HHS Public Access

Author manuscript

Leukemia. Author manuscript; available in PMC 2018 December 09.

Published in final edited form as:

Leukemia. 2019 January ; 33(1): 52–63. doi:10.1038/s41375-018-0174-1.

Drug Targeting of NR4A Nuclear Receptors for Treatment of Acute Myeloid Leukemia

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Abstract

NR4As are AML tumor suppressors that are frequently silenced in human AML. Despite their potential as novel targets for therapeutic intervention, mechanisms of NR4A silencing and strategies for their reactivation remain poorly defined. Here we show that NR4A silencing in AML occurs through blockade of transcriptional elongation rather than epigenetic promoter silencing. By intersection of NR4A-regulated gene signatures captured upon acute, exogenous expression of NR4As in human AML cells with in silico chemical genomics screening, we identify several FDA-approved drugs including dihydroergotamine (DHE) that reactivate NR4A expression and regulate NR4A-dependent gene signatures. We show that DHE induces NR4A expression via recruitment of the super elongation complex to enable elongation of NR4A promoter paused RNA polymerase II. Finally, DHE exhibits AML selective NR4A-dependent anti-leukemic activity in cytogenetically distinct human AML cells in vitro and delays AML progression in mice revealing its potential as a novel therapeutic agent in AML.

Introduction

AML (acute myeloid leukemia) is a heterogeneous disease associated with corruption of normal transcriptional and epigenetic control of myeloid cell differentiation and the emergence of transformed leukemic initiating cells (LICs) with aberrant self-renewal properties capable of sustaining leukemic expansion [12]. Despite substantial improvements in genetic and molecular classification of AML, standard induction chemotherapy using anthracyclines and cytarabine remains a treatment mainstay but mainly targets bulk

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The authors declare no potential conflicts of interest.

leukemic blasts rather than LICs [3]. Although new therapies are urgently needed, genetic, phenotypic and functional heterogeneity among patient LICs presents a major challenge to their development [4, 5–7]. Efficient therapeutic targeting of LICs to eradicate AML therefore requires a detailed understanding of the genetic, epigenetic and molecular pathway dependencies that distinguish AML LICs from normal hematopoietic stem and progenitor cells (HSPCs).

The NR4A subfamily of nuclear receptors consists of three structurally related transcription factors (NR4A1-3) that regulate cell context dependent cell fate decisions in response to extracellular signals including inflammatory, genotoxic, apoptotic and mitogenic stimuli [8, 9]. NR4As are also diverse regulators of hematopoiesis including hematopoietic stem cell maintenance [10–12], T-lymphocyte development and function [13–1718], and monocyte and macrophage maturation and inflammation [19–21].

NR4As also play key roles as tumor suppressors of both myeloid and lymphoid malignancies [1122–24]. In particular, NR4A1 and NR4A3 are functionally redundant tumor suppressors of AML and pre-AML malignancies. Codepletion of NR4A1 and NR4A3 in mice is sufficient to drive AML development while reduction of NR4A1/NR4A3 expression in NR4A1/NR4A3 hypoallelic mice leads to mixed myelodysplastic/myeloproliferative (MDS/MPN) disease [11, 22, 25]. In human patients, NR4A1 and NR4A3 expression is reduced in MDS and silenced in AML bulk blasts and LIC enriched populations irrespective of patient cytogenetics [11, 26, 27]. Further, forced expression of NR4A1 or NR4A3 in human AML cells inhibits their viability and reprograms a subset of gene signatures that distinguish primary human LICs from normal HSCs including suppression of a core oncogenic MYC driven gene signature[25].

Given the widespread silencing of NR4As in AML patients and the sufficiency of their inactivation in causing AML, we hypothesized that NR4A silencing may be an obligate step in AML maintenance and that strategies directed toward NR4A reactivation may be of therapeutic benefit in treatment of AML. Here we address mechanisms of NR4A silencing in AML and we use an integrated systems approach combining NR4A target based genomics data with in silico chemical genomics screening to objectively identify small molecule activators (SMAs) of silenced NR4As. We show how this approach successfully identified the ergot alkaloid, dihydroergotamine (DHE), as a drug inducer of NR4As and NR4A-dependent gene signatures with anti-leukemic efficacy.

Results

To disclose mechanisms of silencing of NR4As in AML cells, we examined the epigenetic status of NR4A1 and NR4A3 promoters. We found that both promoters are devoid of DNA methylation in cytogenetically distinct primary human AML HSPCs and AML cell lines similar to normal human HSPCs (Supplemental Figure S1). Next, we examined the trimethylation status of histone H3 lysine 4 (H3K4me3), a mark associated with active and poised promoters [28, 29]. Using two cytogenetically distinct AML cell lines, Kasumi-1 and MV4-11, representing commonly occurring AML oncogenes (AML-ETO and mixed lineage leukemia (MLL) rearranged human AMLs), we found that both promoters contained high

levels of H3K4me3 that were comparable to levels at the promoter of the highly expressed gene β2 microglobulin (B2M) (Figure 1A). Furthermore, query of publically available databases revealed that promoter enrichment of H3K4me3 at NR4As also extends to normal HSCs and primary HSPCs from AML patients of distinct cytogenetics (Supplemental Figure S2). Consistent with these findings, analysis of the status of RNA Pol II occupancy across NR4A1 and NR4A3 genomic loci revealed that both promoters contain relatively high levels of pre-associated Pol II that is largely dismissed from downstream intragenic regions (Figure 1B). These results indicate that NR4A promoters reside in an open chromatin context and actively recruit Pol II.

