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

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ABSTRACT

Combination treatment has proven effective for patients with acute promyelocytic leukemia, exemplifying the importance of therapy targeting multiple components of oncogenic regulation for a successful outcome. However, recent studies have shown that the mutational complexity of acute myeloid leukemia (AML) precludes the translation of molecular targeting into clinical success. Here, as a complement to genetic profiling, we used unbiased, combinatorial *in vitro* drug screening to identify pathways that drive AML and to develop personalized combinatorial treatments. First, we screened 513 natural compounds on primary AML cells and identified a novel diterpene (H4) that preferentially induced differentiation of FLT3 wild-type AML, while FLT3-ITD/mutations conferred resistance. The samples responding to H4, displayed increased expression of myeloid markers, a clear decrease in the nuclear-cytoplasmic ratio and the potential of re-activation of the monocytic transcriptional program reducing leukemia propagation *in vivo*. By combinatorial screening using H4 and molecules with defined targets, we demonstrated that H4 induces differentiation by the activation of the protein kinase C (PKC) signaling pathway, and in line with this, activates PKC phosphorylation and translocation of PKC to the cell membrane. Furthermore, the combinatorial screening identified a bromo- and extra-terminal domain (BET) inhibitor that could further improve H4-dependent leukemic differentiation in FLT3 wild-type monocytic AML. These findings illustrate the value of an unbiased, multiplex screening platform for developing combinatorial therapeutic approaches for AML.

Introduction

Acute myeloid leukemia (AML) is the most common form of adult acute leukemia. It is characterized by the accumulation of immature myeloid cells with increased self-renewal, apoptotic resistance and dysfunctional differentiation leading to subsequent infections, anemia and bleeding.[1] Although the majority of patients initially respond to the standard of care (cytotoxic chemotherapy), a substantial number of patients will relapse with a 10-year survival of only 20%

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above 60 years of age.[2] The overall poor survival highlights a dire need for better therapies.

During the past years, several targeted therapies have been developed, designed to uniquely target disease-specific molecular events, such as mutant isocitrate dehydrogenase proteins,[3,4] BCR/ABL[5] and PML/RARAα fusion proteins[6] as well as FLT3-ITD mutations.[7] Targeted therapies hold the promise of being superior to standard chemotherapy with increased specificity, improved efficiency, and reduced toxicity/side-effects.[8] However, single-agent targeted therapies have had only moderate clinical success. For instance, targeting mutant IDH2 or FLT3-ITD AML with specific inhibitors produced initial molecular responses, but these promising early results were frequently followed by treatment resistance and relapse.[3,9,10] Relapse and resistance could be explained by the expansion of malignant clones that were not dependent on the targeted mutation,[11,12] indicating that single-agent targeted therapy would not be sufficient for leukemia clearance.

In line with this, combining arsenic salt with standard all-*trans* retinoic acid therapy for acute promyelocytic leukemia enabled simultaneous activation of both RARα-dependent granulocytic differentiation and the PML-dependent apoptosis/senescence pathways.[13-15] This simultaneous targeting of multiple drivers is now the standard treatment for patients carrying PML-RARα rearrangements and results in disease clearance in more than 90% of these patients who previously had poor prognoses.[6] This demonstrated the need for tailored treatment targeting multiple mechanisms driving the disease. However, next-generation sequencing has shown that most cases of AML have a far more complex mutational landscape with an average of 13 mutations per sample (excluding alterations in noncoding regions)[16] as well as complex karyotypes, highlighting the challenge of translating multiple-targeted therapy into clinical success for the treatment of AML.

As a complement to genetic profiling, unbiased *in vitro* drug screening can be used to reveal pathways that drive disease and identify novel therapeutic targets. By combining drug screening and the multiplex power of flow cytometry, we were also previously able to simultaneously identify compounds with cytotoxicity and differentiation-inducing potential, defining a paradigm for high throughput delineation of potential combination therapies.[17] Furthermore, unlike xenograft models, a short culture (<1 week) can efficiently maintain the polyclonality of AML, making *in vitro* models and high throughput drug screening a powerful tool for developing personalized therapies for AML.[18,19]

Here, to identify patient-specific combinatorial treatment options, we improved our stroma co-culture model for mechanistic and combinatorial screening of primary AML cells.[17] We identified H4, a novel, natural compound that induced differentiation of FLT3 wild-type primary AML samples, while FLT3-ITD/mutated AML were found to be resistant to H4 treatment. Using combinatorial screening, we established that H4 induced differentiation by activation of protein kinase C (PKC) signaling and that, for FLT3 wild-type monocytic AML samples, the effect of H4 was further enhanced by a bromo- and extra-terminal domain (BET) inhibitor, demonstrating the potential of unbiased approaches in the development of personalized treatments.

Methods

The methods are described in detail in the *Online Supplementary Material*.

Collection of healthy bone marrow, umbilical cord blood, and acute myeloid leukemia samples

Samples were collected in Swedish hospitals, in accordance with the Declaration of Helsinki and with the approval of the local ethical committees (dnr 2014/596, 826/2004 and 2010/1893-31/2). Mononuclear cells from umbilical cord blood and healthy bone marrow were enriched for $CD34^+$ cells and primary AML samples for mononuclear cells before freezing. The characteristics of the patients' samples are presented in Table 1 and *Online Supplementary Table S1*.

Small molecule libraries

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

Small molecule screening

Small molecule screening has been previously described.[17] Briefly, primary hematopoietic cells were plated on irradiated OP9M2 stromal cells in medium supplemented with human cytokines (stem cell factor, thrombopoietin, FLT3, interleukin 6, and interleukin 3). After 36 to 48 h, compounds were added at the final concentration of 0.5 μM and 10 μM. AML cell lines were treated for 3 days and primary AML samples for 4 days before analysis.

Flow cytometry analysis

Cultured cells were transferred to 96-well round-bottomed plates, washed, and then stained with anti-human monoclonal antibodies (CD11b, CD15, CD64). Cells were analyzed using a FACSCanto II analyzer with a high-throughput unit (Becton Dickinson).

Long-term culture

Every 4 days, the medium was changed completely by adding new medium containing H4 10 μM or DMSO. Cells were immunophenotyped and a volumetric cell count performed using flow cytometry.

Cell cycle inhibition

AML-3 cells were pre-treated for 5 days with palbociclib 5 μM or DMSO before H4 was added with a complete change of medium. On days 5 and 9, cells were anlyzed by immunostaining, volumetric cell count, and cell cycle status using DAPI and PE Mouse Anti-Ki-67 Set (BioLegend).

In vitro treatment, transplantation into NRGS mice and analysis of engraftment

Cultured cells from an equal number of wells per treatment group were transplanted into pre-conditioned (600 cGy radiation) NRGS mice. When mice from the DMSO group showed signs of sickness, mice from both groups were sacrificed. Spleen and bone morrow were collected, stained for human CD45 and CD33 (BioLegend) and analyzed by flow cytometry.

Protein kinase C translocation assay

HEK-293 cells were transfected with plasmids encoding EGFP-tagged full-length PKC[20] and treated with 40 μM of H4. Translocation of PKC-EGFP from the cytoplasm to the plasma membrane was measured using live imaging on a Zeiss 780 confocal laser scanning microscope.

Table 1. Primary acute myeloid leukemia samples and their response to H4.

