Differentiation therapy for the treatment of t(8;21) Acute Myeloid Leukemia using histone deacetylase inhibitors

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KEY POINTS

- HDACi-mediated differentiation therapy is a potent and molecularly rational treatment strategy in t(8;21) AML.

ABSTRACT

Epigenetic modifying enzymes such as HDACs, p300 and PRMT1 are recruited by AML1/ETO, the pathogenic protein for t(8;21) AML, providing a strong molecular rationale for targeting these enzymes to treat this disease. While early phase clinical assessment indicated that treatment with HDAC inhibitors (HDACis) may be effective in t(8;21) AML patients, rigorous pre-clinical studies to identify the molecular and biological events that may determine therapeutic responses have not been performed. Using an AML mouse model driven by expression of AML1/ETO9a we demonstrated that treatment of mice bearing t(8;21) AML with the HDACi panobinostat caused a robust anti-leukemic response, that did not require functional p53 nor activation of conventional apoptotic pathways. Panobinostat triggered terminal myeloid differentiation via proteasomal degradation of AML1/ETO9a. Importantly, conditional AML1/ETO9a deletion phenocopied the effects of panobinostat and other HDACi, indicating that destabilization of AML1/ETO9a is critical for the anti-leukemic activity of these agents.
INTRODUCTION

Modulation of chromatin through histone modification is essential in regulating fundamental biological processes such as gene transcription and DNA repair\(^1\). The biological consequences are determined by the combinatorial pattern of the histone modifications, which is regulated by specific enzymes that are classified into ‘writers’, ‘erasers’ and ‘readers’. Writers, such as histone acetyltransferases (HATs) or histone methyltransferases (HMTs) add distinct chemical modifications to histones, while erasers, including histone deacetylases (HDACs) and histone demethylases, remove these modifications. Lastly, epigenetic readers specifically recognize modified histones and recruit various effector molecules involved in transcriptional and chromatin regulation\(^2\). Lysine acetylation - one of the major regulatory histone modifications - is controlled by the opposing activities of HATs and HDACs\(^3,4\). In addition to modifying gene transcription via histone acetylation, HATs/HDACs also target non-histone proteins affecting their stability, localization and function\(^5,6\). In mammals eighteen HDACs have been identified, which can be divided into four classes based on structure and cellular localization\(^4\). The majority of these HDACs, i.e. class I, II and IV, depend on zinc\(^{2+}\) for their activity, while only class III HDACs (referred to as sirtuins) require NAD\(^+\).

While cancer historically has been viewed as a disease originating from genetic alterations, recent findings have implicated epigenetic aberrations in the initiation and progression of human cancer\(^7\). For example, expression of the tumor-suppressor genes \textit{CDKN2A}, \textit{APC} and \textit{MLH1}, is commonly silenced through promoter hypermethylation, while in hematological cancers histone modifying enzymes such as HDACs and HMTs are aberrantly localized to the genome through recruitment via
oncogenic fusion proteins\textsuperscript{7,8}. The recent discovery of recurrent mutations in genes encoding the DNA methylation regulators \textit{DNMT3A} and \textit{TET2}, the HMT \textit{EZH2} and the HATs \textit{CREBBP} and \textit{EP300} in human tumors further underpin the importance of epigenetic aberrations in tumorigenesis. Unlike genetic mutations, these are potentially reversible, implicating approaches to target epigenetic writers, erasers and readers as promising and feasible strategies for cancer therapy\textsuperscript{2,9}.

Numerous HDAC inhibitors (HDACis) have been developed, the majority of which lack isoform-selectivity and broadly inhibit various zinc\textsuperscript{2+}-dependent HDACs\textsuperscript{10,11}. HDACis demonstrate single-agent clinical activity against various hematological malignancies, including different T-cell lymphomas and acute myeloid leukemia (AML)\textsuperscript{12-18}, which has led to the FDA approval of vorinostat (targeting class I, II and IV HDACs) as well as the more selective HDACi romidepsin (class I HDAC-specific) for treatment of cutaneous T-cell lymphoma. The exact mechanism(s) responsible for the observed HDACi-mediated anti-tumor activity is still largely unclear, and may depend on tumor type and the tumor genetic background.

The AML1-ETO fusion protein produced as a result of the t(8;21) chromosomal translocation is pathogenic for AML in collaboration with secondary mutagenic hits to genes such as \textit{FLT3}, c-\textit{KIT}, \textit{N-RAS} and \textit{K-RAS}\textsuperscript{19}. AML1-ETO recruits a vast array of transcription factors and epigenetic regulatory proteins (e.g. C/EBPa, GATA1, E proteins, HDACs, p300, PRMT1, SON) and complexes (e.g. N-CoR, AETFC)\textsuperscript{20-29} that putatively play important roles in the onset and/or progression of t(8;21) AML. Several studies have proposed the therapeutic potential of HDACis in t(8;21) AML\textsuperscript{30-33}, but some controversy exists regarding the mechanism by which HDACis mediate
their effect. Treatment of A/E-positive Kasumi-1 cells with HDACis \textit{in vitro} leads to tumor cell differentiation, which in some instances is accompanied by apoptosis\textsuperscript{32,33}. In contrast, the single study so far that has tested HDACis \textit{in vivo} for t(8;21) AML indicated that cellular differentiation was not essential for the therapeutic effect observed. That study suggested that HDACi efficacy relied on the extrinsic apoptotic pathway mediated primarily by TRAIL and its cognate receptor (DR5), which are both upregulated by HDACis in a tumor cell-selective manner\textsuperscript{31}. A potential confounding issue with most published studies testing the applicability of HDACis in t(8;21) AML is the use of valproic acid (VPA) as the HDACi. VPA is at best a weak HDACi\textsuperscript{34} resulting in its use at millimolar concentrations to induce histone hyperacetylation, raising concerns about potential off-target effects.

