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DOT1L As a Therapeutic Target for the Treatment of DNMT3A-Mutant Acute Myeloid Leukemia

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• Data from Dnmt3a-null mice implicate Dot1l as a critical mediator of the malignant gene expression program of Dnmt3a-mediated leukemia

• Pharmacologic inhibition of DOT1L exerts potent anti-leukemic activity in DNMT3A-mutant human AML in vitro and in vivo
Abstract

Mutations in DNA methyltransferase 3A (DNMT3A) are common in acute myeloid leukemia and portend a poor prognosis, thus new therapeutic strategies are needed. The likely mechanism by which DNMT3A loss contributes to leukemogenesis is altered DNA methylation and the attendant gene expression changes, however our current understanding is incomplete. We observed that murine HSCs in which Dnmt3a had been conditionally deleted markedly overexpress the histone 3, lysine 79 (H3K79) methyltransferase, Dot1l. We demonstrate that Dnmt3a-null HSCs have increased H3K79 methylation relative to wild-type HSCs, with the greatest increases noted at DNA methylation canyons, which are regions highly enriched for genes dysregulated in leukemia and prone to DNA methylation loss with Dnmt3a deletion. These findings led us to explore DOT1L as a therapeutic target for the treatment of DNMT3A-mutant AML. We show that pharmacologic inhibition of DOT1L suppressed the expression of oncogenic canyon-associated genes and led to dose- and time-dependent inhibition of proliferation, induction of apoptosis, cell cycle arrest and terminal differentiation in DNMT3A-muant cell lines in vitro. We show in vivo efficacy of the DOT1L inhibitor EPZ5676 in a nude rat xenograft model of DNMT3A-mutant AML. DOT1L inhibition was also effective against primary patient AML samples with DNMT3A mutations, reducing colony forming capacity and inducing terminal differentiation in vitro. These studies suggest that DOT1L may play a critical role in DNMT3A-mutant leukemia. With pharmacologic inhibitors of DOT1L already in clinical trials, DOT1L could be an immediately actionable therapeutic target for the treatment of this poor prognosis disease.
Introduction

Mutations of the de novo DNA methyltransferase DNA methyltransferase 3A (DNMT3A) occur in approximately 20% of all adult patients with acute myeloid leukemia (AML). Studies indicate that patients with DNMT3A mutations suffer particularly poor prognosis, indicating novel therapies are needed\(^1\)\(^-\)\(^4\). DNMT3A mutations in AML are almost exclusively heterozygous, and over 50% affect the arginine at amino acid position 882 (R882) in the methyltransferase domain. R882-mutant DNMT3A is a hypomorphic protein that also inhibits the remaining wild-type DNMT3A, dramatically reducing cellular DNA methyltransferase activity\(^5\)\(^,\)\(^6\). However, the exact mechanisms by which DNMT3A loss contributes to leukemogenesis are poorly understood. DNA methylation profiling of DNMT3A-mutant AML samples revealed loci with decreased methylation, but, surprisingly, also a small subset of loci with increased methylation\(^1\)\(^,\)\(^4\)\(^,\)\(^7\)\(^,\)\(^8\). These data suggest the pathologic changes in DNA methylation are mediated by additional, unknown factors. Further, there is a relatively poor correlation between changes in DNA methylation and gene expression, indicating complex regulation of the pathologic gene expression program of DNMT3A-mutant AML.

Conditional ablation of Dnmt3a in the murine hematopoietic system results in a dramatic expansion of hematopoietic stem cells (HSCs), a progressive block in differentiation\(^9\), and priming for malignant transformation\(^10\)\(^,\)\(^11\). Whole genome bisulfite sequencing of Dnmt3a\(^/-\) HSCs revealed that the borders of expansive undermethylated regions, termed methylation canyons, are hotspots for DNA methylation loss, which leads to expansion of the canyon\(^12\). Canyons that expand with Dnmt3a deletion are highly enriched for genes dysregulated in human leukemia, including HOX genes\(^12\), suggesting these sites are important in leukemogenesis. Analysis of The Cancer Genome Atlas (TCGA) data confirmed many of these sites have methylation loss in DNMT3A-mutant AML\(^8\)\(^,\)\(^12\), and many canyon-associated genes, including HOX genes, are significantly changed in DNMT3A-mutant AML\(^4\)\(^,\)\(^8\)\(^,\)\(^12\).
In addition to the DNA methylation changes in Dnmt3a−/− HSCs, ChIP-seq and RNA-seq data revealed evidence of perturbations of histone modifications. Given the known functional interaction between DNA methylation and histone modifications, these alterations were intriguing. The observed overexpression of the histone 3, lysine 79 (H3K79) methyltransferase, Dot1l (disrupter of telomere silencing 1-like), was especially interesting, as DOT1L plays a critical role in leukemia with MLL-rearrangements. Pharmacologic inhibition of DOT1L has shown promising pre-clinical activity in MLL-rearranged leukemia and is now being tested in adult and pediatric clinical trials. MLL rearrangements rarely co-occur with DNMT3A mutations in AML. The mutual exclusion of these lesions and the overexpression of Dot1l in our murine model led us to hypothesize that MLL-rearrangements and DNMT3A mutations are distinct epigenetic aberrations that converge on a common mechanism, resulting in dysregulated gene expression mediated by H3K79 methylation. We therefore explored the role of DOT1L in DNMT3A-mediated leukemia and evaluated DOT1L as a therapeutic target for the treatment of this poor prognosis disease.