Sample	Subtype	Relapse	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
AML-1	M4	Yes	Normal	ITD	DNMT3A, NPM1	23.2%*	23.8%*	1.39	1.02 / 0.98
AML-2	M2	No	Normal	WT	NPM1, TET2	31.4%*	47.4%*	0.88	0.95 / 1.00
AML-3	M5	Yes	Complex	WT	TP53	20.8%	62.9%	0.73	1.09 / 1.27
AML-4	M1	No	Normal	ITD	DNMT3A, NPM1	14.6%*	19.0%*	1.02	0.89 / 0.78
AML-5	M0	No	Normal	WT	DNMT3A, IDH1, NOTCH1, SRSF2	14.8%*	7.6%*	0.62	0.82 / 0.89
AML-6	Unspecified	No	Complex	WT	TP53, RUNX1, NRAS	65.2%	73.1%	0.04	0,.86 / 1.15
AML-7	Unspecified	No	Complex	WT	NPM1, IDH2, RUNX1, PHF6	28.3%*	57.7%*	0.64	0.91 / 0.77
AML-8	M5	No	Normal	ITD	DNMT3A, NPM1, SMC3, PTEN	23.1%	34.9%	1.18	1.02 / 1.01
AML-9	M5	NK	NK	WT	NI	15.9%*	45.0%*	0.73	0.97 / 1.05
AML-10	M5	NK	Normal	Mut	DNMT3A, NPM1, TET2	28.8%	29.6%	0.69	0.96 / 0.90
AML-11	M5	No	NK	Mut	CEBPA	25.4%	35.2%	0.49	0.98 / 1.30
AML-12 (PDX)	M4	No	Normal	WT	NPM1, DNMT3A, IDH2	64.3%	75.5%	0.82	0.97 / 0.98
AML-13	M1	NK	Normal	WT	NI	22.3%	25.0%	1.09	1.02 / 1.00
AML-14	M1	NK	Complex	WT	DNMT3A	38.8%	64.4%	0.22	0.93 / 1.06
AML-15	M3	NK	Normal	Mut	NPM1, CEBPA, PML-RARA	21.5%	29.3%	1.03	1.01 / 1.11
AML-16	M0	Yes	Normal	WT	TP53, DNMT3A, STAG2	69.8%	72.4%	0.66	0.97 / 1.03
AML-17	NOS	NK	Normal	ITD	TET2, KRAS, WT1	78.5%	78.3%	0.13	0.80 / 0.81
AML-18	MD	NK	Normal	WT	NPM1, CEBPA	76.3%	86.9%	0.25	0.93 / 0.88
AML-19	M2	NK	Normal	ITD	NI	44.8%	47.6%	0.48	1.00 / 0.94
AML-20	M1	No	NK	ITD	NI	64.5%*	72.5%*	0.94	0.91 / 0.94
Ctrl UCB (MNC)	NA	NA	NA	NA	NA	11.6%	18.0%	1.06	1.03 / 1.09
Ctrl UCB ($CD34^+$ MNC)	NA	NA	NA	NA	NA	12.7%	18.3%	0.32	1.01 / 0.99
Ctrl BM ($CD34^+$ MNC)	NA	NA	NA	NA	NA	32.5%	24.9%	1.18	1.98 / 0.99

AML: acute myeloid leukemia; DMSO: dimethylsulfoxide; MFI: mean fluorescent intensity; SSC: side scatter; FSC: forward scatter; ITD: internal tandem duplication; WT: wild-type; Mut: mutated; NK: not known; NI: not identified; NA: not applicable; PDX: patient-derived xenograft; Ctrl: healthy control; UCB: umbilical cord blood; MNC: mononuclear cells; BM: bone marrow.

Immunostaining of protein kinase C phosphorylation

Cells were treated for 30 min with H4 10 μM or 40 μM and then cytospun onto polysine adhesion slides and stained with primary anti-PKCα phospho S657 + Y658 and anti-PKCε phospho S729 (Abcam) antibodies for 1 h at room temperature followed by secondary anti-rabbit antibody (Abcam). Nuclei were stained with Hoechst 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).

Results

Small natural product screening identified compounds with toxic or myeloid differentiation potential on acute myeloid leukemia cells

To identify novel targets for combinatorial treatment, we performed a primary small molecule screen using 513 purified natural products selected for their structural diversity and the potential for further drug refinement. We screened three primary AML samples with different subtypes and genotypes (AML-1 to -3; Table 1 and *Online Supplementary Table S1*) as well as healthy bone marrow cells using our co-culture platform (Figure 1A).[17,21] The drug response was evaluated 4 days after treatment at two concentrations (0.5 μM and 10.0 μM) using flow cytometry and compared to control (DMSO-treated) AML cells as well as to healthy bone marrow cells. We identified 44 compounds with a biological effect which we divided into three categories: cytotoxic (reduction of volumetric cell count by at least 50%), cytotoxic and myeloid differentiation potential (increase in CD11b expression) or myeloid differentiation potential without cytotoxicity (Figure 1B, C and *Online Supplementary Figure S1A*). Within each category, we identified molecular groups with similar structures. Molecules that belonged to amaryllidaceae and indole alkaloids were mainly toxic, while rotenoids and cardiac glycosidases were mainly toxic and differentiating (Figure 1C). Finally, diterpenes mainly demonstrated the potential to induce differentiation of primary leukemic cells without being toxic to healthy bone marrow cells. This group was thus selected for further characterization as a potential therapeutic option with fewer side effects than chemotherapy (Figure 1C).

H4 is a diterpene with the potential to differentiate primary FLT3 wild-type acute myeloid leukemia cells

Next, we selected the most potent diterpene, a novel jatrophane called H4, which was originally isolated from the plant *Euphorbia paralias* (Figure 2A).[22] In the initial screen, H4