Herein we used a recently developed tractable mouse model of t(8;21) AML\textsuperscript{35} that is to identify the molecular and biological events that underpin the therapeutic effects of HDACi. We focused on panobinostat, which has strong inhibitory activity at low nanomolar concentrations against class I, II and IV HDACs\textsuperscript{34,36}. In this transplantable model, AML develops as a result of the coexpression of AML1/ETO\textsuperscript{9a} (A/E\textsuperscript{9a})\textsuperscript{37} and oncogenic Nras (Nras\textsuperscript{G12D}). In A/E\textsuperscript{9a};Nras\textsuperscript{G12D}-driven leukemia HDAC inhibition triggered proteasomal degradation of AE/9a leading to terminal differentiation, subsequent decrease in tumor burden and therapeutic efficacy. To our knowledge, this is the first definitive evidence that HDACi-mediated differentiation of t(8;21) AML cells results in a clear therapeutic benefit. Moreover, we have identified the biological and molecular processes that underpin the therapeutic effects of HDACis in this setting.
MATERIALS AND METHODS

Experimental animals and materials
C57BL/6 and C57BL/6.SJL-Ptprca mice were purchased from The Walter and Eliza Hall Institute of Medical Research and Animal Resources Centre respectively. Nzeg-enhanced GFP (eGFP) mice were obtained from Prof. Klaus Matthaei (Australian National University). All mice were used in accordance with the institutional guidelines of Peter MacCallum Cancer Centre. Panobinostat was provided by Novartis and prepared as a 2mg/ml solution in 5% dextrose/dH2O (D5W). Cytarabine was obtained from the Peter MacCallum Cancer Centre and further diluted in PBS to a 12.5mg/ml solution.

Fetal liver cell isolation, retroviral transduction and transplantation
E13.5-15 fetal liver cells were cultured in the presence of IL-3, IL-6 and SCF. For production of retrovirus-containing supernatant, Phoenix packaging cells were transfected with MSCV-AML1/ETO9a-IRES-GFP (A/E9a), MSCV-luciferase-IRES-NrasG12D, MSCV-MLL-AF9-IRES-VENUS (M/A), MSCV-MLL-ENL-IRES-GFP (M/E), MSCV-Bcl-2-IRES-mCherry, TREtight-dsRed-IRES-AML1/ETO9a or MSCV-NrasG12D-IRES-tTA expression constructs. Supernatants were mixed in a 1:1 ratio and used to transduce fetal liver cells in RetroNectin (TaKaRa Bio)-precoated 6-well plates. Two days after the last viral hit, 1x10^6 total cells per recipient were injected i.v. into lethally irradiated C57BL/6 mice.

Monitoring leukemia
Whole-body bioluminescent imaging (BLI) was assessed with an IVIS100 imaging system (Caliper LifeSciences). Mice were injected intraperitoneally with 50mg/kg D-Luciferin (Caliper LifeSciences), anesthetized with isoflurane, and imaged for 2min
after a 15min incubation following injection. Blood (~30µl) was obtained from the retro-orbital sinus. White cell counts were measured using an Advia 120 automated hematology analyzer (Bayer Diagnostics) and percentage of GFP-positive or dsRed-positive cells were analyzed by flow cytometry. At terminal disease stage, mice were sacrificed and leukemia cells were isolated from bone marrow (femur) and spleen. Single cell suspensions were prepared and cells were cryopreserved in FCS/10% DMSO.

**In vivo experiments**

Leukemia cells (1x10^6) were transplanted into non-irradiated (M/E;Nras^{G12D} and M/A;Nras^{G12D} leukemias) or sub-lethally irradiated mice (A/E9a;Nras^{G12D} leukemias) by i.v. injection. Treatment was initiated once leukemia was clearly established as demonstrated by visible bioluminescence and/or when there were 5-20% GFP-positive cells in peripheral blood. Mice were treated daily with panobinostat (25mg/kg, five consecutive days per week i.p. injection for one week followed by 15mg/kg for three to four weeks) or vorinostat (200mg/kg, seven consecutive days per week i.p. for one week followed by 150mg/kg for three to four weeks). This dosing schedule of panobinostat was well tolerated by recipient mice (Supplementary Fig 1A). Control mice received the equivalent volume of vehicle. Mortality events from advancing leukemia were recorded for the analysis of therapeutic efficacy.

For short-term drug response studies, mice received 25mg/kg panobinostat, 100mg/kg cytarabine or equivalent volume of vehicle by i.p. injection once daily for indicated period prior to harvesting of spleen and bone marrow.

For inducible knockdown studies, mice received 2mg/ml doxycycline via drinking water (2% saccharose) and food two weeks after reconstitution of mice with transduced Nzeg-eGFP fetal liver cells.
**Flow cytometry**

Cell suspensions were incubated in red cell lysis buffer (150mM NH₄Cl, 10mM KHCO₃, 0.1mM EDTA) and washed twice in FACS staining buffer (PBS supplemented with 1% FCS and 0.02% NaN₃). Cells were pre-incubated with anti-CD16/CD32 (2.4G2) and stained on ice with antibodies specific for c-Kit (CD117) and Mac-1 (CD11b) (both BD Biosciences) in FACS staining buffer for 30min. Data were collected on a FACSCanto II flowcytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star).

**Cell Culture**

Kasumi cells were cultured in RPMI-1640 + 10% Fetal calf serum (FCS) while A/E9a were cultured in a high-glucose version of DMEM supplemented with 10% FCS, Primary human t(8;21) cells were cultured at a starting density of 7.5 × 10⁵ cells/ml in serum-free media consisting of IMDM supplemented with 20% BIT (Stem Cell Technologies), 100ng/ml SCF, 100ng/ml FLT3 ligand, 100ng/ml TPO and 20ng/ml IL-6 (all Peprotech) and stimulated with panobinostat for 6 or 24h at the indicated concentration.

**Western Blot analysis and Immunoprecipitation**

Cell lysates were separated by SDS/PAGE and transferred onto PVDF membranes (Millipore). Membranes were blocked with 5% non-fat milk in TBS/0.1% Tween-20 at room temperature and incubated overnight with antibodies against AML1 (Cell Signaling Technology, 4336), HDAC1 (Cell Signaling Technology, 5356), p16 (Santa Cruz, M-156), p21 (Santa Cruz, F-5), phosphorylated RB (BD Biosciences, 554136), GFP (Invitrogen, A6455), histone H3 (Abcam, AB1791), acetyl-histone H3 (Millipore, 06-599), Hsp90 (Stressgen, SPA830), GAPDH (Abcam, AB9484) or beta-actin (Sigma, A2228) at 4°C. Membranes were developed using appropriate
horseradish peroxidase (HRP)-coupled secondary antibodies (Dako) and enhanced chemiluminescence (GE Healthcare).