METHODS

Murine model

Animal procedures were approved by the Animal Care and Use Committee of Baylor College of Medicine. Dnmt3a-KO in C57Bl/6 CD45.2 Dnmt3afloflo-Tamoxifen-cre and control Dnmt3aWTWT-Tamoxifen-cre mice were induced by 5 daily intraperitoneal injections of tamoxifen (1mg/0.1mL corn oil/mouse/day). Eight weeks later, bone marrow was harvested for transplantation and transplanted (1x10^6 per mouse) into lethally irradiated syngeneic CD45.1 recipients.

ChIP-seq

Four months after transplantation, recipient mice were sacrificed, and pooled bone marrow hematopoietic stem cells (HSCs) from Dnmt3aKO and control mice were purified using c-Kit
magnetic enrichment (AutoMACS; Miltenyi Biotec) followed by gating on live cells and sorting for lineage+, Sca1+, CD48, and CD150+ cells (FACSAria; BD Biosciences; antibodies from Becton Dickinson). ChIP-seq was performed on purified HSCs after chromatin cross-linking with 1% formalin followed by cell lysis in SDS buffer. DNA was fragmented by sonication and ChIP performed using anti-H3K79me2 (ab3594; Abcam). Eluted DNA was used to prepare a library (Illumina ChIP-seq kit) and then sequenced on an Illumina HiSeq (100-base paired-end). Raw reads were quality trimmed (Trimgalore) and mapped (mm9)(Bowtie 2.0.6). One tag from each unique position was examined to eliminate signal generated by clonal amplification. Danpos 2.22 was used to assess enrichment of H3K79me2; signal was normalized to total library size and non-specific input background was subtracted. The Danpos profile function (default parameters) was used to analyze H3K79me2 distribution flanking regions of interest: signal centered on Refseq transcription and translation (coding) start sites; average signal over under-methylated regions and DNA methylation canyons normalized to constant length.

**Cell Culture and reagents**

Human leukemia cell lines OCI AML3 and OCI AML2 were provided by Mark Minden, Ph.D. (Ontario Cancer Institute). Cells were grown in RPMI-1640 (Invitrogen) plus 10% FBS, 1% L-glutamine, and 1% pen strep, at 37°C in 5% CO₂. Cell lines were validated by the short tandem repeat method. For *in vitro* experiments, we utilized DOT1L inhibitors SYC-52221 and EPZ00477723 (Epizyme, Inc). For *in vivo* studies EPZ-567622 was used (Epizyme).

**Mass spectrometric analysis**

Nuclear isolation, utilizing a 250 mM sucrose nuclear isolation buffer, followed by acid extraction of histones (0.4 N sulphuric acid) was as previously described26. Histones were derivatized using propionic anhydride approximately27. Peptide samples were analyzed by targeted nano-LC-MS (ThermoFisher U3000 nanoLC and Orbitrap XL mass spectrometer) utilizing pseudo
selected reaction monitoring\textsuperscript{26}. Skyline v3.1 was used for data analysis (MacCoss Lab\textsuperscript{28}); transitions used for fragment ion quantitation are shown in Supplemental Table S1.

Cell proliferation, viability, and colony formation assays

Exponentially growing cells were plated in triplicate in 24-well plates (2x10\textsuperscript{5}/mL; final volume 1mL). For dose-dependent assays, cells were incubated in increasing concentrations of SYC522\textsuperscript{21} or EPZ004777\textsuperscript{23} or DMSO control. For time-dependent assays, cells were incubated in 3 µM EPZ004777\textsuperscript{23} or DMSO control. Every 2-3 days media and compound was replaced and cells were split to 2x10\textsuperscript{5}/mL. At each replating viable cells number was determined (trypan blue). Total cell number is expressed as split-adjusted viable cells per mL. Analysis of apoptosis, cell cycle and cellular differentiation and gene expression changes were performed as described in Supplemental Methods. Viably frozen primary AML samples obtained from MD Anderson Cancer Center or Texas Children's Cancer Center under IRB approved protocols, were thawed quickly and placed in fresh RPMI 1640 plus 10% FBS, 1% L-glutamine, and 1% pen strep. After recovery for 2-3 hours, viable cells were counted and plated in triplicate (5,000 viable cells/plate) in methylcellulose media (H4034; StemCell Technologies) with 3 µM EPZ00477 or DMSO vehicle control. After 12-14 days, plates were scored for colony number and morphology. Cells were isolated from plates, stained for CD45, CD14, CD13 and analyzed by flow cytometry. Cell morphology was examined by H&E staining of cytospins (Cytopro).