To objectively identify chemicals that can reactivate expression of silenced NR4As and restore their transcriptional activity, we used NR4A-regulated gene signatures in Kasumi-1 AML cells to conduct an *in silico* screen of the Connectivity Map (CMap) database [30]. This generated 1229 connectivity scores in HL-60 AML cells. We examined 3 metrics of NR4A connectivity to identify chemicals for secondary *in-vitro* screening: NR4A-dependent upregulated genes connectivity, NR4A-dependent downregulated genes connectivity, and connectivity to upregulation of NR4A1 and NR4A3 mRNAs. Given the limitation of CMap HL60 data (e.g., no replicates), we used a simple non-statistical percentile ranking cutoff (80%) which yielded a list of 12 chemicals to pursue in empirical secondary screening (Figure 2A and Supplemental Figure S3). Visualization of NR4A connectivity with the 12 chemical signatures by both heatmap depiction (Supplemental Figure S3A) and CMap rank-ordered plotting (Supplemental Figure S3B) revealed significant connectivity with few conversely regulated genes.

Next, we examined the ability of the 12 chemicals to induce NR4A protein expression and suppress cell viability in Kasumi-1 AML cells. We found that 6 of the 12 top chemicals induced measureable NR4A protein (Figure 2A) and also exhibited varying degrees of cell growth inhibition (Figure 2B). Since Alprostadil (ALP), a prostaglandin, and the ergot alkaloid, dihydroergotamine (DHE) performed best in these assays, we confirmed that both drugs are potent dose-dependent activators of NR4A1 and NR4A3 gene transcripts in Kasumi-1 and MV4-11 cells (Figure 2C). Thus, our integrative screen successfully identifies chemicals that induce NR4A expression and compromise the viability of human AML cells.

To understand how DHE and ALP facilitate NR4A activation, we monitored Pol II occupancy of the NR4A1 and NR4A3 loci in response to drug treatment using ChIP analysis. We found that both drugs elicited a moderate increase in bound Pol II at the NR4A1/3 promoter but stimulated significant increases in Pol II binding to intragenic regions of both genes (Figure 3A). This indicated that both drugs function as positive regulators of transcriptional elongation of promoter paused Pol II across the NR4A1 and NR4A3 genes in AML cells.

Promoter clearance and elongation competence of Pol II require phosphorylation of Ser5 (S5-P) and Ser2 (S2-P) at its C-terminal domain (CTD). To examine the mechanisms by which DHE and ALP promote elongation competence of Pol II, we first investigated the phosphorylation status of Pol II CTD at the NR4A promoters and intragenic regions. We found low levels of S5-P and S2-P under basal conditions that were strongly induced upon

treatment with ALP or DHE (Figure 3B,C). Pol II activation and processivity were also associated with acquisition of intragenic histone H3K36 trimethylation (H3K36Me3), a histone mark of transcription elongation, while high basal levels of promoter associated H3K4me3 activation mark were unchanged (Figure 3D,E).

Phosphorylation of Pol II is accomplished by several cyclin-dependent kinases (CDKs) that are components of a Pol II-associated preinitiation complex (PIC) composed of general transcription factors (GTFs) and Mediator complex, bridging transcription factors to Pol II and the super elongation complex (SEC) which is required for productive transcription elongation. CTD phosphorylation of Pol II at Ser2 is accomplished by the CDK9 catalytic subunit of positive transcription elongation factor b (P-TEFb), a component of the SEC complex critical for Pol II transcriptional elongation [31]. CTD phosphorylation of Ser5 is deposited by CDK7, a kinase subunit of TFIIH and also by CDK8, a kinase component of the Mediator complex that also recruits P-TEFb to the promoter upon stimulus induction of IEG transcription elongation [323334, 35]. Consistent with increases in S5-P and S2-P, we also found significant increases in NR4A1 promoter bound transcriptional elongation factors including CDK9 and CDK8 (Supplemental Figure 4A,B) Further, both ALP and DHE dependent induction of NR4As was inhibited by the CDK9 antagonist, NVP-2 while constitutive expression of B2M was unaffected (Supplemental Fig 4C,D). Together these data indicate that DHE and ALP-dependent induction of NR4A expression occurs via regulation of transcription elongation and involves recruitment of CDK8 and P-TEFb leading to activation of elongation competent Pol II.

We previously demonstrated that rescue of NR4A expression in human AML cells (including Kasumi-1) inhibits their proliferation and leads to acute transcriptional regulation of a gene expression signature (GES) that includes suppression of a core oncogenic Myc signature that is common to AML LICs [25]. We therefore asked whether chemical inducers of NR4As could mimic the cellular and molecular responses of AML cells observed after NR4A rescue in Kasumi-1 cells. We found that exposure to either ALP or DHE was sufficient to suppress cell viability in a dose-dependent manner (Figure 4A,B). Both drugs also strongly reduced AML colony-forming potential demonstrating potent long-term suppression of AML cell proliferation (Figure 4C).