For immunoprecipitations, cell lysates were precleared by incubation with protein A or G sepharose beads (GE Healthcare) for 30 min, washed in lysis buffer, incubated with anti-AML-1 or anti-Hsp90 antibodies for 30 min and then crosslinked to protein A or G sepharose beads at 4°C overnight. Beads were washed 5 times with lysis buffer before addition of 5x denaturing loading buffer, heating at 95°C and analysis by SDS-PAGE.

**Quantitative RT-PCR**

RNA was isolated using commercially available kit (Qiagen). Synthesis of cDNA was performed following standard protocols using MMLV Reverse Transcriptase (Promega). Quantitative RT-PCR was performed using SYBR Green (Applied Biosystems) method in a 384-well format using the ABI Prism7900HT (Applied Biosystems). For quantification, the Cₜ values were obtained and normalized to the Cₜ values of HPRT gene. Fold changes in expression were calculated by the 2⁻DDCT method.

**Histology**

Mice femora were fixed in 10% neutral buffered formalin before decalcification using formic acid and embedding in paraffin. TUNEL staining was performed using the Apoptag Peroxidase In Situ Apoptosis Detection Kit (Chemicon) kit. Sections were counterstained using hematoxylin and images were recorded with a Zeiss microscope and a 20x lens. For cytospins, bone marrow cells were stained with Romanowski stain solution.
Statistical analysis

Kaplan-Meier survival curves were created and survival of mice (using a log-rank test) was statistically analyzed with GraphPad Prism software (GraphPad software).

All other statistical analyses (using a two-tailed unpaired t test) were also performed with this software.
RESULTS

A/E9a;NrasG12D-driven leukemia is susceptible to the HDACi panobinostat

Recipient mice transplanted with fetal liver cells transduced with retroviral vectors encoding A/E9a (coexpressing GFP), and NrasG12D (coexpressing firefly luciferase) developed AML. Leukemic cells expressed A/E9a (Figure 1A), were GFP-positive (Figure 1B) and expressed c-Kit (CD117), but not Mac-1 (CD11b) or other lineage markers (Figure 1B and data not shown). Consistent with the previously identified interaction between A/E and HDACs20,21,38-40, immunoprecipitation assays confirmed the interaction between HDAC1 and A/E9a in primary A/E9a;NrasG12D leukemic cells (Figure 1C). Similar experiments were performed to determine if HDAC2 and -3 also associated with A/E9a, and while HDAC2 immunoprecipitated with A/E9a, no physical interaction was detected between HDAC3 and A/E9a (data not shown). Treatment with panobinostat had no acute effect on the interaction of HDACs with A/E9a (Figure 1C and data not shown).

C57BL/6 mice transplanted with leukemia cells from a single A/E9a;NrasG12D leukemia mouse demonstrated a clear reduction in tumor burden following panobinostat treatment (Figures 1D-F) resulting in a pronounced survival benefit (Figure 1G). Similar therapeutic responses with panobinostat were observed in C57BL/6 mice transplanted with cells from a different A/E9a;NrasG12D leukemia mouse (Supplemental Figure 1B). Treatment of wild type C57BL/6 mice with panobinostat using the same treatment regimen as used for the treatment of tumor-bearing mice resulted in a slight decrease in peripheral white blood counts and little or no weight loss or other signs of toxicity (unpublished observations). Moreover, vorinostat, an FDA-approved HDACi also mediated a reduction in tumor burden and...
prolonged survival of A/E9a;NrasG12D tumor-bearing mice (Supplemental Figures 1C-D). We additionally determined if chronic exposure to panobinostat in vivo altered the phenotype of A/E9a;NrasG12D tumors or induced acquired resistance to the compound. A/E9a;NrasG12D tumors harvested from tumor-bearing mice treated with panobinostat for 4 cycles were assessed by flow cytometry for GFP expression and expression for c-Kit and Mac-1. As shown in supplemental figure 1E, cells chronically exposed to panobinostat retained their immature AML phenotype. Moreover, mice transplanted with GFP-positive A/E9a;NrasG12D tumors harvested from mice that had received eight cycles of panobinostat treatment and subsequently treated with panobinostat showed a robust therapeutic response (Supplemental Figure 1F), similar to mice bearing A/E9a;NrasG12D tumors not previously exposed to panobinostat.

To determine whether panobinostat was also effective in other AML subtypes, we generated leukemias in mice coexpressing the MLL fusion proteins MLL/ENL (M/E) or MLL/AF9 (M/A) and NrasG12D. Recipient mice were transplanted with leukemic cells and mice bearing secondary transplanted tumors were treated with panobinostat resulting in a marginal effect on leukemia progression and survival (Figures 1H-I and Supplemental Figures 2A-C).

Response to panobinostat is independent of a functional p53 pathway

Standard treatment for AML patients currently consists of cytarabine in combination with an anthracycline such as doxorubicin. Many standard chemotherapeutics rely on an intact p53 pathway for an optimal therapeutic response and we have shown that p53-deficient A/E9a;NrasG12D leukemias were refractory to standard chemotherapy (Supplemental Figure 3A). We previously showed that the apoptotic
and therapeutic effects of HDACis in a B-cell lymphoma model occurred in the absence of wild type p53, and we wished to investigate the response of p53-deficient A/E9a;Nras<sup>G12D</sup> leukemias to panobinostat. Panobinostat reduced the leukemic burden and significantly prolonged the survival of mice bearing highly aggressive A/E9a;Nras<sup>G12D</sup>/p53<sup>/−</sup> leukemias (Figures 2A-C, Supplemental Figure 3B). These results suggest that HDACis may provide a therapeutic option in p53-deficient AML that is commonly resistant to conventional induction therapy.