Nude Rat xenografts

\textit{In vivo} studies were conducted after review by the animal care and use committee at Charles River Discovery Research Services (Durham, NC). OCI-AML3 cells were implanted subcutaneously into the right flank of female athymic nude rats (Hsd:RH-Foxn1nu, Harlan Laboratories, Inc.). EPZ-5676 was delivered by continuous IV infusion via a catheter surgically implanted in the femoral vein of each rat. Animals were separated into either an efficacy or
PK/PD cohort. Both cohorts were dosed by continuous IV infusion with 35 or 70 mg/kg/day of EPZ-5676. A control group received continuous IV infusion of the vehicle, 5% hydroxypropyl-β-cyclodextrin (HPBCD) in saline. Efficacy was determined after 21 days of drug treatment followed by a 7-day drug holiday. Animals assessed for PK/PD were dosed for 14 days and euthanized following the completion of infusion. Rats were weighed and tumors calipered twice weekly. At the completion of the study, animals were euthanized and tumor tissue collected in an RNAase-free environment, bisected, snap frozen in liquid nitrogen, pulverized and stored at -80 °C. Effects on H3K79 methylation and ELISA and gene expression by RTqPCR were performed (Supplemental Methods).

Statistics

Student’s t-test and one-way ANOVA were used for statistical comparisons where appropriate.

RESULTS

**Dot1l mRNA expression and H3K79 methylation are increased in Dnmt3a-null HSCs**

Analysis of previously performed RNA-seq of HSCs isolated from *Dnmt3a*-null mice compared to wild-type mice of various ages\(^2^9\) revealed that *Dot1l* was overexpressed in the *Dnmt3a*-null HSCs relative to wild-type HSCs (**Fig 1A and 1B**). Given the aberrant expression of this histone methyltransferase, we examined whether Dot1L-induced H3K79 methylation was also altered in *Dnmt3a*-null HSCs compared to wild-type controls, and if these alterations were associated with altered DNA methylation. We previously reported that the edges of large undermethylated regions, termed DNA methylation canyons, are hotspots for DNA methylation loss in *Dnmt3a*-null HSCs. However, only a portion of these canyons lose methylation and expand with Dnmt3a loss, and a close association between canyon DNA methylation changes and the associated histone marks was identified\(^1^2\). Expanding canyons are characterized by the presence of the activating H3K4 tri-methyl (me3) mark and absence of the repressive histone mark H3K27me3\(^1^2\), suggesting that Dnmt3a is particularly important in maintaining DNA methylation specifically at canyons with activating histone marks and active gene transcription. We
speculated that H3K79me may be another key component of this activating histone signature. To determine if Dot1l-induced H3K79me was altered in Dnmt3a-null HSCs, we performed ChIP-seq for H3K79me2 and aligned these data with existing whole genome DNA methylation data from Dnmt3a-null HSCs. We found that with Dnmt3a loss, the levels of H3K79me2 were markedly increased at transcription start sites, protein coding start sites, and at undermethylated regions (UMRs), including a substantial increase specifically at DNA methylation canyons (Fig. 1C). We then looked at the association between H3K79me2 and canyon dynamics with Dnmt3a loss. We found that among canyons, H3K79 is essentially absent at canyons that gain DNA methylation with Dnmt3a loss, is present at relatively low density in canyons that show little change with Dnmt3a loss, and densely coats those canyons that expand with Dnmt3a loss. Additionally, the most substantial gains in H3K79me2 in Dnmt3a-null HSCs occur at expanding canyons, such as the canyon associated with the Gata2 gene (Fig. 1D and 1E). This strong correlation between H3K79me and altered DNA methylation suggests a functional interaction.

**DOT1L-induced H3K79 methylation is increased in DNMT3A-mutant AML**

Based on our murine findings, we postulated that DOT1L might play a critical role in human DNMT3A-mutant AML. To explore this hypothesis, we used DNMT3A-mutant human cell lines OCI AML3, which harbor the most common and well-characterized type of DNMT3A mutation, the dominant-negative acting R882 mutation, and OCI AML2 cells which have a non-R882 mutation that is functionally uncharacterized. As controls, we used the MLLr cell line, THP1, and KG-1 cells that have wild-type DNMT3A and MLL. The relative methylation of H3K79 was determined by mass spectrometry, which demonstrated decreased unmethylated H3K79 and increased H3K79me1 and H3K79me2 in the OCI AML3 DNMT3A-mutant cells compared to DNMT3A wild-type cells (Fig. 2A-C, respectively, and Suppl. Fig. S1). These results are consistent with the overexpression of Dot1l and increased H3K79me density observed in our murine Dnmt3a-null model, despite the fact that mRNA expression was not increased in these
In a separate experiment, the non-R882 Dnmt3a-mutant cell line OCI AML2 showed increased H3K79 methylation by mass spec, though these data need to be confirmed (Suppl. Fig. S1).