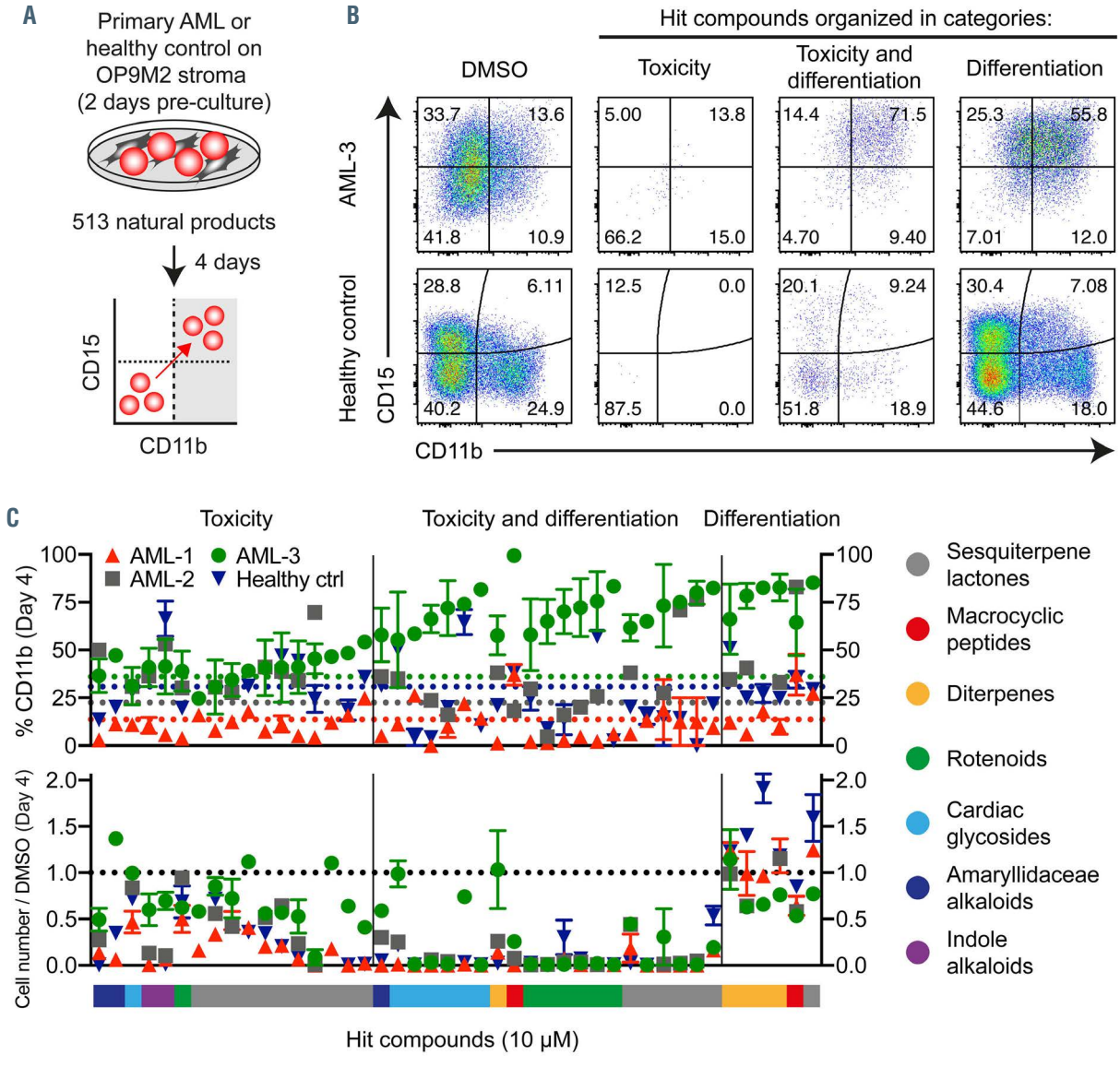


Figure 1. Screening of small natural products identifies compounds that are toxic to acute myeloid leukemia cells or have myeloid differentiation potential. (A) Primary acute myeloid leukemia (AML) cells and control cells (CD34⁺ bone marrow cells) from healthy volunteers were cultured for 2 days on irradiated stromal cells and then treated with 513 natural product compounds at the concentrations of 10 µM and 0.5 µM. Four days later, cells were collected and assayed by flow cytometry to measure their viability (volumetric cell count) and myeloid differentiation (CD11b and CD15 expression). (B) An illustrative example of a hit compound from each of the three categories: toxicity, toxicity and differentiation, and differentiation. (C) Hit compounds were categorized into biological response groups and then on molecular structure. In the 'differentiation' category: CD11b >55% (AML-3) and cell number/DMSO ratio >0.5 (all samples). In the 'toxicity and differentiation' category: CD11b >55% (AML-3) and cell number/DMSO ratio <0.5 (any sample). In the 'toxicity' category: CD11b <55% (AML-3) and cell number/DMSO ratio <0.5 (any samples). The baseline expression of CD11b for each sample is indicated with a dotted line in a designated color. Data are shown as mean ± standard error of mean from independent experiments, healthy control n=2, AML-1 n=2-3, AML-2 n=1, AML-3 n=2-3. DMSO: dimethylsulfoxide, Ctrl: control.

was specifically efficient in differentiating the AML-3 sample, which was isolated from a patient with chemotherapy-resistant AML (Table 1 and *Online Supplementary Figure S1B*). A dose-response experiment demonstrated that 10 µM was the optimal concentration to induce myeloid differentiation (CD11b⁺ cells: DMSO, 20%, H4, 65%) with May-Grünwald Giemsa staining showing an apparent reduction in nuclear to cytoplasmic ratio, supporting the induction of myeloid differentiation (Figure 2B). The relative decrease to DMSO in cell number at the 10 µM concentration on day 4 was not due to increased apoptosis or necrosis throughout the 4-day culture (Figure 2C). In line with this, absolute cell count during long-term culture (4 weeks) of AML-3 together with H4 demonstrated an initially increased proliferation of the treated cells followed by a sharp decline in cell number after 8 days in culture, while the control cells exposed to DMSO continued to expand (Figure 2D). In addition, the proportion of CD11b⁺ cells increased with continuous treatment with H4, further supporting that H4 induces differentiation of AML cells (*Online Supplementary Figure S2A*). However, since leukemia-propagating cells (leukemia stem cells) are known to be quiescent and thought to be resistant to chemotherapy, we next assessed the potential of H4 to induce differentiation of non-cycling cells, G_0-arrested cells, by pre-treating AML-3 cells with the cell cycle inhibitor palbociclib[23] for 5 days (*Online Supplementary Figure S2B*). Even with the continuous addition of palbociclib together with H4, the upregulation

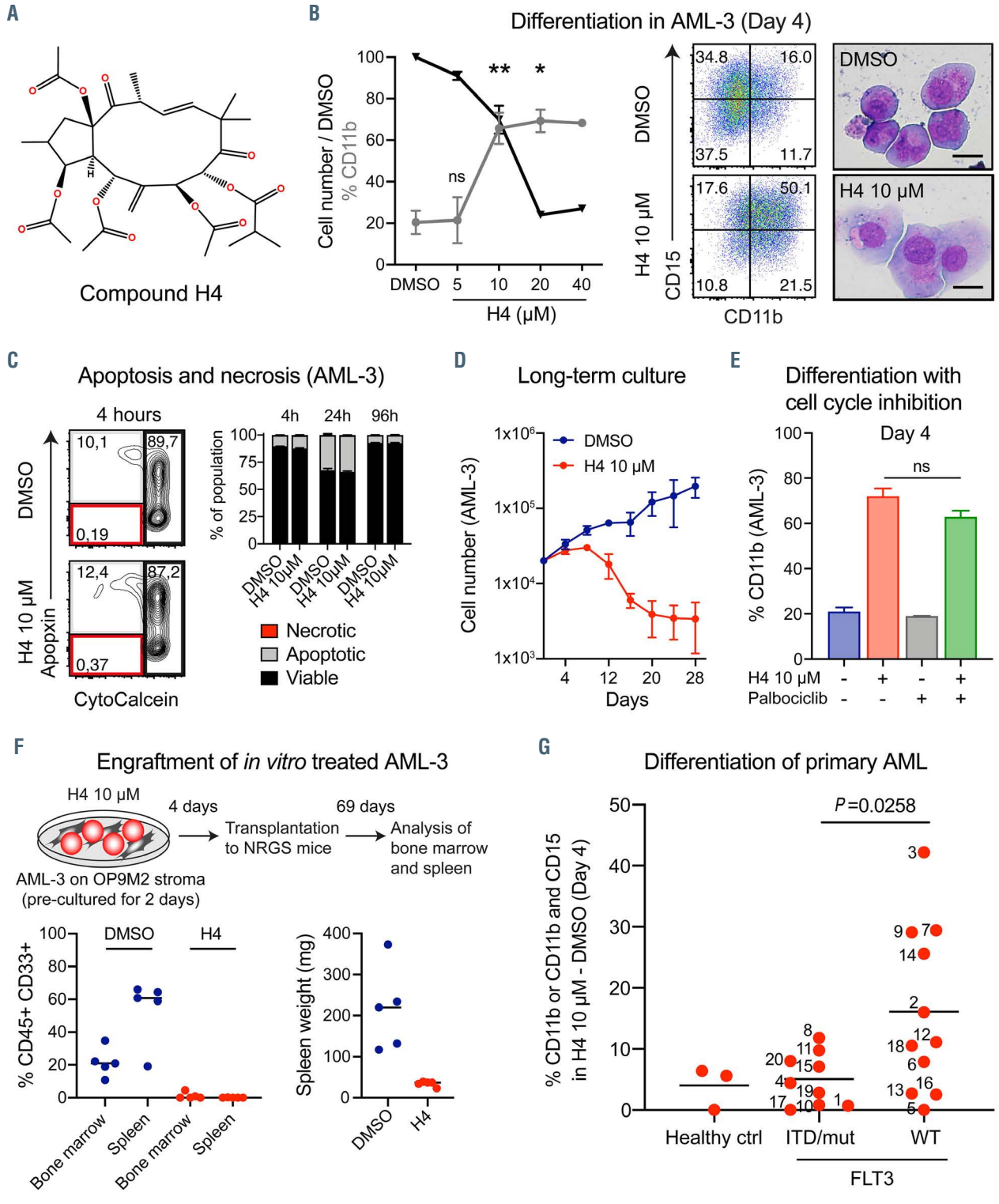
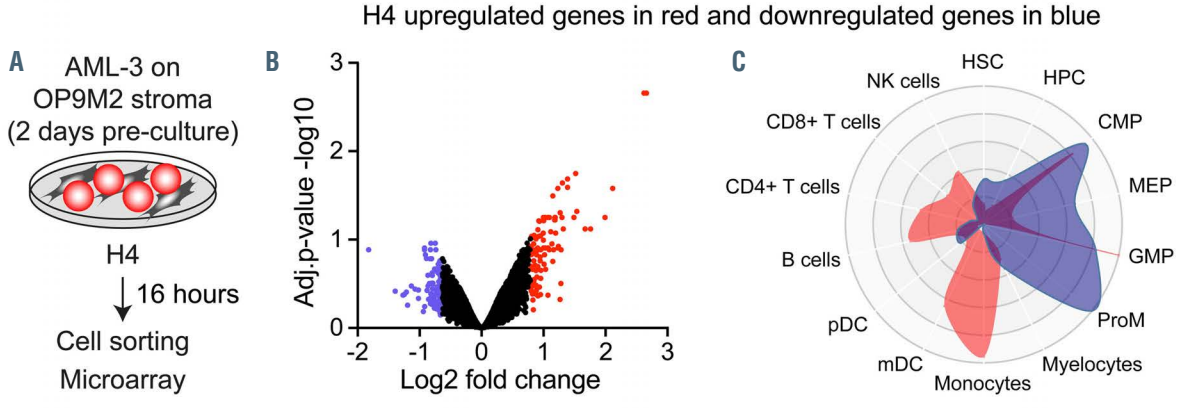