We next asked whether both drugs can regulate the expression of NR4A target genes. Using a subset of NR4A target genes regulated upon forced expression of NR4As in Kasumi-1 cells [36] (Figure 4D), we found that acute exposure to DHE or ALP (6h) mimicked NR4As in their ability to regulate the expression of these NR4A target genes including repression of c-MYC (Figure 4E,F). Finally, to determine whether the responses to DHE and ALP were dependent on reactivation of NR4As, we examined the consequences of siRNA-mediated depletion of all 3 NR4A family members (siNR4A1-3). Using a pool of scrambled siRNA controls or siNR4A1-3, we achieved selective siNR4A1-3 mediated knockdown of NR4A induction in response to DHE and ALP (Figure 4G, Supplemental Figure S5). Both DHE and ALP dependent AML cell growth inhibition were significantly overcome by NR4A knockdown (Figure 4H,I). Further, siNR4A1-3 mediated knockdown of NR4As strongly abrogated drug dependent regulation of NR4A-dependent target genes (Figure 4J) but had

no off target effects on the basal level of expression of these genes in the absence of drug dependent induction of NR4As (Supplemental Figure S6).

To address the AML subtype selectivity of NR4A activating drugs, we examined the growth suppressive effects of DHE on a range of established human AML cell lines representing diverse cytogenetic backgrounds. We focused on DHE since it is an FDA approved drug for treatment of migraine and has the potential for rapid repurposing for AML intervention. Dose responsive suppression of AML cell growth by DHE was observed to varying degrees in the majority of cell lines tested including HL60 (Myc amplified), OCI-AML3 (NPM1 mutated) and those containing mixed lineage leukemia (MLL) fusions, MLL-AF9 (MOLM-13, MOLM14) and MLL-AF4 (MV4-11) that represent more aggressive chemotherapy-resistant AML disease (Figure 5A). In contrast, DHE failed to suppress the growth of NB4 (PML-RARa) AML cells indicating that DHE-dependent cell growth suppression was cell selective (Figure 5A). This lack of growth suppression of NB4 cells by DHE correlated with a lack of DHE induction of NR4As and suppression of the NR4A target c-Myc in contrast to DHE responsive cell lines (Supplemental Fig S7). Using MV4-11 and MOML14 cells, we found that DHE also strongly suppressed their long-term colony forming potential (CFU) (Figure 5B). The growth suppressive effects of DHE also extended to patient derived primary AML samples of distinct cytogenetics (Supplemental Table S1) but DHE had no effects on the growth of normal CD34+ cells (Figure 5B and Supplemental Figure S8 and 9). Thus, the growth inhibitory effects of DHE are selective to AML cells.

To address the anti-leukemic efficacy of DHE in vivo we used a xenograft mouse model of disseminated MLL-rearranged human AML. 2×10^6 MOLM14 cells were injected i.v. into the tail vein of NOD/SCID/gamma (NSG) immunocompromised mice, and mice were injected twice daily i.p. with vehicle (n=7) or DHE at two doses, 0.1mg/kg (n=7) or 1.0mg/kg (n=8) and monitored for overall NSG mouse survival. Dissemination of AML was confirmed at necropsy by bone marrow and peripheral blood smears (Supplemental Figure S10A–B). Treatment with DHE led to a dose-dependent increase in overall mouse survival that was significant at both doses of DHE (Figure 6A). Further, analysis of bone marrow (BM) engraftment of MOML14 derived human CD45+ blasts and the myeloid differentiation marker, human CD11b at necropsy revealed that DHE treatment leads to a decrease in BM tumor burden as well as increased myeloid differentiation of human AML blasts (Figure 6B and C).

Finally, to address the in vivo effects of DHE on normal hematopoietic cell development, we examined the effects of daily exposure of normal mice to the high dose (1.0mg/kg) of DHE over a 30-day period. Chronic exposure of mice to DHE had no significant effects on frequencies of bone marrow HSPCs (Supplemental Figure S10A–D) or mature blood cells (Supplemental Table S2) relative to vehicle treated mice. Thus, the effects of DHE in vivo are selective to AML cells.

Discussion

We have revealed a novel approach for restoring expression of silenced NR4As in AML cells by integrating NR4A regulated GES with *in-silico* chemical genomics screening. This

approach identified chemicals that restore NR4A expression, regulate NR4A-dependent gene signatures and exhibit anti-leukemic activity against cytogenetically distinct AML subtypes. In the absence of drug stimulation, silenced NR4A1 and NR4A3 promoters reside in open chromatin with recruited RNA Pol II that is stalled in an elongation incompetent state. Accordingly, chemical inducers of NR4As act not through promoter activation, but rather through recruitment of transcription elongation complex components to activate elongation competence of promoter stalled RNA Pol II. Together, our results disclose mechanisms of NR4A silencing and reactivation in AML, identify NR4A reactivating drugs with potential for drug repositioning for treatment of AML patients, and provide a general strategy for discovering chemical modulators of transcription factors.