Pharmacologic DOT1L inhibition reduces cellular H3K79me and oncogenic HOX gene expression in DNMT3A mutant AML cells

Given H3K79 methylation is increased in both murine Dnmt3a\textsuperscript{-/-} HSCs and human DNMT3A-mutant AML cells, we hypothesized that H3K79me may contribute to an oncogenic gene expression program, and therefore H3K79me inhibition might lead to anti-leukemic activity by inhibiting the expression of leukemogenic genes. To explore this possibility, we tested the efficacy of pharmacologic DOT1L inhibition \textit{in vitro} using two specific DOT1L inhibitors; SYC-522\textsuperscript{21}, and EPZ004777\textsuperscript{23}. These structurally similar compounds both work by binding the DOT1L-nucleosome complex and competitively inhibiting the binding of the methyl donor, S-adenosylmethionine\textsuperscript{21,23,24}; they have comparable potency and specificity. We treated the DNMT3A-mutant human AML cell lines OCI AML2 and OCI AML3 with SYC-522 or EPZ004777\textsuperscript{23}. DOT1L inhibitor treatment resulted in a dose- and time-dependent reduction in H3K79me\textsubscript{2} (Suppl. Fig. S2, Fig. 3A). We then evaluated the impact of DOT1L inhibition on the expression of leukemogenic genes. We examined HOXA and HOXB cluster genes, as a number of these genes are overexpressed in DNMT3A-mutant leukemia\textsuperscript{4,8}. We also examined changes in the expression of the HOX co-factor gene, MEIS1, which is expressed in most DNMT3A-mutant AML samples, because decreased MEIS1 expression has been correlated to responses to DOT1L inhibition in MLL-rearranged leukemia\textsuperscript{22,23}. HOX and MEIS1 genes were also of interest, as each is associated with a H3K79me\textsubscript{2}-coated DNA methylation canyon in murine Dnmt3a\textsuperscript{-/-} HSCs. Treatment with 3µM of EPZ004777 reduced the expression of HOXA9 and MEIS1 in the OCI AML2 cells (Fig. 3B) and the expression of MEIS1 and predominantly HOXB cluster genes in the OCI AML3 cells (Fig 3C). The expression of GAPDH was not
affected by DOT1L inhibitor treatment, indicating this decreased expression was not due to a general inhibitory effect on gene expression (Fig. 3B and 3C). These data suggest that reduction of H3K79me by pharmacologic inhibition of DOT1L can specifically reverse the oncogenic gene expression program of DNMT3A-mutant AML.

**Treatment with pharmacologic inhibitors of DOT1L inhibits the cellular proliferation of DNMT3A-mutant AML cell lines in a dose- and time-dependent fashion**

We hypothesized that the decreased leukemogenic gene expression would result in anti-leukemic effects in the DNMT3A-mutant cell lines. We therefore examined the impact of DOT1L inhibition on cellular proliferation. Cells were treated with SYC-522 or EPZ004777. We counted and re-plated cells at a constant cell concentration in fresh drug- or vehicle-containing media every 2-3 days. We first treated the DNMT3A mutant and OCI AML2 and OCI AML3 cells with escalating doses of both SYC-522 and EPZ004777 for 10-14 days. Both compounds inhibited growth in a dose-dependent fashion, but the effects were more profound in the OCI AML3 cells compared to the OCI AML2 cells (Fig. 4A, Suppl. Fig. S3A). To fully analyze the time-dependent impact of DOT1L inhibitor treatment on DNMT3A mutant AML cells, we performed proliferation assays for 14 days treating the DNMT3A-mutant cell lines with 3 µM EPZ004777 or vehicle control. We also included the DOT1L inhibitor-sensitive MLLr cell line, MV411, as a positive control, and the MLL- and DNMT3A-wild-type cell line, HL60, as a negative control.

There was no impact on the growth of the HL60 cells, and only modest slowing of growth of the non-R882 mutant OCI AML2 cells, whereas the proliferation of the R882 DNMT3A-mutant OCI AML3 cells was profoundly inhibited from around 7 days of treatment, comparable to the growth kinetics of MV411, the MLLr cell line (Fig. 4B). These results indicate that inhibition of DOT1L effectively inhibits the growth of DNMT3A-mutant cell lines, albeit with differences between cell lines with differing DNMT3A mutations.
Treatment with pharmacologic inhibitors of DOT1L induces apoptosis, cell cycle arrest and terminal differentiation of DNMT3A-mutant AML cell lines

