cell were treated for 20 min with H4 40 µM. GFP is shown in the left field and brightfield in the right.

Supplemental Video 4: H4 translocate PKCε-GFP to the plasma membrane in HEK-293T cells.
Cell were treated for 30 min with H4 40 µM. GFP is shown in the left field and brightfield in the right.