Common mechanisms of tumor suppressor gene (TSG) silencing in cancers involve transcriptional repression through DNA hypermethylation and/or deposition of repressive histone marks primarily within the promoter regions of TSGs [37–39]. The reversible nature of this repression has fueled development of drugs capable of inhibiting key factors involved in establishment of repressive chromatin, including DNA methyltransferases and histone deacetylases as a therapeutic strategy for epigenetic reactivation of silenced TSGs [40–42]. However, profiling of genomic Pol II occupancy in mammalian cells has revealed that promoter proximal pausing of Pol II is a common regulatory step in transcriptional elongation of a large number of genes, including stimulus-responsive immediate early genes [29, 31, 35, 43]. Further, comparative genome-wide analysis of the H3K4me3 epigenome in normal and tumor tissues has revealed that TSG expression in normal cells is often associated with a broad pattern of trimethylation of histone H3K4 across the promoter and TSS of these genes. In cancer cells, however, H3K4me3 at TSGs is frequently retained but more tightly restricted to the proximal promoter region, and this restricted H3K4me3 pattern is associated with TSG repression [44]. These findings predict that additional mechanisms of TSG silencing may operate post recruitment of Pol II to active promoters at the elongation stage of transcription. Consistent with these findings, we observed that the promoter regions of silenced NR4As in AML cells are hypomethylated and contain active promoter marks (H3K4me3) with promoter proximally paused Pol II and therefore are unresponsive to epigenetic drugs targeted at reactivation of epigenetically silenced promoters.

Integration of NR4A GES data with chemical genomics screening provides a powerful objective approach to discover new drugs that reactivate NR4A TSGs in AML cells. This approach has several key advantages: First, it identifies chemicals inducers of NR4As that we can utilize as molecular tools to disclose mechanisms of NR4A silencing and reactivation in AML. Second, the NR4A-regulated GES is predictive of therapy, in that it corresponds to compromised AML cell viability. Third, by searching for chemicals that mimic the NR4A-dependent GES in contrast to simply inducing NR4A1 and NR4A3 mRNAs, we select for chemicals that recapitulate the tumor suppressive activities of NR4As in AML cells. Finally, since many of the top chemicals are FDA-approved drugs, the approach facilitates rapid reassignment of old drugs to new indications.

NR4A TSGs comprise subclass of nuclear receptors that are stimulus responsive immediate early genes (IEGs). Recent studies showed that IEG induction is primarily controlled at the level of transcription elongation [35, 43, 45, 46]. Similarly, NR4A activating drugs, alprostadil

and DHE rapidly induce NR4A expression via recruitment of P-TEFb to NR4A promoters, resulting in activation of elongation competence of Pol II via phosphorylation at Serine 2. Although NR4As respond to a variety of extracellular stimuli in a cell context-dependent manner under normal physiological conditions [8], NR4A induction in AML cells was drug selective. Out of over 1000 chemicals interrogated in CMap database only 6 chemicals, several of which were structurally related ergot alkaloids (supplemental Figure S9), scored positive for NR4A induction and GES connectivity.

In addition to inducing NR4A expression, the top scoring chemicals, DHE and alprostadil, regulated GES that displayed strong connectivity with NR4A regulated GES, including suppression of MYC which directs a core oncogenic signature in cytogenetically diverse human AML cells [47]. Further, both drugs suppressed the growth of human AML cells in an NR4A dependent manner, indicating that GES connectivity is a reliable predictor of anti-leukemic response. We focused on DHE as it is FDA-approved for treatment of severe migraine and represents an ergot alkaloid drug class that demonstrated high connectivity with NR4A GES. DHE exhibited dose-dependent growth inhibitory activity against several cytogenetically distinct human AML cells lines and primary AML patient samples, including those representing MLL fusions which are highly dependent on MYC and represent aggressive chemotherapy resistant AMLs [48, 49]. Importantly, the growth inhibitory activity of DHE was AML selective and DHE did not affect normal hematopoietic cell development. The lower effective dose of DHE in vivo (0.1mg/kg i.p. twice daily) is >200-fold lower than the lethal dose in mice (i.v. 44mg/kg, http://www.drugs.com/pro/d-h-e-45.html). Using the body surface area (BSA) recommended method of dose translation from mouse to humans [50], this represents a dose of approximately 0.01mg/kg daily in humans and is within the maximum dosage recommended for humans (0.012mg/kg per day). Thus DHE displays anti-leukemic efficacy in vivo at clinically relevant doses. DHE acts in the central nervous system (CNS) through binding to several serotonergic, dopaminergic and adrenergic G protein coupled receptors [51]. Members of these receptor families are expressed in hematopoietic progenitors and regulate their mobilization [52–55]. While the individual contributions of these receptors to the NR4A activating and anti-leukemic effects of DHE remain to be defined, analysis of the acute effects of DHE exposure on NR4A induction and MYC repression in primary patient samples together with preclinical screening in patient-derived xenografts should provide a powerful approach to stratify potential AML patient responders.

Materials and Methods

Human AML cell lines and Patient Samples

HL-60, Kasumi-1, MV4-11, MOLM-13, MOLM14, OCI-AML3, THP1 and NB4 cells were purchased from ATCC. Primary AML patient samples were collected after informed consent at Texas Children’s Hospital in accordance with the Declaration of Helsinki and institutional review board regulations. Normal cord blood CD34+ stem/progenitor cells were obtained from AllCells.