We next sought to determine the specific mechanism of DOT1L inhibitor-induced cytotoxicity in DNMT3A mutant AML cells. By annexin-V binding (AVB) flow cytometry assay, DOT1L inhibition with either SYC-522 or EPZ004777 led to a dose-dependent induction of apoptosis in both OCI AML2 and OCI AML3 cells, though higher doses were required in the OCI AML2 to achieve significant apoptosis (Suppl. Figs. S3B and S4A, respectively). Treatment with 3 µM EPZ004777 led to substantial induction of apoptosis in the OCI AML3 cells in a time-dependent fashion beginning around 5 days of treatment, somewhat earlier than observed in the MV411 cell line (Fig. 5A). The HL60 cells had no induction of apoptosis and the OCI AML2 cells experienced minimal induction of apoptosis that plateaued at 7 days of therapy (Fig 5A). We also examined the impact of DOT1L inhibitor treatment on cell cycle kinetics by flow cytometry for DNA content. Both OCI AML2 and OCI AML3 cells experienced cell cycle arrest with increased percentages of cells in sub-G1 and decreased percentages in S and G2/M phases in a dose- and time-dependent fashion (Suppl. Fig. S4B and Fig. 5B, respectively). Additionally, both DNMT3A-mutant cell lines had evidence of induced differentiation with increased expression of the mature monocyte marker CD14, equivalent to the effects of DOT1L inhibition seen in the MV411 cells (Fig. 5C).

DOT1L inhibition inhibits tumor growth in a nude rat AML xenograft

To test the in vivo efficacy of DOT1L inhibition on human DNMT3A-mutant AML, we utilized a nude rat xenograft model in which OCI AML3 cells were injected subcutaneously, forming a leukemic tumor. After tumor engraftment, the rats (n=8 per treatment cohort) were treated with continuous intravenous infusion of vehicle control or EPZ-5676, a DOT1L inhibitor currently being tested in phase I clinical trials that is structurally similar to EPZ004777 but with improved pharmacokinetic properties and increased potency. Rats were treated at doses of either
35mg/kg/day or 70mg/kg/day via continuous IV infusion for 21 days followed by a 7-day drug holiday. Both doses of EPZ-5676 led to significant reduction of H3K79me2 (Fig. 6A), and resulted in repression of MEIS1 and HOXB3 expression, consistent with in vitro results (Fig. 6B). Tumor growth was inhibited in a dose-dependent fashion reaching statistical significance in the 70mg/kg/day cohort (Fig. 6C). These results indicate that DOT1L inhibition is effective in vivo for the treatment of DNMT3A-mutant AML.

DOT1L inhibitor treatment selectively inhibits the colony-forming capacity and induces differentiation of primary patient samples with DNMT3A mutations

While our therapeutic experiments utilizing human AML cell lines allowed us to explore the efficacy of DOT1L inhibition in DNMT3A-mutant leukemia, given the potential confounding variables inherent to cell lines, we sought to test our hypothesis using primary patient samples.

Viably frozen human primary samples were plated in methylcellulose media plus 3 µM EPZ00477 or DMSO vehicle control (Patient sample characteristics provided in Supplemental Table S2). Samples with MLL aberrations and most samples with DNMT3A mutations had reduced colony-forming capacity (CFC), whereas normal human cord blood CD34+ cells and primary AML patient samples lacking MLL aberration and DNMT3A mutation had no change with treatment (Fig. 7A). Given the differences in R882 mutant and non-R882 mutant cells in our cell line experiments, we examined DNMT3A-mutant samples by mutation type. The average CFC of R882-mutations was significantly reduced compared to AML samples wild-type for both DNMT3A and MLL and normal CD34+ cord blood cells, similar to the average reduced CFC of MLL aberrant samples (Fig. 7B). Only 2 samples with non-R882 DNMT3A mutation were analyzed, therefore no definitive conclusions about the responsiveness of this genotype can be reached.

Treatment with EPZ004777 also induced differentiation of DNMT3A-mutant samples with increased expression of the mature monocyte marker CD14 compared to vehicle-treated
controls (Fig. 7C, Suppl. Fig. S5A). Histologic evaluation of the isolated cells also showed evidence of monocytic differentiation with reduced nuclear to cytoplasmic ratio, increased granules in the cytoplasm and condensation of the nuclei (Suppl. Fig. S5B). These results indicate that pharmacologic DOT1L inhibition reduces cellular proliferation and promotes differentiation of primary AML patient samples with DNMT3A mutations.

**Discussion**

DOT1L plays a critical role in leukemia with MLL-rearrangements\textsuperscript{17-20,32}, and is now being explored as a therapeutic target for patients with these genetic alterations\textsuperscript{21-25}. Here, we report a possible role for DOT1L in leukemias with mutations of DNMT3A. Given the high prevalence of DNMT3A mutations across a variety of hematologic malignancies, and the fact that in most clinical studies DNMT3A mutations are associated with a particularly poor prognosis\textsuperscript{1,3,4,33-35}, identifying a novel therapeutic target is of substantial clinical impact. Our data suggest that DOT1L may be an immediately actionable target in DNMT3A-mutated AML.