Figure 2. H4 is a jatrophane with differentiation potential for primary FLT3 wild-type acute myeloid leukemia cells. (A) Molecular structure of the natural compound H4. (B) The optimal concentration of H4 was 10 μM for primary AML-3 cells. The data are shown as mean ± standard error of mean from independent experiments, DMSO (n=5), H4 5 μM (n=2), H4 10 μM (n=6), H4 20 μM (n=2), H4 40 μM (n=1) (**P<0.01, *P<0.05, ns not significant, using one-way analysis of variance). On the right, representative change in cell morphology and myeloid differentiation markers after treatment with H4 10 μM. Scale bars represent 10 μm. (C) No detected increase in the percentage of viable, apoptotic and necrotic AML-3 cells after treatment with H4 μM. The data are shown as mean ± standard deviation (SD) of technical replicates. (D) Long-term culture of AML-3 cells on OP9M2 stroma cells. The data are shown as mean ± SD of technical replicates. (E) H4 induced differentiation of AML-3 cells that had been pretreated for 5 days with the CDK4/6 cell cycle inhibitor palbociclib, after 4-day combination treatment with the CDK4/6 cell cycle inhibitor palbociclib 5 μM. The data are shown as mean ± SD of technical replicates. (ns not significant, using an unpaired two-tailed *t*-test). (F) Engraftment levels of *in vitro* treated human AML3 cells transplanted into mice. (G) H4 triggered the strongest differentiation response in FLT3 wild-type acute myeloid leukemia samples. The data are shown with the average response from each sample labeled with their respective sample number for easier identification. (significance test using an unpaired two-tailed *t*-test with the Welch correction). DMSO: dimethylsulfoxide; AML: acute myeloid leukemia; Ctrl: control; ITD: internal tandem duplication; mut: mutated.

of CD11b expression was similar to that with H4 alone, suggesting that quiescent cells can also be differentiated by H4 (Figure 2E). Finally, to further evaluate the effect on leukemia stem cell function, AML-3 cells were treated for 4 days *in vitro* and transplanted into irradiated NRGS mice (DMSO control, 34,200 cells per mouse *vs.* H4-treated, 18,800 cells per mouse, 97% viability in both groups). Ten weeks after transplantation, the control mice had to be



D

H4 induced states and processes

Hallmark GSEA	NES	FDR q-val
MYC TARGETS V2	2.46	<0.0001
INTERFERON ALPHA RESPONSE	2.18	<0.0001
TNFA SIGNALING VIA NFKB	1.98	<0.0001
OXIDATIVE PHOSPHORYLATION	1.98	<0.0001
IL2 STAT5 SIGNALING	1.91	<0.0001
MYC TARGETS V1	1.86	<0.0001
INTERFERON GAMMA RESPONSE	1.82	0.0003
UNFOLDED PROTEIN RESPONSE	1.80	0.0009
INFLAMMATORY RESPONSE	1.59	0.0142
UV RESPONSE UP	1.58	0.0165
REACTIVE OXIGEN SPECIES PATHWAY	1.54	0.0220