Connectivity Map (CMAP) Analysis

The NR4A1 minimal signature used to query CMAP was generated from our previously published NR4A1 regulated GES obtained in Kasumi-1 cells and defined as those probes whose q-value = 0.0 when subject to Rank Product Analysis (RPA) between NR4A1 and EGFP arrays [25]. This represents the most conservative, and statistically rigorous NR4A signature (Supplemental Table S3). Probes comprising the NR4A minimal GES were mapped to CMAP's Affymetrix U133A microarray platform [30]. Querying CMAP resulted in 1229 connectivity scores with chemical signatures derived from HL60 AML cells. We used 3 independent metrics of connectivity: NR4A Up Gene connectivity, NR4A Down Gene connectivity and NR4A mRNA upregulation. Chemical signatures were classified as NR4A-connected signatures if they scored above the 80th percentile by all three metrics (Supplemental Table 3). DOWN connectivity scores are negative values. Thus, a low percentile ranking (<20th) was used in NR4A Down Gene signature analysis.

Cellular Growth Assays

Cell viability was assessed using CellTiter Cell Proliferation Assay (Promega). For colony-forming assays, 5,000 cells (MV4-11 and MOLM14), 500 cells (CD34+ human cord blood) or 10,000 – 1,000,000 cells (primary AML) were plated in triplicate in MethoCult H4100+10% FBS (MV4-11 and MOLM14) or H4434/H4534 (CD34+ cord blood and primary AMLs) (StemCell Technologies) and scored for CFUs after 7–14 days.

Chromatin Immunoprecipitation

ChIP-qPCR analysis was carried out as previously described [36]. Details are in Supplemental Methods.

Xenograft Models of Human AML

MOLM14 cells (2×10^6) were injected into the tail vein of female (6–8 week) NOD.*Cg-Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mice (The Jackson Lab, Bar Harbor, ME). Mice were treated twice daily with vehicle (n=8 mice), 0.1mg/kg DHE (n=7 mice) or 1.0mg/kg DHE (n=8 mice), sacrificed when moribund and bone marrow (BM) cellularity was determined by manual counts. Blood smears and bone marrow cytospins were stained with Wright–Giemsa Stain (Sigma, St Louis, MO, USA). Tumor burden in BM was measured by flow cytometry of human CD45+ cells and myeloid differentiation analyzed using human CD11b. Mouse experiments were approved by the BCM Animal Care and Use Committee.

Statistical Analysis

Mean values from experimental triplicates were analyzed for statistical significance using independent Student's *t*-test (*p*-values of <0.05). For mouse survival experiments, statistical significance of mean latency of survival differences from Kaplan Meier plots was calculated using a Log-rank (Mantel-Cox) test using the *survival* R library.

Additional details are in Supplemental Methods

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by RO1 CA160747 from National Institutes of Health to OMC and by the Cell Sorting and Flow Cytometry shared resource of the Dan L. Duncan Comprehensive Cancer Center with funding from National Cancer Institute grant (P30CA125123). We acknowledge the joint participation by Adrienne Helis Melvin Medical Research Foundation through its direct engagement in the continuous active conduct of medical research in conjunction with Baylor College of Medicine. We also thank Dr. Terzah Horton and the Leukemia Research Interest Group at Texas Children's Hospital for providing primary patient AML samples. Author contributions: SPB, RPD and SGC carried out experiments including Cmap chemical genomics integration, ChIP-experiments, bisulfite sequencing, and cell growth assays. PRF did flow cytometry analysis. LN did transplantation assays. PN, MSR and PRF were responsible for colony forming assays on primary AML samples. SPB and OMC conceived the strategy, designed experiments and wrote the paper.

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Statement of significance

A chemical genomics strategy identifies dihydroergotamine as a novel activator of silenced NR4A tumor suppressors with repositioning potential for treatment of acute myeloid leukemia.