In many MLL-rearranged leukemias, DOT1L is believed to contribute to leukemogenesis via the fusion partners, including AF9, AF10 and ENL, which normally interact with DOT1L\textsuperscript{17,18,20}. The fusions lead to aberrant recruitment of DOT1L and the H3K79me mark to the promoters of MLL target genes such as *MEIS1* and *HOX* cluster genes, ultimately leading to their constitutive expression. However, recent evidence indicates that DOT1L and its co-factor AF10 may play a critical role in regulating HOX gene expression via H3K79me2 in a subset of additional leukemias without such MLL fusions, including AMLs with partial tandem duplication of MLL, NUP98-NSD1 fusions and IDH mutations\textsuperscript{18,32,36,37}. We have identified DNMT3A-mutant AML as an additional distinct subset of leukemia in which DOT1L contributes to leukemogenesis via MLL fusion-independent mechanisms.

Using our murine *Dnmt3a*-null model, we identified a potential functional interaction between H3K79me2 and altered DNA methylation that may explain the site specification in
DNMT3A-mutant AML. In murine HSCs, H3K79me2 is highly enriched in DNA methylation canyons\textsuperscript{12}, and is most prominent in canyons that expand with Dnmt3a loss. Expanding canyons coated by H3K79me2 are highly enriched for genes aberrantly expressed in hematologic malignancies, including HOX cluster genes\textsuperscript{12}. We previously reported that these sites are also characterized by the activating histone mark H3K4 tri-methylation and lack of the repressive H3K27 tri-methyl mark\textsuperscript{12}. We expect H3K79me2 is one critical component of an activating histone signature that dictates where in the genome DNMT3A is most critical for maintaining DNA methylation. When DNMT3A function is lost, by deletion in a murine model or mutation in human AML, DNA methylation at these sites is eroded. We have further shown that in the Dnmt3a-null HSCs, hypomethylated canyons gain additional H3K79 methylation. These observed perturbations of both DNA methylation and covalent histone modifications including H3K79 methylation likely result in the aberrant expression of the associated genes, including HOX cluster genes. Additional work to fully define the mechanistic role of DOT1L in DNMT3A-mutant leukemia is ongoing.

We hypothesized that DOT1L-induced H3K79me might be a therapeutic target in human DNMT3A-mediated hematologic malignancies. Our results support this hypothesis, with pharmacologic DOT1L inhibitors leading to inhibition of cellular proliferation, induction of apoptosis, cell cycle arrest and terminal differentiation in DNMT3A-mutant cell lines and primary patient samples. These effects were comparable to MLL-rearranged cell lines and patient samples. We observed variability in responses between two DNMT3A-mutant cell lines, similar to differences observed in responses of cell lines with different MLL-rearrangements\textsuperscript{22,23,25}. The differences observed in our assays may be attributable to biologic differences between the type of DNMT3A mutations present in each respective cell line. OCI AML3 cells harbor a heterozygous mutation at the R882 position\textsuperscript{30,31}, which results in a hypomorphic protein that exerts dominant negative activity\textsuperscript{5,6}. Conversely, OCI AML2 cells have a hemi-/homo-zygous non-R882, catalytic domain DNMT3A mutation that has not been functionally characterized\textsuperscript{30}.  

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An additional contributing factor to the variability in response could be the presence of other acquired genetic or epigenetic aberrations in the cell lines. In addition to the *DNMT3A* mutation, the OCI AML3 cell line also harbors a mutation in the nucleophosmin gene (*NPM1*). Both *DNMT3A* and *NPM1* mutations have been associated with overexpression of *HOX* cluster genes and altered DNA methylation\textsuperscript{4,6,8,38-40}, however, as two of the most commonly co-occurring mutations in human AML\textsuperscript{7}, it is difficult to ascertain if these biologic features are attributable to the *DNMT3A* mutation or the *NPM1* mutation, or are secondary to the combined effect of these likely cooperative events. We cannot fully exclude the possibility that a portion of the therapeutic effects of DOT1L inhibition observed in the OCI AML3 cell lines is secondary to the presence of the *NPM1* mutation. However, we did not see a clear difference in responses of *DNMT3A*-mutant primary AML patient samples based on the *NPM1* status, with most *DNMT3A* mutant samples showing a significant response regardless of *NPM1* status. Furthermore, our initial data indicating a link between aberrant DNA methylation and H3K79 methylation come from our *Dnmt3a*-null murine model, which has wild-type *Npm1*. Together, these observations suggest that the majority of therapeutic effect noted with DOT1L inhibitor therapy is attributable to the biology of the *DNMT3A* mutation.