**Induction of apoptosis is dispensable for a therapeutic response to panobinostat**

Based on previous studies showing a direct link between HDACi-induced tumor cell apoptosis and pre-clinical efficacy, we generated A/E9a;Nras<sup>G12D</sup> leukemias with defined genetic alterations in both the extrinsic (knockout of either TRAIL or its receptor DR5) and the intrinsic (overexpressing Bcl-2, Supplemental Figure 4A) apoptotic pathways. Although Dr5 expression increased upon treatment with panobinostat (Figure 3A), A/E9a;Nras<sup>G12D</sup> leukemias with deletion of Dr5 (Figure 3B-C) or Trail (Supplemental Figure 5A-B) retained profound sensitivity to HDACi treatment. Importantly, the potent anti-leukemic activity of panobinostat was comparable in independently-derived DR5<sup>/−</sup> or TRAIL<sup>/−</sup> A/E9a;Nras<sup>G12D</sup> leukemias (Figure 3D and Supplemental Figures 5C-D and 6). These data provide definitive evidence that a functional TRAIL pathway is not required for HDACi to mediate a robust therapeutic effect against t(8;21) AML.

Overexpression of Bcl-2 (Supplemental Figure 4A) also had no effect on the sensitivity of A/E9a;Nras<sup>G12D</sup> leukemias to panobinostat in vivo (Figures 4A-B). Mice bearing these leukemias treated with panobinostat clearly had a survival benefit over
vehicle-treated mice (Figure 4C and Supplemental Figure 4B). These data imply that
tumor cell apoptosis was not the primary biological response of A/E9a;Nras\(^{G12D}\)
leukemias to panobinostat. Consistent with this observation, little or no TUNEL-
positive tumor cells were detected in leukemia-bearing mice following treatment with
panobinostat (Figure 4D and Supplemental Figure 7). In contrast, treatment with
cytarabine led to a substantial increase in TUNEL-positive tumor cells (Figure 4D and
Supplemental Figure 7). Despite their failure to undergo apoptosis in response to
panobinostat, A/E9a;Nras\(^{G12D}\) leukemias were reduced in spleen, bone marrow and
peripheral blood within 3-7d post treatment (Figure 4E). Hence, while
A/E9a;Nras\(^{G12D}\) leukemias can undergo apoptosis in response to certain anti-cancer
agents, our data strongly implicate a non-apoptotic primary effector mechanism for
panobinostat.

Panobinostat induces differentiation of A/E9a;Nras\(^{G12D}\) AML.

Treatment of primary A/E9a;Nras\(^{G12D}\) leukemias with panobinostat in vitro resulted in
a reduction in the number of cells in S-phase (Figure 5A) and biochemical changes
characteristic with a block in cell cycle at the G1/S checkpoint including increased
expression of p21\(^{WAF1/CIP1}\) and p16\(^{INK4A}\), and hypophosphorylation of pRb (Figure
5B). These changes in cell proliferation were concomitant with a decrease in
expression of c-Kit following treatment with panobinostat for 24 or 48h (Figure 5C).
No change in c-Kit expression was seen following treatment of A/E9a;Nras\(^{G12D}\) cells
with etoposide (Figure 5C) demonstrating the specific effect of panobinostat.
Concomitant with panobinostat-mediated induction of tumor cell cycle arrest and
decreased expression of c-Kit an increase in mRNA for the pro-myeloid
differentiation transcription factors *PU-1*, *GATA-2*, *SCL* and *C/EBP alpha* was observed (Figure 5D).

To confirm that panobinostat was inducing tumor cell differentiation the effect of panobinostat on A/E9a;Nras\(^{G12D}\) leukemias *in vivo* over a 7d time-course demonstrated increased levels of *GATA-2*, *SCL* and *C/EBP alpha* in GFP-positive A/E9a;Nras\(^{G12D}\) leukemic cells 72h after exposure to the compound (Figure 5E and Supplemental Figure 8). Flow cytometry demonstrated a time-dependent decrease of GFP-positive A/E9a;Nras\(^{G12D}\) leukemic cells expressing c-Kit (Figure 5F) concomitant with an increase in cells expressing the myeloid differentiation marker Mac-1 (Figure 5G). Histological analysis revealed that A/E9a;Nras\(^{G12D}\) leukemic cells from panobinostat-treated leukemic mice displaying a more granulocyte/macrophage-like appearance (Figure 5H).

**Panobinostat mediates proteasome-dependent degradation of A/E9a.**

It has previously been observed that treatment of human Kasumi-1 cells with the class I-selective HDACi romidepsin resulted in apoptosis and reduced expression of A/E\(^{33}\). Treatment of A/E9a;Nras\(^{G12D}\) leukemic blasts with panobinostat *in vitro* for as little as 6h caused a marked decrease in expression of A/E9a concomitant with histone H3 hyperacetylation (Figure 6A). Importantly, there was no change in expression of GFP, which in our experimental system is coexpressed with A/E9a (Figure 6A), and there was no substantial change in the levels of A/E9a mRNA over a 24h time-course (data not shown). We next treated Kasumi-1 cells with panobinostat and demonstrated a similar decrease in A/E with kinetics similar to those observed using the mouse A/E9a;Nras\(^{G12D}\) leukemias (Figure 6B). In addition, primary human t(8;21) AML
cells treated with panobinostat also showed time-dependent decrease in A/E (Figure 6C, D). These data demonstrate that panobinostat induces degradation of full length A/E and the A/E9a fusion protein with similar kinetics. Co-treatment of A/E9a;NrasG12D leukemic cells with panobinostat and the proteasome inhibitor MG-132 partially rescued the panobinostat-mediated decrease in A/E9a (Figure 6E), suggesting that panobinostat triggered proteasome-mediated decay of A/E9a. A/E has previously been shown to be a client protein for the molecular chaperone Hsp9033, suggesting that HDACi-mediated hyperacetylation of Hsp90 may be sufficient to mediate degradation of A/E9a. Treatment of A/E9a;NrasG12D leukemic blasts in vitro with the Hsp90 inhibitor 17-AAG resulted in a rapid loss in expression of A/E9a (Figure 6F), supporting previous studies demonstrating that A/E is a client protein of Hsp9033 and that disruption in the association of A/E9a with Hsp90 may trigger its degradation. Immunoprecipitation assays showed that A/E9a physically interacted with Hsp90, which was diminished upon treatment with panobinostat (Figure 6G) and marginally reduced following treatment with 17-AAG (Supplemental Figure 9). Taken together, these data demonstrate that A/E9a is an Hsp90 client protein and treatment with panobinostat promotes dissociation of A/E9a from Hsp90 and supports a model in which hyperacetylation of Hsp90 results in subsequent ubiquitination and proteasomal degradation of A/E9a.