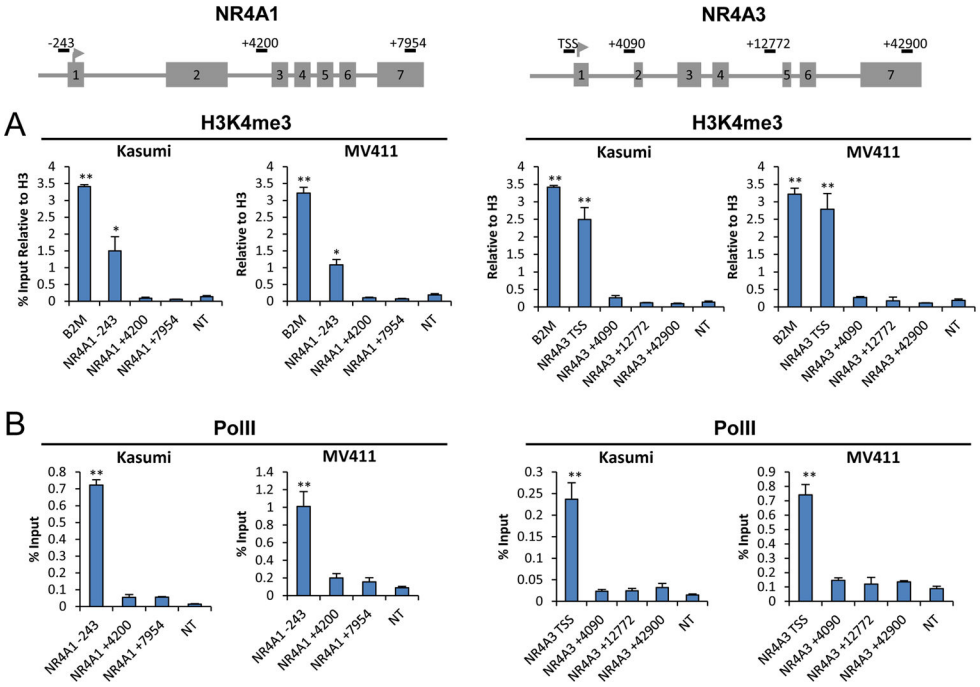

Figure 1. NR4A Promoters Reside in Open Chromatin

(A) H3K4Me3 enrichment at NR4A1 and NR4A3 promoters compared to B2M. (B) Pol II occupancy at NR4A1 and NR4A3 loci. Gene loci numbers are relative to TSS. **p<0.01, *p<0.05 compared to non-transcribed control (NT).

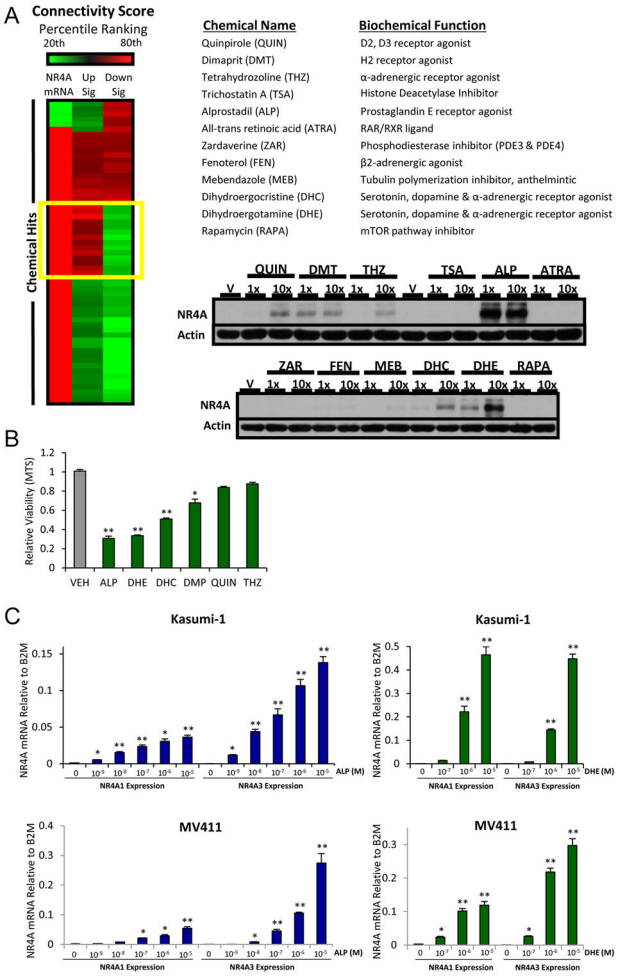

Figure 2. Integration of an NR4A Activation Signature with CMAP Identifies Chemical Activators of NR4As with Anti-leukemic Activity

(A) Heatmap showing top scoring chemicals with percentile ranking above 80% (highlighted in yellow) according to three independent connectivity scores: (1) NR4A mRNA induction, (2) NR4A UPregulated genes and (3) NR4A DOWNregulated genes connectivity. Chemical identities of top 12 chemicals. Western blot for NR4A protein in Kasumi-1 cells at 1x and 10x levels used in CMAP (10uM and 100uM, respectively). (B) Growth inhibitory activity of top chemicals (10uM) in Kasumi-1 cells (48h). (C) Alprostadil (ALP) and dihydroergotamine (DHE) are dose dependent activators of NR4A1 and NR4A3 mRNAs in Kasumi-1 and MV4-11 cells. **p<0.01, *p<0.05 compared to vehicle controls.

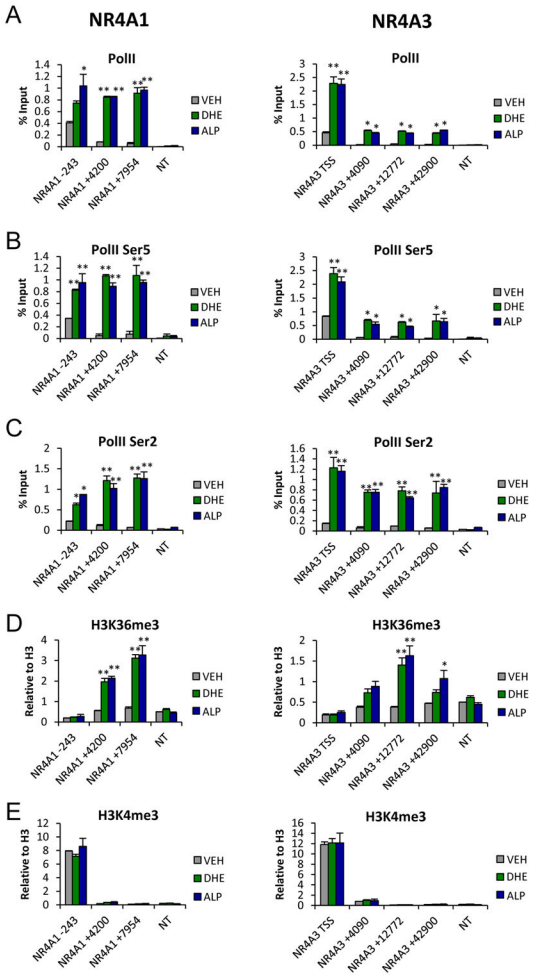

Figure 3. Chemical Inducers of NR4As Activate Transcriptional Elongation of the NR4A Loci