In summary, based on novel observations from our *Dnmt3a*-KO murine model, we hypothesized that DOT1L may play a role in *DNMT3A*-mutant human leukemia, and therefore may represent a therapeutic target. Our *in vitro* and *in vivo* work with both cell lines and primary patient samples support this hypothesis, and provide the pre-clinical rationale for possible clinical investigation of pharmacologic DOT1L inhibitors for *DNMT3A*-muant leukemia. Ongoing adult and pediatric phase I clinical trials of the DOT1L inhibitor, EPZ-5676, for patients with *MLL*-rearranged hematologic malignancies could greatly facilitate the translation of these findings into clinical investigations for patients with *DNMT3A* mutations in the near term. Ultimately, if our results are validated, DOT1L inhibitors could be incorporated into the multi-agent therapeutic regimen for the treatment of this relatively refractory group of patients.
Authorship and Disclosures

Contribution: R.E.R. designed and performed experiments, analyzed results, made figures and wrote the paper; B.R. analyzed the data and made a figure; M.L., M.J, A.R., J.R. C.A.C., S.R.D., S.S., and T.C., designed and performed experiments; L.D. and Y.S., synthesized and provided DOT1L inhibitor compounds; M.A, provided patient samples; and W.L. helped analyze the data; and M.A.G. designed the experiments and wrote the paper. All authors commented on the edited manuscript.

Conflict of Interest Disclosure: C.T.C and S.R.D. are employees of Epizyme, Incorporated.

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Data access

Reviewers may access a track hub for H3K79me2 in the mouse genome (mm9) at:
http://genome.ucsc.edu/cgi-bin/hgTracks?db=mm9&hubUrl=http://dldcc-web.brc bcm.edu/lilab/benji/RaRau/k79.hub.txt
REFERENCES

Figure Legends

Figure 1: Dnmt3a<sup>-/-</sup> HSCs are characterized by increased Dot1L expression and increased H3K79 methylation. A) RNA-seq signal density tracks of mRNA expression of Dot1L in murine Dnmt3a-null (3aKO_RNA) hematopoietic stem cells (HSCs) compared to HSC from wild-type HSCs from 4-, 12- and 24-month old mice (m04_RNA, m12_RNA, m24_RNA respectively), B) Average FPKM value of Dot1L in wild-type vs. Dnmt3aKO HSCs. C) Average normalized signal density of H3K79me2 at transcription start sites, protein coding start sites (Coding Start Site), undermethylated regions (UMR) and DNA methylation canyons in Dnmt3aKO HSCs (red) and wild-type HSCs (black). D) Representative DNA methylation canyon that expands with Dnmt3a deletion (DNA methylation, red; Canyon, extend grey bar) and associated H3K79me2 in wild-type HSCs (blue) and Dnmt3aKO HSCs (dark red). E) Average normalized H3K79me2 signal at DNA methylation canyons that expand with Dnmt3a deletion (3aKO expand, red; WT expand, gold), canyons that do not change with Dnmt3a deletion (3aKO no change, green; WT no change teal), and canyons that contract with Dnmt3a deletion (3aKO contract, blue; WT contract, purple).

Figure 2: DOT1L-induced histone 3 lysine 79 methylation is increased in DNMT3A-mutant human AML cell lines.

Relative level of A) unmethylated H3 lysine 79, B) mono-methylated H3K79, and C) di-methylated H3K79 measured by mass spectrometry in three AML cell lines KG1, THP1 and OCI AML3. Data is from a single biological replicate and three technical replicates. Error bars show standard deviation. P value was determined using unpaired two-way t-test. **, P<0.01; *, P<0.05; ns, not statistically significant.
Figure 3: Pharmacologic DOT1L inhibition reduces cellular H3K79me2 and oncogenic gene expression in DNMT3A mutant AML cells. A) Immunoblot analysis of cellular H3K79me2 in OCI AML2 and OCI AML3 cells after treatment with 3 µM EPZ004777. B) Relative expression determined by quantitative RT PCR of leukemogenic HOXA9, MEIS1, HOXB3, HOXB8 genes and the housekeeping gene GAPDH in OCI AML 2 and OCI AML3 cells treated with 3 µM EPZ004777 or vehicle control for 14 days.

Figure 4: EPZ004777 treatment inhibits the proliferation of DNMT3A-mutant human AML cell lines. A) Growth of OCI AML2 and OCI AML3 cells treated with increasing concentrations of EPZ004777. B) Growth curves of HL60, MV411, OCI AML2 and OCI AML3 cells treated with 3 µM EPZ004777 or vehicle control for 14 days. Numbers are plotted on logarithmic scale. Assays were done in triplicate. Error bars represent standard deviation.