**Inducible deletion of A/E9a phenocopies the effect of panobinostat.**

To determine if genetic deletion of A/E9a phenocopied the effects of panobinostat we established a model of t(8;21) driven by doxycycline-inducible expression of A/E9a and constitutive expression of oncogenic Nras. Mice were transplanted with Nzeg-eGFP fetal liver cells transduced with two retroviral vectors, one encoding dsRed-
linked A/E9a under the control of a tetracycline response element (TREtight) promoter and the other encoding Nras$^{G12D}$ coexpressing the “Tet-off” tetracycline transactivator (tTA), which shuts off the expression TRE-regulated genes in a doxycycline-dependent manner. Primary GFP-positive, dsRed-positive Tet-off A/E9a;Nras$^{G12D}$ leukemias were established in mice which were then treated with doxycycline or vehicle. *In vivo* exposure to doxycycline efficiently silenced A/E9a and dsRed protein (Figure 7A and Supplemental Figure 10A-C), leading to a substantial survival advantage compared to vehicle-treated mice (Figure 7B). Mice that relapse while being treated with doxycycline present with morphological features of A/E9a;Nras$^{G12D}$ leukemia including the presence of GFP-positive, dsRed-positive cells in the peripheral blood and bone marrow (unpublished observations) which suggests that the Tet-off system used to express A/E9a in a regulated manner is not 100% effective in a small proportion of cells and these cells eventually grow out to kill the mouse.

As seen following *in vivo* treatment of mice bearing A/E9a;Nras$^{G12D}$ leukemias with panobinostat, genetic depletion of A/E9a was accompanied by a decrease in c-Kit-positive cells (Figure 7C), an increase in Mac-1-positive cells (Figure 7D), and an increase in genes (*M/CSF receptor, CEBPalpha* and *PU.1*) associated with myeloid differentiation (Figure 7E). Moreover, histological analysis revealed that A/E9a;Nras$^{G12D}$ leukemic cells harvested from doxycycline-treated leukemic mice assumed a differentiated granulocyte/macrophage-like morphology similar to that observed following treatment with panobinostat (Supplementary Figure 10D). These data demonstrate that a reduction in the expression of A/E9a in primary leukemias phenocopies the molecular, biological and therapeutic effects of panobinostat treatment.
DISCUSSION

Aberrant recruitment of HDACs by A/E provides a strong molecular rational to use HDACis to treat t(8;21) AML. Initial studies aimed to address this hypothesis predominantly used in vitro assays, a single human cell line (Kasumi) and a weak HDAC inhibitor (valproic acid) that needs to be used at millimolar concentrations to induce histone hyperacetylation. While these studies hinted that an HDACi may be effective in this setting, therapeutic insights were lacking and it was unclear if tumor cell apoptosis or differentiation was the primary effector mechanism of HDACis. Herein, we describe the therapeutic, biological and molecular effects of the HDACis vorinostat and panobinostat in an established and transplantable mouse model of t(8;21) AML. Panobinostat was effective in reducing tumor burden in A/E9a;NrasG12D leukemia-bearing mice, whereas those transplanted with leukemias expressing MLL/ENL;NrasG12D or MLL/AF9;NrasG12D were more resistant to panobinostat therapy. Interestingly, the primary biological response to panobinostat in A/E9a;NrasG12D leukemia was induction of differentiation rather than apoptosis, preceded by degradation of the A/E9a fusion protein through the release of A/E9a from Hsp90. Furthermore, we used an inducible A/E9a expression system to demonstrate that established A/E9a;NrasG12D leukemias were dependent on A/E9a for their long-term survival, and that genetic depletion of A/E9a phenocopied the biological and therapeutic effects of panobinostat. These results provide important insight into the working mechanism of HDACis in A/E9a;NrasG12D-driven AML, highlight the continued dependence of A/E9a;NrasG12D leukemias on the presence of A/E9a and imply that targeting of this oncogenic fusion protein and using its expression as a biomarker for response to HDACis may have important implications for future clinical development.
APL driven by the PML/RARα fusion protein is a primary example in which the relationship between clinical outcome and therapy-induced degradation of a single oncoprotein is evident. Although treatment of t(15;17) APL with retinoic acid (RA) results in differentiation and leukemia regression, this monotherapy rarely leads to prolonged remissions. Combination with arsenic however clearly improves clinical efficacy of RA, and the key determinant to this clinical success may be the loss of leukemia-initiating cells through the degradation of PML/RARA. Similar improvements in therapeutic responses have also been observed for the combination of RA with VPA, which correlated with more pronounced degradation of PML/RARA. Our data show that in A/E9a;Nras<sup>G12D</sup>-driven leukemia, treatment with panobinostat as monotherapy results in a therapeutic response that is associated with myeloid differentiation and degradation of A/E9a. We noted that upon cessation of panobinostat treatment, mice relapsed with disease that was pathologically and phenotypically similar to the original disease. We posit that using the regime undertaken in this study, panobinostat was unable to induce differentiation of all A/E9a;Nras<sup>G12D</sup> tumors and mice therefore subsequently relapsed. This is supported by our studies demonstrating that panobinostat remained fully effective in mice transplanted with A/E9a;Nras<sup>G12D</sup> cells harvested from mice previously treated with panobinostat (Supplemental figure 1E). Moreover, FACS analysis of A/E9a;Nras<sup>G12D</sup> cells harvested from mice treated with vehicle or panobinostat for 4 weeks revealed no change in hemopoietic cell surface markers (Supplemental figure 1D). Finally, we have performed studies showing transplantation of only one A/E9a;Nras<sup>G12D</sup> tumor into recipient mice was sufficient to induce fatal disease in recipient mice.
Depletion of A/E9a in established leukemias phenocopied the effect of panobinostat, supporting a model in which HDACi-mediated degradation of A/E9a is the primary biological event involved in the elimination of leukemic cells and the therapeutic response observed. As expression of other oncogenes, such as Nras<sup>G12D</sup>, was maintained in the experimental systems used, this implies that A/E9a;Nras<sup>G12D</sup> AMLs are addicted to A/E9a and that other compounds such as Hsp90 antagonists that indirectly target A/E9a may also be therapeutically effective. Interestingly and in contrast to A/E9a, MLL fusion proteins do not require Hsp90 as a chaperone and as such expression levels of MLL fusion proteins are not affected upon treatment with 17-AAG<sup>53</sup>. Given the mechanism of action of panobinostat in A/E9a;Nras<sup>G12D</sup> AMLs, this may explain why the therapeutic responses observed in our MLL fusion protein-expressing AMLs were relatively poor.