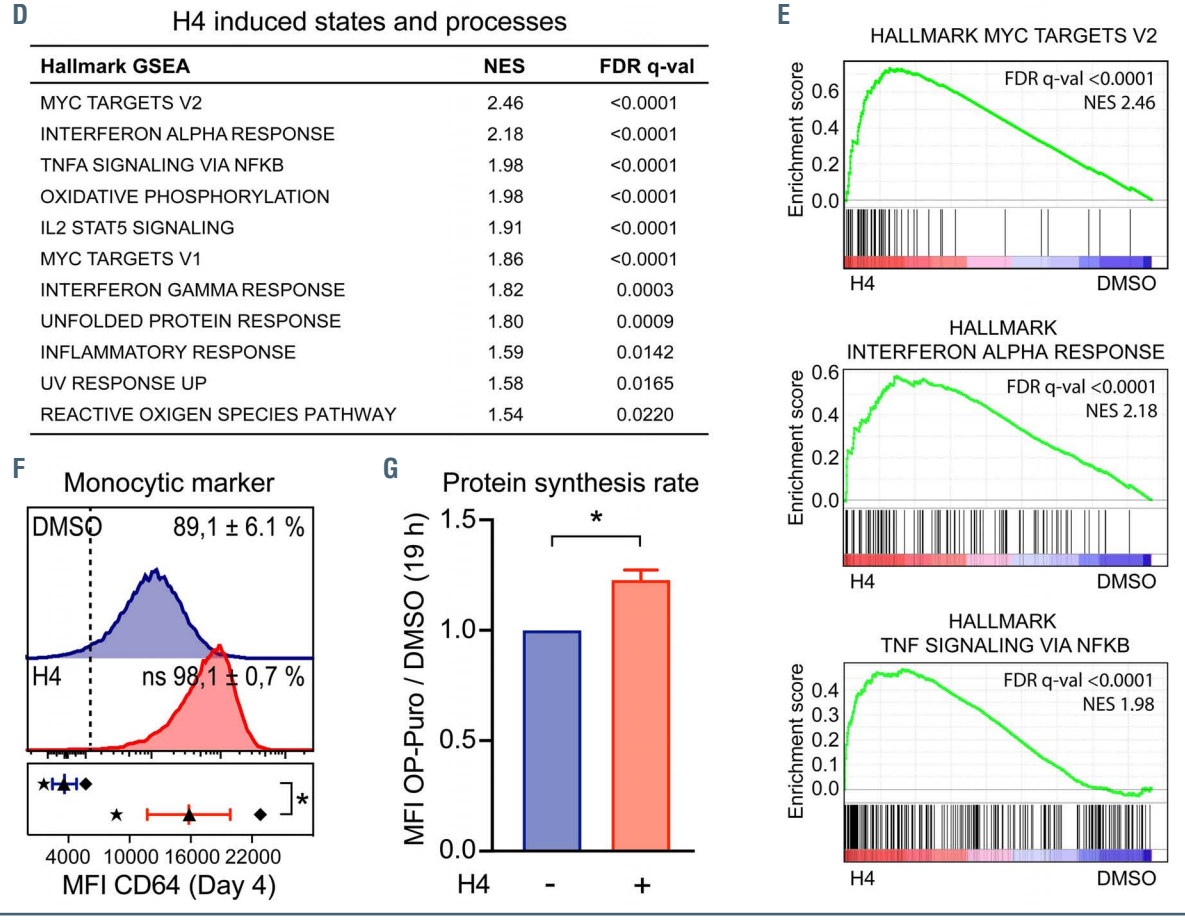


Figure 3. H4 treatment activates the gene expression program of monocytic differentiation. (A) Gene set expression analysis (GSEA) of AML-3 cells after 16 h of treatment with H4 10 μM. (B) The 100 most upregulated genes in red and the 100 most downregulated genes in blue after treatment with H4 compared to dimethylsulfoxide. (C) H4 treatment triggered a gene expression program of monocytic differentiation with the 100 most upregulated genes associated with monocytic lineage in red and the 100 most downregulated genes associated with myeloid progenitors in blue. Visualized using CellRadar software (developed by G. Karlsson's Laboratory, Lund University; unpublished data from Dhapola *et al.*) based on gene expression data from normal human hematopoiesis (HemaExplorer) found at the BloodSpot database.[24] (D) H4 treatment led to changes in gene expression associated with MYC targets, inflammatory response, and reactive oxygen species pathway. (E) The three most significant Hallmark GSEA plots after treatment with H4. (F) H4 10 μM triggered the upregulation of the monocytic CD64 marker in AML-3 cells after 4 days of treatment. The data shown are mean ± standard error of mean (SEM) from three independent experiments designated with individual symbols in the lower graph (*P<0.05, ns not significant, using an unpaired two-tailed t-test). (G) The translation rate was increased by H4 10 μM treatment of AML-3 cells (measured at 19 h of treatment). The data are shown as mean ± SEM of two independent experiments (*P<0.05, using an unpaired two-tailed t-test). HSC: hematopoietic stem cells; HPC: hematopoietic progenitor cells; CMP: common myeloid progenitors; MEP: megakaryocyte-erythroid progenitors; GMP: granulocyte-macrophage progenitors; ProM: promyelocytes; mDC: myeloid dendritic cells; pDC: plasmacytoid dendritic cells; NK: natural killer; NES: normalized enrichment score; FDR: false discovery rate; DMSO: dimethylsulfoxide; MFI: mean fluorescent intensity.

euthanized because of the development of AML, while only two out of five mice transplanted with H4-treated cells had leukemic cells, at barely detectable levels of engraftment (>0.02% engraftment in spleen and bone marrow) (Figure 2F and *Online Supplementary Figure S2C*). These results demonstrate that H4 can induce differentiation of primary leukemia-propagating cells.

To further investigate the leukemia-differentiating potential of H4, we next assessed the response of an additional 17 (20 in total) primary samples and seven AML cell lines. Four of the 20 primary samples and two of the cell lines responded with increased CD11b expression (> 20% increase) (Figure 2G, Table 1, *Online Supplementary Table S1*, *Online Supplementary Figure S3A, B*), still without any major toxicity recorded for the tested samples (Table 1, *Online Supplementary Figures S2D* and *S3B*). For healthy control cells ($CD34^+$ bone marrow cells, $CD34^+$ peripheral mononuclear cells and peripheral mononuclear cells), only a small increase in CD11b was detected, without altered cell morphology, size, or granularity (Figure 2G, Table 1, *Online Supplementary Figure S2E-F*). Next, to establish a link between drug response and genetic aberrations, the samples were grouped based on their FLT3 status, which revealed a significant correlation to drug response in primary samples lacking *FLT3* mutations (Figure 2G), with a similar trend in AML cell lines (*Online Supplementary Figure S3C*). These data suggest that the FLT3-ITD/FLT3 mutations confer resistance to the effect of H4 and that the therapeutic potential is effective in patients with normal *FLT3* alleles. In addition, two of the four responding primary samples (with >20% increase in CD11b) and the three strongest responding AML cell lines (Figure 2G, Table 1 and *Online Supplementary Figure S3A, B*), were diagnosed as acute monocytic leukemia (AML subtype M5 according to the French-American-British [FAB] classification) suggesting a favorable drug response for AML blocked at the monoblastic stage. Taken together, we identified H4 as a differentiating agent of FLT3 wild-type AML.

H4 treatment activates the gene expression program of monocytic differentiation

To further characterize H4-induced leukemic differentiation, we performed global gene expression profiling on primary AML-3 cells 16 h after treatment (Figure 3A). The genes most significantly modulated (42 unique ID, adjusted *P*-value $-\log_{10} >1$) were mainly upregulated, indicating that H4 treatment leads to transcriptional activation rather than repression (Figure 3B). Next, by comparison of the genes with the greatest fold change in expression to those in a dataset of normal human hematopoiesis[24] (using CellRadar, developed by G. Karlsson's laboratory at Lund University; unpublished data from Dhapola *et al.*) we found that the upregulated genes were associated with monocyte maturation and downregulated genes with myeloid progenitor cell identity (Figure 3B, C), supporting that H4 induces leukemic differentiation towards monocytes. In line with this, gene set enrichment analysis (GSEA) also showed strong enrichment for processes involved in monocytic differentiation and M1 polarization such as interferon-α/γ, tumor necrosis factor-α signaling via NF-κB, and reactive oxygen species (Figure 3D, E).[14,25] In addition, among the upregulated genes, we identified FcγRI (CD64), which is known to be specifically expressed by monocytes and macrophages.[26] The upregulation was confirmed at the protein level, with there being a 4-fold change in CD64 protein levels 4 days after treatment in comparison to the level in control experiments with DMSO (Figure 3F). Furthermore, gene ontology analysis of the upregulated genes demonstrated significant enrichment of genes coding for ribosomal biogenesis and translational elongation (*Online Supplementary Table S2*). In line with this, AML-3 cells treated for 19 h with H4 showed increased incorporation of OP-Puro, indicating an increased rate of protein translation (Figure 3G). Collectively, these findings demonstrate that H4 can alter the leukemic molecular program of primary AML to induce leukemic differentiation towards the monocytic lineage.