Figure 5: EPZ004777 treatment induces apoptosis, cell cycle arrest and terminal differentiation in DNMT3A-mutant human AML cells. HL60, MV411, OCI AML2 and OCI AML3 cells were treated with 3µM EPZ004777 or DMSO vehicle control for 14 days. Cells were re-plated at a constant concentration in fresh drug-containing media every 2-3 days. A) Left, representative flow cytometry histograms of annexin V binding (AVB) for OCI AML2 and OCI AML3 cells on day 14 of treatment with DMSO vehicle control (blue) or EPZ004777 (red). Right, and quantification of % of EPZ004777-treated cells AVB+ minus % AVB+ vehicle control-treated cells. B) Representative flow cytometry plots of PI DNA content cell cycle analysis for OCI AML2 cells and OCI AML3 cells (left panels) with quantification of experiments (right panel) C) Representative flow plots of CD14 cell surface expression of OCI AML 2 and OCI AML 3 cells treated with DMSO vehicle control or EPZ004777 for 14 days after gating out PI+ dead cells (left panels) and quantification of percentage of CD14+ cells treated with 3 µM EPZ004777 or
vehicle control at specified time points (right panel). All assays were done in triplicate. Error bars represent standard deviation.

**Figure 6:** *In vivo* efficacy of pharmacologic DOT1L inhibition in *DNMT3A*-mutant AML. H3K79me2 levels in acid-extracted histones as measured by ELISA in A) OCI AML3 subcutaneous tumors and bone marrow from vehicle control-treated animals or animals treated with 35 or 70 mg/kg/day EPZ-5676 administered via continuous IV infusion. H3K79me2 levels were normalized to those of total histone H3 in the same sample and are plotted as a percent of the mean H3K79me2 level in tissue from the vehicle-treated group, which is set at 100%. Horizontal lines represent the mean percent H3K79me2 values for each group. (N=5 animals per cohort) B) Relative expression of MEIS1 and HOXB3 in OCI AML3 subcutaneous tumors in vehicle control treated mice and mice treated with 35 or 70 mg/kg/day EPZ-5676 for 21 days plotted as a percent of the mean transcript level in tumors from the vehicle treated group which is set at 100%. Horizontal lines represent the mean percent transcript level in each group. (N=5 animals per cohort) C) Volume of OCI AML3 subcutaneous tumors over time in vehicle control treated animals and animals treated with 35 or 70 mg/kg/day EPZ-5676 administered via continuous IV infusion for 21 days (N=8 animals per cohort). Error bars represent standard deviation.

**Figure 7:** DOT1L inhibitor treatment selectively inhibits the *in vitro* growth of and induces terminal differentiation of primary patient samples with *DNMT3A* mutations. A) Relative colony forming units (CFU) of normal cord blood CD34+ cells and primary AML samples wild-type for both *DNMT3A* and *MLL* (left panel), primary AML samples with *MLL* anomalies (middle panel), and primary AML samples with *DNMT3A* mutations (right panel, *, non-R882 *DNMT3A* mutation) treated with DMSO vehicle control or 3 µM EPZ004777. Assays performed in triplicate, error bars represent SEM. B) Average change in colony forming capacity of primary patient samples treated with 3 µM EPZ004777 compared to DMSO vehicle treated control. Error bars represent SEM C) Flow cytometry analysis of CD14 expression of primary AML cells with
DNMT3A mutation isolated from plates after treatment with DMSO vehicle control or 3 µM EPZ004777.
Figure 2. Rau

A

B

C

% Signal Intensity

H3K79me0

H3K79me1

H3K79me2

KG1 THP1 OCI AML3

KG1 THP1 OCI AML3

KG1 THP1 OCI AML3

** ns

* ns

* **
Figure 3. Rau

A

[Image of Western blot analysis showing H3K79me2 and H3 proteins over time for OCI AML2 and OCI AML3]

B

[Graph showing relative expression of HOXa9, MEIS1, and GAPDH for OCI AML2 with control (DMSO) and 3μM EPZ treatment]

C

[Graph showing relative expression of HOXa9, MEIS1, HOXb1, and GAPDH for OCI AML3 with control (DMSO) and 3μM EPZ treatment]
Figure 4. Rau

A

OCI AML2

DMSO
- 0.75 μM
- 1.5 μM
- 3 μM
- 6 μM
- 12 μM
- 24 μM

Days

OCI AML3

DMSO
- 0.375 μM
- 0.75 μM
- 1.5 μM
- 3 μM
- 6 μM
- 12 μM
- 24 μM

Days

B

HL60

DMSO
- 3 μM EPZ

Days

OCI AML2

DMSO
- 3 μM EPZ

Days

MV411

DMSO
- 3 μM EPZ

Days

OCI AML3

DMSO
- 3 μM EPZ

Days
Figure 6. Rau

A

Tumor

Bone Marrow

% Control

Vehicle 35 70

EPZ-5676 mg/kg/day

% Control

Vehicle 35 70

EPZ-5676 mg/kg/day

C

Tumor Volume (mm³)

EPZ-5676 Infusion

Vehicle 35 mg/kg/day CI X 21d

70 mg/kg/day CI X 21d

P < 0.05

Days

B

HOXB3

MEIS1

% Control

Vehicle 35 70

EPZ-5676 mg/kg/day

% Control

Vehicle 35 70

EPZ-5676 mg/kg/day