MV4-11 cells were treated for 1hr with 10uM DHE or 1uM ALP followed by ChIP-qPCR analysis of (A) Pol II, (B) Pol II S5P, (C) Pol II S2P, (D) H3K36me3 and (E) H3K4me3 at NR4A1 and NR4A3 loci. Gene loci numbers are relative to TSS.**p<0.01, *p<0.05 compared to vehicle controls. NT = non-transcribed control.

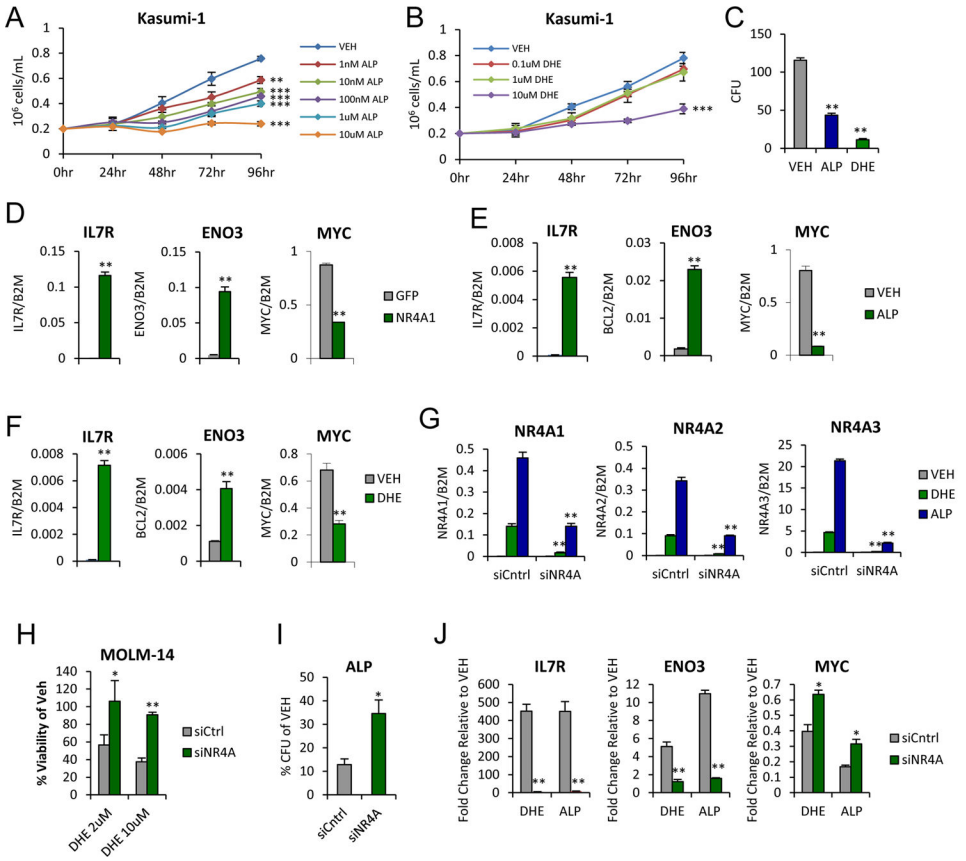

Figure 4. Chemical Inducers of NR4As inhibit AML cell proliferation and restore NR4A-dependent transcriptional activity

(A–B) Cell growth of Kasumi-1 cells treated with indicated doses of (A) ALP or (B) DHE. (C) AML colony forming units (CFUs) at 12 days after treatment with vehicle, 1uM ALP or 10uM DHE. (D) NR4A-dependent target gene regulation at 6 hours after NR4A1 rescue. (E–F) or treated with (E) 1uM ALP or (F) 10uM DHE. (G) siRNA-mediated suppression of drug-induced NR4A expression. (H) siRNA-mediated knockdown of NR4As restores MOLM-14 cell viability in liquid culture when treated for 48 hours with either 2 or 10uM DHE. (I) siRNA-mediated knockdown in Kasumi-1 partially overcomes ALP-dependent inhibition of AML colony forming ability. (J) Drug-dependent regulation of NR4A target genes in Kasumi-1 is compromised by siRNA-mediated NR4A knockdown. **p<0.01, *p<0.05 compared to vehicle (C–F) or siCntrl (G–J).