Previous studies proposed that tumor-specific induction of the TRAIL death receptor pathway was responsible for the anti-tumor effects of HDACis<sup>31,46</sup>. Consistent with those studies, we found that Dr5 was transcriptionally activated following treatment of A/E9a;Nras<sup>G12D</sup> leukemias with panobinostat. However, A/E9a;Nras<sup>G12D</sup> leukemias with genetic knockout of either Dr5 or Trail were equally sensitive to panobinostat treatment as unmodified A/E9a;Nras<sup>G12D</sup> leukemias providing definitive genetic evidence that the TRAIL signaling pathway plays no role in mediating the anti-tumor response to HDACis in A/E9a-driven leukemia. In contrast to our previous studies using the Eμ-myc model of B cell lymphoma, which demonstrated a direct link between activation of the intrinsic apoptosis pathway and therapeutic efficacy<sup>43,44</sup>, we saw no evidence of apoptosis following treatment of A/E9a tumors with panobinostat. These findings indicate that the molecular and biological effector mechanisms of
HDACis identified for one tumor type may not be directly translatable to another and highlight the importance of testing anti-cancer drugs in physiologically relevant and genetically tractable pre-clinical models of disease. Panobinostat treatment also provided a significant survival benefit to mice bearing A/E9a;Nras\textsuperscript{G12D}/p53\textsuperscript{--} leukemias however the survival benefit was less pronounced than that observed using A/E9a;Nras\textsuperscript{G12D} with wild type p53. We and others have demonstrated that a functional p53 is not required for HDACis to induce tumor cell apoptosis (see ref 10 and references therein). However, it is possible that p53 is required for HDACis to induce a robust differentiation response as has been recently demonstrated for the treatment of MLL-ENL/Hras\textsuperscript{V12D}-driven AML treated with DNA damaging agents\textsuperscript{54}.

Although A/E has been shown to be a direct target of HATs and in fact the transforming activity of A/E is dependent of p300-mediated acetylation of the fusion protein\textsuperscript{27}, we saw no change in the acetylation status of A/E9a following HDACi treatment (data not shown). Our finding that HDACi-induced degradation of A/E9a in primary leukemic cells is mediated through release of A/E9a from the molecular chaperone Hsp90 is consistent with previous observations using HDACi in the human t(8;21) cell line Kasumi-1\textsuperscript{33}. It has been proposed that the chaperone activity of Hsp90 is regulated through deacetylation by HDAC6\textsuperscript{55} and HDACi-induced hyperacetylation of Hsp90 then leads to release and subsequent proteasome-mediated degradation of client proteins such as A/E9a. However, it is controversial whether HDAC6-specific deacetylation of Hsp90 plays an important role in stabilizing A/E9a protein. Using romidepsin, a very weak inhibitor of HDAC6, we still observed degradation of A/E9a (data not shown). This suggests that inhibition of class I HDACs is sufficient to
mediate degradation of A/E9a and is consistent with recent studies by us and others demonstrating that class I HDACs can regulate the acetylation status of Hsp90\textsuperscript{56}.

In summary, we demonstrated that the therapeutic efficiency of HDACis in A/E9a AML is mediated by degradation of A/E9a and subsequent differentiation of leukemic cells along the myeloid lineage. Our data showing therapeutic responses in leukemias with knockout of \textit{p53}, \textit{Dr5} or \textit{Trail}, or overexpressing the anti-apoptotic protein Bcl-2, provides preclinical evidence that panobinostat will be effective for the treatment of t(8;21) AML and will have a clear advantage over conventional therapeutic agents in leukemias that have acquired resistance toward apoptosis, such as leukemias deficient for p53. Our findings, further supported by the positive responses previously observed in t(8;21) patients treated with HDACi\textsuperscript{14}, indicate that HDACi-mediated differentiation therapy is an attractive and molecularly rational treatment strategy for this type of cancer.
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Author contribution: M.B. designed research, performed research, collected, analyzed and interpreted data, performed statistical analysis, and wrote the manuscript; I.V., B.P.M., J.M.S., M.G., K.S., A.B. and J.S. performed research and collected, analyzed and interpreted data; J.Z. designed research and contributed vital new reagents; A.R.R, P.A., G.J.O. and S.W.L. contributed vital new reagents; R.W.J. designed research, interpreted data and wrote the manuscript.

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Figure Legends

Figure 1: The HDACi panobinostat demonstrates therapeutic efficacy in a mouse model of A/E9a;Nras^{G12D}- but not M/E;Nras^{G12D}-driven AML.

(A) Western blot analysis of whole-cell lysates prepared from cell lines K562 and Kasumi-1 and spleen cells (spl) isolated from a wild-type (WT) and an A/E9a;Nras^{G12D} leukemia recipient mouse using an antibody to AML1 (upper panel). Membrane was stripped and reprobed for GAPDH as loading control (bottom panel).

(B) Flow cytometry analysis of c-Kit and Mac-1 expression in GFP-positive spleen cells isolated from an A/E9a;Nras^{G12D} leukemia recipient mouse. A representative flow cytometry plot is shown.