H4 induces leukemic differentiation through activation of protein kinase C

To identify candidate signaling pathways enabling H4-driven leukemic differentiation, we screened cells from patient AML-3 with H4 (at 10 μM) in combination with 176 compounds (Selleck Chem L3000) with defined targets (tested at 0.05 μM, 0.5 μM, and 10.0 μM) (Figure 4A). Several molecules had an inhibitory effect on H4-dependent CD11b upregulation, with an enrichment of compounds targeting the RAF/MEK/ERK signaling pathway together with JNK and glutaminase 1 (GLS1), all of which are downstream of PKC (Figure 4B and *Online Supplementary Figure S4A, B*).[27,28] In line with this, the addition of the specific PKC inhibitor GF109203X[29] prevented CD11b upregulation and the morphological changes of H4-treated AML-3 cells (Figure 4C and *Online Supplementary Figure S4C*), as well as in the AML cell lines THP-1 and MM6 (*Online Supplementary Figure S5A*). H4 treatment of 293T cells transfected with PKC-EGFP fusion protein led to a rapid translocation (within 3 min) from the cytoplasm to the plasma membrane indicative of direct activation of both conventional PKCα and β, as well as novel ε and δ isoforms[30] (Figure 4D, E and *Online Supplementary Video S1-4*). Additionally, H4 treatment of FLT3-wild type primary AML-3 cells and the FLT3-ITD AML cell line MOLM-13 led to increased endogenous phospho-PKC activation of both novel and conventional isoforms 30 min after H4 treatment (Figure 4F). Notably, the PKC inhibitor efficiently prevented CD11b upregulation by H4 in the FLT3-mutated AML cell line MM6, while the inhibitors of ERK and MEK only had limited effects on H4-induced differentiation. This is in contrast to AML-3 and THP1 where the inhibitors of ERK and MEK efficiently prevented H4-induced differentiation (*Online Supplementary Figure S5*). Furthermore, the FLT3 inhibitor quizartinib reduced the H4-induced differentiation in both FLT3 wild-type AML-3 cells and THP1 cells while quizartinib was toxic to the FLT3-mutated MM6 cell line as expected (Figure 4B and *Online Supplementary Figure S5*). These data support the concept that, mechanistically, H4 relies on activating PKC to promote leukemic differentiation while downstream signaling may be affected differently depending on inter-sample differences. Furthermore, these data underline the robustness of our model as a high throughput platform for mechanistic studies on primary cells.

Combinatorial screening identified a BET inhibitor as an enhancer of H4-induced differentiation

To identify molecules and pathways that can synergize with H4, we analyzed the above combinatorial screen for compounds that enhanced H4-driven CD11b upregulation of primary AML-3 cells (Figure 5A). Among the top candi-

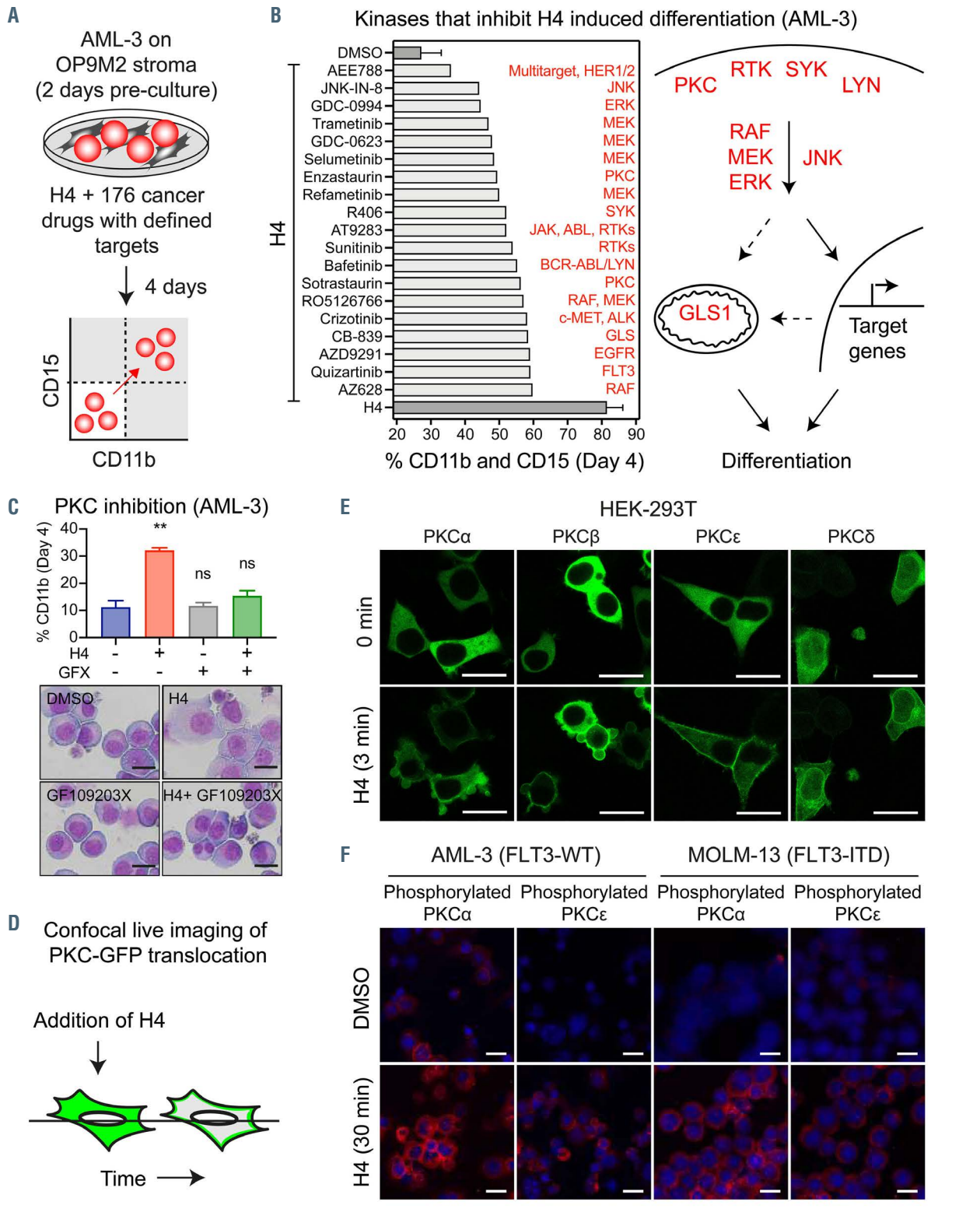


Figure 4. H4 induces leukemic differentiation through activation of PKC. (A) Primary AML-3 cells were treated with 176 compounds (5 μM, 0.5 μM, and 0.05 μM) with defined targets alone and in combination with compound H4 (10 μM). (B) Inhibition of the RAF/MEK/ERK and JNK signaling pathway inhibited H4-induced differentiation together with inhibition of kinases upstream (PKC, RTK, SYK, and LYN) and downstream of the pathway (GLS1). Compounds were omitted from the analysis if their toxicity reduced cell numbers more than 75% relative to the number following treatment with H4. The data are shown as mean ± standard deviation of technical replicates. The schematic diagram on the right shows kinase targets of the most potent compounds and how they relate to each other in signaling pathways. (C) Inhibition of PKC (GF109203X 4 μM) confirmed that compound H4 (10 μM) was unable to differentiate AML-3 cells without intact PKC signaling. Representative examples of May-Grünwald Giemsa staining are shown (scale bars represents 10 μm) and the data are mean ± standard error of mean from two independent experiments (**P<0.01, ns not significant, using one-way analysis of variance). (D) Schematic illustration of the PKC translocation assay, which is an essential step in PKC activation. HEK-293T cells were transfected with GFP-tagged PKCα, βII, ε, and δ, and treated with 40 μM of H4 for 20 min. Confocal images were taken every 5 s. (E) H4 triggers the translocation of PKC from the cytosol to membranes. Representative examples of confocal images at 0 and 3 min are shown. Scale bars represent 20 μm. (F) H4 triggers phosphorylaton of PKCα and PKCε in primary AML-3 cells and the AML cell line MOLM-13. Representative examples of confocal images at 30 min of treatment are shown (MOLM-13 PKCα 40 μM of H4 and the rest with 10 μM). Scale bars represent 40 μm. PKC: protein kinase C; AML: acute myeloid leukemia.