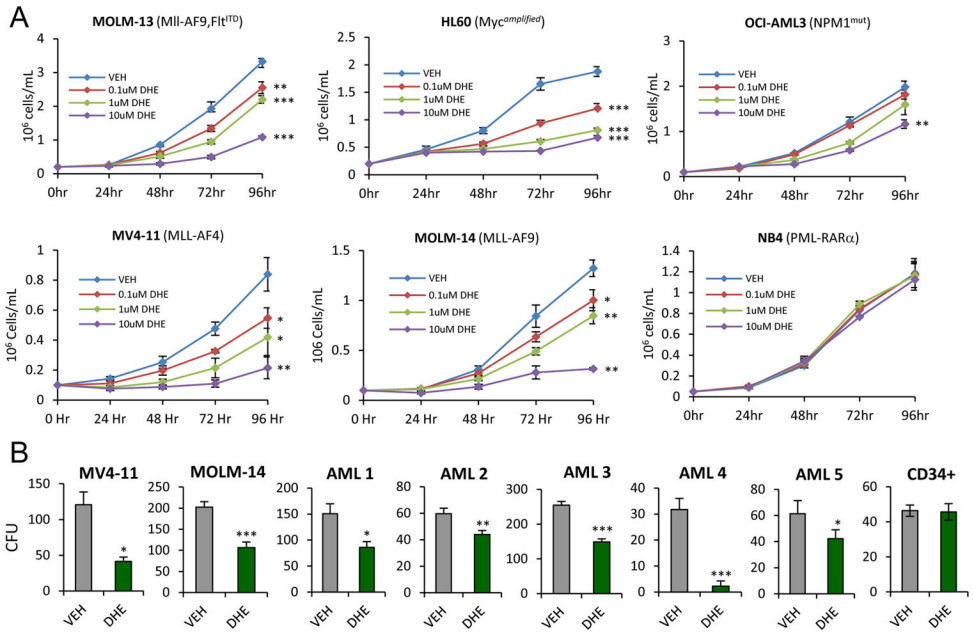

Figure 5. DHE suppresses viability of cytogenetically distinct AML cell lines and primary AML samples but not normal CD34+ cells

(A) HL-60, OCI-AML3, MV4-11, MOLM13, MOLM14 and NB4 cells counts at indicated doses of DHE. (B) CFUs 7 days after treatment of MV4-11 or MOLM14 cells and 10–14 days after treatment of primary AMLs or normal CD34+ cells with vehicle or 10uM DHE. *p<0.05, **p<0.01, ***p<0.001 compared to vehicle.

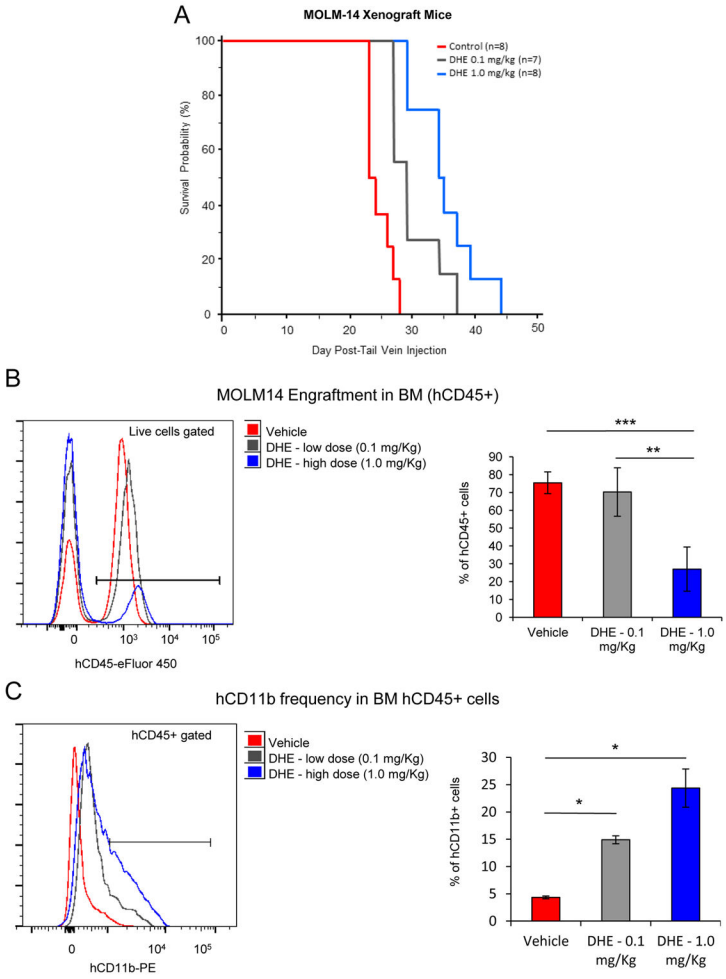

Figure 6. DHE delays AML progression in mice

(A) Kaplan-Meier survival plots of mice treated with vehicle (n=8), 0.1mg/kg DHE (n=7) or 1.0mg/kg DHE (n=8) twice daily (p=0.002, DHE 0.1mg/kg, P<0.001, DHE1.0mg/kg). (B) BM frequency of CD45+ cells at necropsy. (C) CD11b levels in CD45+ gated cells. Results expressed as mean ± SD, n=4 (Vehicle), n=3 (DHE 0.1 mg/Kg), n=4 (DHE 1.0 mg/Kg), * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.