(C) Immunoprecipitation/western blot analysis of the interaction between A/E9a and HDAC1 in A/E9a;Nras^{G12D} leukemic cells treated with DMSO (D) or 16nM panobinostat (P) for 6h. A control mouse immunoglobulin G (IgG) and antibody to AML1 (AML) were used for immunoprecipitation; antibodies to HDAC1 and AML1 were used for western blotting. The results shown are representative of 3 independent experiments.

(D-F) Total white blood cells (WBC), flow cytometry analysis of leukemic cells in peripheral blood and bioluminescent imaging of C57BL/6 mice bearing A/E9a;Nras^{G12D} tumors treated with panobinostat or vehicle using the standard treatment regimen. In (D and E) each data point represents an individual mouse and horizontal bars represent mean value (***, p<0.0001).

(G) Kaplan-Meier survival curves of treated A/E9a;Nras^{G12D} leukemia recipient mice following initiation of therapy (n=6 for vehicle, n=5 for panobinostat; median survival benefit 84d, p=0.0006). Dotted line indicates final day of treatment.

(H) Bioluminescent imaging of M/E;Nras^{G12D} leukemia recipient mice treated with panobinostat or vehicle using the standard treatment regimen.
Meier survival curves of treated M/E;Nras<sup>G12D</sup> leukemia recipient mice following initiation of therapy (n=6 for vehicle, n=6 for panobinostat; median survival benefit 6d, p=0.0015).

**Figure 2: Response to panobinostat is independent of a functional p53 pathway.**

(A,B) Flow cytometry analysis of leukemic cells in peripheral blood and bioluminescent imaging of A/E9a;Nras<sup>G12D/p53<sup>-/-</sup></sup> leukemia recipient mice treated with panobinostat or vehicle using the standard treatment regimen. In (A) each data point represents an individual mouse and horizontal bars represent mean value (***, p<0.0001). (C) Kaplan-Meier survival curves of treated A/E9a;Nras<sup>G12D/p53<sup>-/-</sup></sup> leukemia recipient mice following initiation of therapy (n=10 for vehicle, n=8 for panobinostat; median survival benefit 21d, p<0.0001). Dotted line indicates final day of treatment.

**Figure 3: Extrinsic apoptotic pathway is dispensable for therapeutic response of panobinostat.**

(A) Quantitative real-time PCR of Dr5 mRNA levels in spleen cells (>80% GFP-positive cells) isolated from A/E9a;Nras<sup>G12D</sup> leukemia recipient mice treated with panobinostat (25mg/kg) or vehicle (D5W) for 4h. Mean value of two individual samples is shown. (B,C) Flow cytometry analysis of leukemic cells in peripheral blood and bioluminescent imaging of A/E9a;Nras<sup>G12D/DR5<sup>-/-</sup></sup> leukemia recipient mice treated with panobinostat or vehicle using the standard therapy regimen. In (B) each data point represents an individual mouse and horizontal bars represent mean value (***, p<0.0001). (D) Kaplan-Meier survival curves of treated
A/E9a;Nras\(^{G12D}/DR5^{-/-}\) leukemia recipient mice following initiation of therapy (n=10 for vehicle, n=10 for panobinostat; median survival benefit 45d, p<0.0001). Dotted line represents final day of treatment.

**Figure 4: Intrinsic apoptotic pathway is dispensable for therapeutic response to panobinostat.**

(A,B) Flow cytometry analysis of leukemic cells in peripheral blood and bioluminescent imaging of A/E9a;Nras\(^{G12D}/Bcl-2\) leukemia recipient mice treated with panobinostat or vehicle using the standard therapy regimen. In (A) each data point represents an individual mouse and horizontal bars represent mean value (***, p<0.0001). (C) Kaplan-Meier survival curves of treated A/E9a;Nras\(^{G12D}/Bcl-2\) leukemia recipient mice following initiation of therapy (n=12 for vehicle, n=8 for panobinostat; median survival benefit 63d, p<0.0001). Dotted line represents final day of treatment. (D) Analysis of apoptotic cells via TUNEL staining. Staining was performed on bone marrow isolated from A/E9a;Nras\(^{G12D}\) leukemia recipient mice treated with panobinostat (25mg/kg) or cytarabine (100mg/kg) for the indicated time. Sections are representative of three (panobinostat) or two (cytarabine) mice per time point. Dark brown cells indicate TUNEL-positive cells. (E) Flow cytometry analysis of GFP-positive leukemic cells in indicated tissue isolated from A/E9a;Nras\(^{G12D}\) leukemia recipient mice treated with panobinostat (25mg/kg) or vehicle (D5W, 250µl) for three days. Data is combined from two individual experiments. Each data point represents an individual mouse and horizontal bars represent mean value (blood: **, p=0.004; spleen: **, p=0.0022 and p=0.0012 respectively; bone marrow: ***, p<0.0001).
Figure 5: Panobinostat treatment of A/E9a;Nras^{G12D} leukemic cells triggers differentiation.