dates were histone deacetylase inhibitors as well as CPI-203 (Figure 5B and *Online Supplementary Figure S6*), a well-known BET inhibitor of BRD4.[31] Notably, while displaying little to no differentiating effect on its own, CPI-203 enhanced the H4-driven increase of CD11b expression, but more strikingly led to a 22-fold increase of the CD11b protein intensity compared to 6-fold with H4 alone (Figure 5C, *Online Supplementary Figure S7*, *Online Supplementary Table S3*). In additional primary samples, the combination enhanced the differentiating effect of H4 on two out of two FLT3 wild-type AML-M5 samples and in one out of three FLT3-mutated AML-M5 primary samples (Figure 5D, *Online Supplementary Figure S7* and *Online Supplementary Table S3*). The AML-M5 cell lines followed the same pattern, with THP1 (FLT3 wild-type) showing a clear response while MM6 and MOLM-13 (FLT3 mutated) did not respond to the addition of CPI-203 (*Online Supplementary Figures S7* and *S8*, *Online Supplementary Table S3*). Finally, the combination of H4 and the BET inhibitor had no additional effect on the other primary samples regardless of FAB-type or of FLT3 mutation status, except the cell line HL60 (FLT3 wild-type) which responded to the combination (Figure 5D, *Online Supplementary Figure S8* and *Online Supplementary Table S3*). The healthy control also responded to the combination with an increase of CD11b^{+} cells. However, the increase was still below the 20% threshold, and no morphological changes were detected (Figure 5D, E, *Online Supplementary Figure S7* and *Online Supplementary Table S3*). Taken together, our findings indicate that among the H4-sensitive primary samples, the combination with the BET inhibitor predominantly affects monocytic (M5) AML.

GSEA of upregulated genes in H4-treated AML-3 cells revealed strong enrichment of MYC target genes (Figure

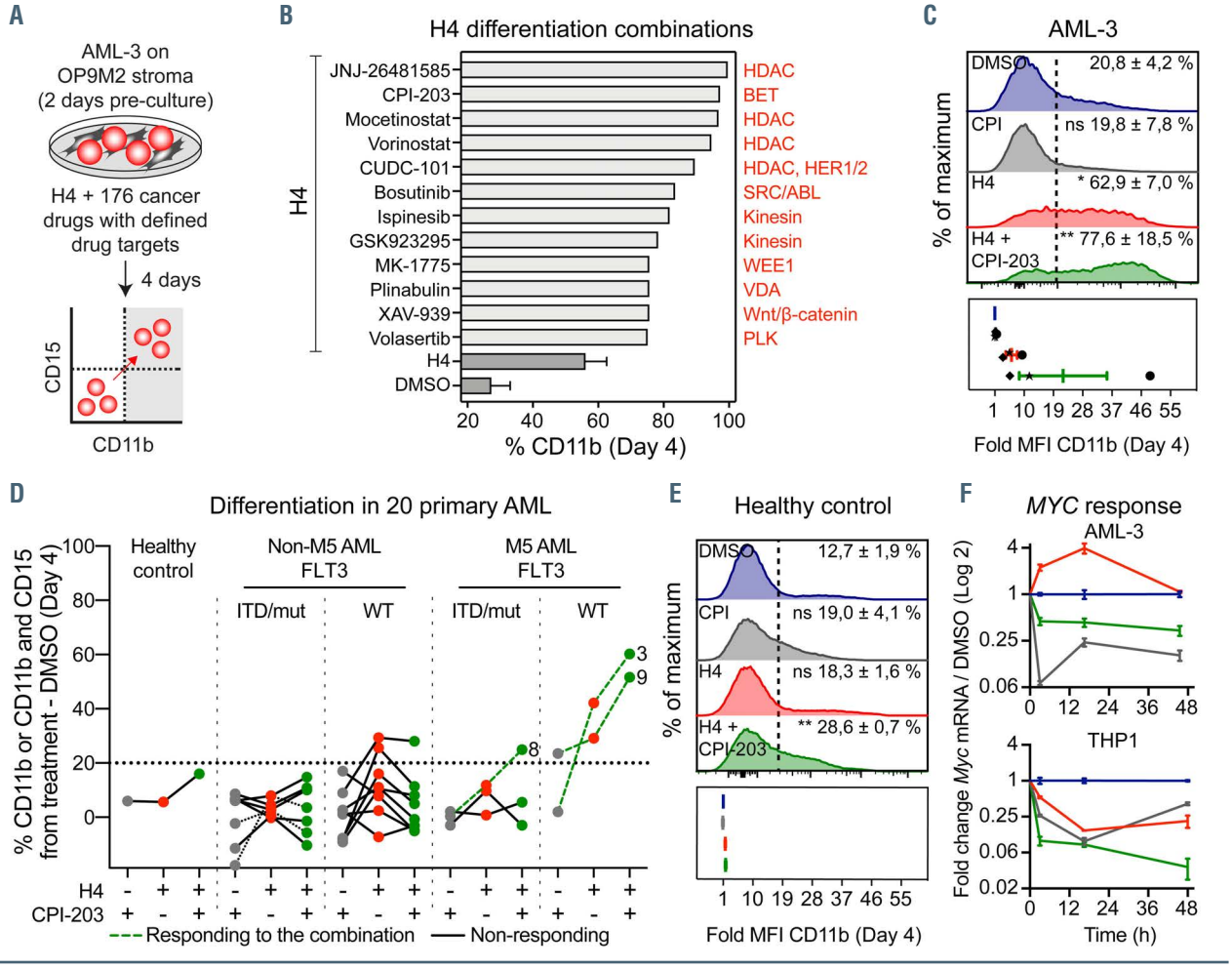


Figure 5. Combinatorial screen identified a BET inhibitor as an enhancer of H4-induced differentiation. (A) Cells from primary AML-3 were treated with 176 compounds (5 μM, 0.5 μM, and 0.05 μM) with defined targets alone and in combination with compound H4 (10 μM). After 4 days, the combinatorial differentiation response was assayed by increased expression of CD11b using flow cytometry. (B) The most potent compounds in combination with H4 are shown in the concentration that did not reduce cell number more than 75% relative to treatment with H4 alone (the data are shown as mean ± standard deviation [SD] of technical replicates). (C) Representative histogram plots showing the combinatorial differentiation effect of H4 (10 μM) and CPI-203 (0.05 μM) in AML-3 cells. Lower graphs show fold-change of mean fluorescent intensity (MFI) of CD11b relative to dimethylsulfoxide (DMSO). The data are shown as mean ± standard error of mean (SEM) from independent experiments designated with individual symbols. (*P<0.05, **P<0.01, ns not significant, using one-way analysis of variance [ANOVA]). (D) Combining H4 (10 μM) and the BET inhibitor CPI-203 (0.05 μM) enhanced differentiation in two out of two FLT3 wild-type and one out of three FLT3-ITD/mutated monocytic AML-M5 samples, while none of 15 samples of other M-types responded to the combination. The healthy control was CD34^{+} umbilical cord blood. Responders (green dashed line) to the combination had to exceed the 20% cutoff (dotted line) in increased CD11b expression to be categorized as responding. (E) As in panel (C). Healthy control (CD34^{+} umbilical cord blood) n=3. (**P<0.01, ns not significant, using one-way ANOVA). (F) *MYC* was dispensable for the differentiation response in primary-monocytic AML-3 cells and the monocytic THP1 cell line. Cells were treated with H4 10 μM and CPI-203 0.05 μM alone and in combination. *MYC* levels are displayed as fold-change compared to DMSO for each time-point. The data are shown as mean ± SD of technical replicates.DMSO: dimethylsulfoxide; ITD: internal tandem duplication; mut: mutated; WT: wild-type: MFI: mean fluorescent intensity.