(A) Cell cycle analysis of A/E9a;Nras^{G12D} leukemic cells treated in vitro with vehicle or 16nM panobinostat for the indicated time. Percentage of cells in S-phase (EdU-positive) was determined by flow cytometry. Mean values of two independent experiments are shown; error bars represent s.d. (B) Western blot analysis of whole-cell lysates prepared from A/E9a;Nras^{G12D} leukemic cells treated in vitro with vehicle (D) or 16nM panobinostat (P) for the indicated time using antibodies to p16^{INK4A}, p21^{WAF1/CIP1} and phosphorylated RB. Beta-actin served as loading control. The results shown are representative of 3 independent experiments. (C) Flow cytometry analysis of c-Kit expression in A/E9a;Nras^{G12D} leukemic cells treated in vitro with 16nM panobinostat for the indicated time. Mean values of three independent experiments are shown; error bars represent s.d. (D) Quantitative real-time PCR of relative mRNA levels of target genes in A/E9a;Nras^{G12D} leukemic cells treated in vitro with 16nM panobinostat for 24h. Results were normalized to HPRT mRNA. Mean value of 3 to 6 individual experiments is shown and error bars represent s.d. (E) Quantitative real-time PCR of relative mRNA levels of target genes in GFP-positive splenocytes isolated from A/E9a;Nras^{G12D} leukemia recipient mice 72h after initiation of treatment with panobinostat (25mg/kg) or vehicle (D5W). Results were normalized to HPRT mRNA. Mean value of 3 to 5 individual samples is shown and error bars represent s.e.m. (F,G) Flow cytometry analysis of (F) c-Kit and (G) Mac-1 expression in GFP-positive bone marrow cells isolated from A/E9a;Nras^{G12D} leukemia recipient mice treated with panobinostat (25mg/kg) or vehicle (D5W) for three days. Data is combined from two individual experiments. Each data point represents an individual mouse and horizontal bars represent mean value (in F: *, p=0.0248 and ***, p=0.0026).
p=0.0009; in G: *, p=0.0292). (H) Light microscopy of May-Grunwald/Giemsa-stained bone marrow cells isolated from A/E9a;Nras^{G12D} leukemia recipient mice cells treated with panobinostat (25mg/kg) or vehicle (D5W, 250µl) for five days. Imaging was performed with a 60x objective. Representative images of five biological replicates are shown (scale bar=10µm). GFP-positive cells isolated from 5 day vehicle-treated mice (left panel) demonstrate immature blast morphology, including a fine rim of agranular basophilic cytoplasm with a round to oval nucleus containing ‘open chromatin’ and one or more prominent nucleoli (arrowheads). In contrast, GFP-positive cells from 5 day panobinostat-treated mice (right panel) show features of maturation including a reduction in the nuclear: cytoplasmic ratio, condensation of nuclear chromatin and infrequent nucleolation. Frequent coarse azurophilic cytoplasmic granules (arrowheads) indicate myeloid differentiation.

Figure 6: Panobinostat induces degradation of A/E9a and A/E.

(A) Western blot analysis of whole-cell lysates prepared from A/E9a;Nras^{G12D} leukemic cells treated in vitro with DMSO vehicle (D) or 16nM panobinostat (P) for the indicated time using antibodies to AML1, GFP and acetylated histone H3. Beta-actin served as loading control. The results shown are representative of at least 3 independent experiments. (B) Western blot analysis of whole-cell lysates prepared from Kasumi cells treated in vitro with DMSO vehicle (D) or 8nM panobinostat (P) for the indicated time using antibodies to AML1 and acetylated histone H3. Total histone H3 and Beta-actin served as loading controls. The results shown are representative of at least 3 independent experiments. (C) Western blot analysis of whole-cell lysates prepared from primary t(8;21) AML cells treated in vitro with DMSO (D) or indicated concentrations (nM) panobinostat (P) for 6h
using an antibody to AML1 (upper panel). Membrane was stripped and reprobed for p42 as loading control (bottom panel). (D) Western blot analysis of whole-cell lysates prepared from a different primary t(8;21) AML sample as that shown in (C) treated \textit{in vitro} with DMSO (D) or 8nM panobinostat (P) for the indicated time using an antibody to AML1 (upper panel). Membrane was stripped and reprobed anti-acetyl-tubulin antibody (bottom panel). (E) Western blot analysis of whole-cell lysates prepared from A/E9a;Nras$^{G12D}$ leukemic cells treated \textit{in vitro} with DMSO vehicle (D), 16nM panobinostat (P) for 24h with addition of 5µM MG132 for the final 4h (lanes 1 and 2) using antibodies to AML1, acetylated histone H3, ubiquitin and GFP. The results shown are representative of at least 3 independent experiments. (F) Western blot analysis of whole-cell lysates prepared from A/E9a;Nras$^{G12D}$ leukemic cells treated \textit{in vitro} with vehicle (V), 16nM panobinostat (P) or 100nM of the Hsp90 inhibitor 17-AAG for 24h using antibodies to AML1. Beta-actin served as loading control. The results shown are representative of at least 3 independent experiments. (G) Western blot analysis of the interaction between A/E9a and Hsp90 in A/E9a;Nras$^{G12D}$ leukemic cells treated with vehicle (V) or 16nM panobinostat (P) for 4h. A control mouse immunoglobulin G (IgG) and antibody to Hsp90 were used for immunoprecipitation; antibodies to AML1 and Hsp90 were used for western blotting. The results shown are representative of at least 3 independent experiments.

\textbf{Figure 7: Inducible deletion of A/E9a phenocopies the effect of panobinostat.}

(A) Western blot analysis of whole-cell lysates prepared from GFP-positive spleen cells isolated from Tet-off A/E9a;Nras$^{G12D}$ leukemia recipient mice 72h after
initiation of treatment with doxycycline or vehicle using an antibody to AML1. Beta-actin served as loading control. The results shown are representative of 2 independent experiments. (B) Kaplan-Meier survival curves of treated Tet-off A/E9a;NrasG12D leukemia recipient mice following initiation of therapy (n=5 for vehicle, n=4 for doxycycline; median survival benefit 42d, p=0.0051). (C,D) Flow cytometry analysis of c-Kit and CD11b expression in donor GFP-positive spleen cells isolated from Tet-off A/E9a;NrasG12D leukemia recipient mice at the indicated time after initiation of treatment with doxycycline (2mg/kg) or vehicle. Each data point represents an individual mouse and the mean value +/- SE from two separate experiments is shown (*, p<0.001). (E) Mice bearing Tet-off A/E9a;NrasG12D leukemias were treated with vehicle or doxycycline (dox) for 5 days. Tumors were harvested and expression of the indicated genes was determined by quantitative real-time PCR. Results (mean and s.e.m.) shown are from tumors harvested from five individual recipient mice for each treatment regimen.
Bots et al. Figure 2

A

% GFP⁺ cells

***

time of treatment (d)

B

d0

vehicle

panobinostat


d14

C

Percent survival

veh.

pan.

Time post start treatment (d)
Bots et al. Figure 6

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Relative expression of A/E9a
Differentiation therapy for the treatment of t(8;21) acute myeloid leukemia using histone deacetylase inhibitors