3D, E). As BET inhibitors, such as CPI-203, are known to downregulate *MYC* expression,[32] we next investigated the role of *MYC* in H4-dependent differentiation of primary AML-3 cells. While *MYC* expression increased upon the addition of H4, treatment with CPI-203 alone or in combination efficiently downregulated *MYC* (Figure 5F), indicating that upregulation of *MYC* is dispensable in H4-induced differentiation of AML-3 cells. Notably, the M5 FLT3 wild-type leukemic cell line THP1 responded with *MYC* downregulation upon H4 treatment, while the combination with CPI-203 further suppressed *MYC* expression (Figure 5F). These results suggest that the effect of H4 can be enhanced with the BET inhibitor CPI-203 in a *MYC*-independent process. We also provide supporting evidence that the monoblastic nature of AML is a potential prognostic marker of drug sensitivity to H4 and CPI-203. Overall, our study highlights the power and robustness of multiplexed screening on individual primary samples and demonstrates that mutation agnostic combined pathway-targeting can lead to efficient leukemic differentiation.

Discussion

The 10-year survival of patients with AML between 55 and 64 years is as low as 20%.[2] These patients often develop co-morbidities, and their treatment must be chosen with care and often terminated in advance. Therefore, new and more AML-specific treatments with fewer side effects are greatly needed. However, due to the underlying diversity in the regulation of AML, the "*one size fits all*" strategy will be challenging to pursue, and the key to success is to identify subpopulations of patients with similar responses to selective treatments. Thus, better ways to develop patient-specific treatments with good translation from research to the clinic are needed. In this study, we performed a screen of the effects of natural products on primary AML cells using a co-culture system that simulates the microenvironment in the bone marrow. The screen identified a novel natural compound, called H4, which induced differentiation of FLT3 wild-type AML. Furthermore, using a combinatorial screen with H4 and molecules with defined targets, we identified the mechanism of H4 and determined that PKC activation, together with BET inhibition, further promoted the induction of leukemia differentiation. This demonstrates that our strategy based on a multiplex screening platform can be used for developing combined targeted therapy as well as for gaining mechanistic insights into leukemic regulation.

The novel macrocyclic diterpene identified in this study belongs to the jatrophane class of compounds, previously unknown for its effect on AML. Jatrophanes structurally resemble phorbol molecules known as PKC activators.[33] In line with this, H4 activated PKC followed by the downstream MAPK-ERK/JNK pathways that have previously been shown to be crucial to the induction of monocytic differentiation by additional agents such as vitamin D3, iron-chelating drugs, reactive oxygen species-inducing agents, as well as other PKC agonists.[34-38] Furthermore, the clinical potential of activating this pathway was demonstrated in a retrospective case-control study of AML patients treated with vitamin D3 and an iron-chelating agent which resulted in induced blast cell differentiation and increased overall survival.[38,39] Hence, further studies are warranted to evaluate H4 as a clinical candidate, including defining the therapeutic window *in vivo*.

H4-induced differentiation was found to be dependent on FLT3 status, in that FLT-ITD or FLT3 mutations inhibited the differentiation-inducing capacity of H4, with the strongest responders being monocytic (M5) FLT3 wild-type leukemias. This indicates that the predicted response is dependent on both the genetic aberrations and in what stage in the hierarchy the differentiation is arrested. The mechanism by which FLT3-ITD or FLT3 mutant signaling inhibits the effect of H4 is not clear, but since these mutations are often seen as secondary mutations, they may be central in the leukemic transformation and thus directly control the differentiation block.[40] As such, several essential myeloid transcription factors have been shown to be directly regulated by FLT-ITD such as RUNX1, PU.1 and CEBP/α, which may confer resistance to PKC signling.[41,42] Since FLT-ITD and FLT3 mutations are among the most common aberrations in AML (being present in around 30% of all AML patients)[7] this could limit the use of H4 clinically. However, in FLT3-ITD or FLT3-mutated AML, it would be interesting to evaluate if H4 combined with FLT3 inhibitors or molecules specifically targeting the downstream effector genes to FLT3 such as CKI,[43] could unlock the differentiation block, allowing differentiation also in these leukemias.

Gene expression analysis revealed that inflammatory signaling was the primary cellular response to H4, which, together with the upregulation of CD64, suggests monocytic differentiation towards non-classical inflammatory monocytes by M1 polarization.[44,45] We also observed a strong activation of *MYC* and MYC target genes. Consistent with M1 polarization, MYC is involved in metabolic reprogramming, leading to increased use of glutamine, whose process, when inhibited, also reduced the effect of H4.[46,47] However, we and others found that *MYC* is downregulated in leukemic cell lines upon PKC activation,[35] suggesting that the differentiation response in cell lines and primary cells can be mechanistically different. This points to the importance of using individual AML samples for mechanistic studies.

Combinatorial treatment is a strategy to overcome therapy resistance to directed therapy.[13,15] Nevertheless, identifying combinations based on mutational profile alone is difficult, especially in the context of complex karyotypes. In contrast, using our platform, we could rapidly evaluate hundreds of combinations on primary patients' cells, revealing the combination of CPI-203 (a BET4 inhibitor) and H4. Intriguingly, the combination of PKC agonists with BET inhibitors is an effective strategy to activate latent human immunodeficiency virus (HIV) in CD4 T cells and monocytes.[48] Studies on HIV also showed that the combination of BET and PKC enabled optimal efficacy at a lower concentration than using the individual molecules, which is important for reducing potential side effects. In line with this, the optimal concentration for CPI-203 in our study was 50 nM, which is non-toxic for healthy bone marrow cells, and below the concentrations used in other studies in which CPI-203 was given alone.[49,50]

Our combination not only improves the effect of H4 but also potentially improves its clinical potential by removing unwanted side effects such as *MYC* expression. Furthermore, it would be interesting to determine whether cancer patients with specific *MYC* amplifica-

tions or dysregulated expression of *MYC* as part of their tumor mechanism may also benefit, as seen in chronic myeloid leukemia.[50]

In summary, our work highlights the importance of working with primary human samples in mechanistic studies and drug screening. We also show the value of combinatorial treatment to develop novel mutation agnostic therapeutic approaches for AML. Finally, our finding that a combination of PKC activation and BET inhibition promotes increased differentiation in FLT3 wild-type AML provides strong support for further investigation towards clinical use.

Disclosures

No conflicts of interest to disclose.

Contributions

SH, AB, EK and MM contributed to the conception and design of the study; SH, AB, and LS contributed to the development of the methodology; SH, AB, PB, RS, FE and MM analyzed and interpreted the data; SH, AB, LS, PB, CS, TF, CL, SL, GJ, FE and MM wrote and reviewed the manuscript; LS, PB, SP-T, CS, TF, RS, CL, SL and GJ provided technical or material support; AB, FE and MM supervised the study.

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