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

Rachel E. Rau¹,², Benjamin Rodriguez²,⁶, Min Luo³,⁴,⁵, Mira Jeong³,⁴,⁵, Allison Rosen³,⁴,⁵, Jason H. Rogers¹, Carly T. Campbell⁸, Scott R. Daigle⁸, Lishing Deng⁷, Yongcheng Song⁷, Steve Sweet⁹, Timothy Chevassut¹⁰, Michael Andreeff¹¹, Steven M. Kornblau¹¹, Wei Li²,⁶, and Margaret A. Goodell¹,²,³,⁴,⁵

¹Department of Pediatrics, Baylor College of Medicine and Texas Children’s Hospital, Houston, TX
²Dan L. Duncan Cancer Center, Baylor College of Medicine, Houston, TX
³Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX
⁴Stem Cells and Regenerative Medicine Center, Baylor College of Medicine, Houston, TX
⁵Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, TX
⁶Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX
⁷Department of Pharmacology, Baylor College of Medicine, Houston, TX
⁸Epizyme, Inc., Cambridge, MA
⁹Genome Damage and Stability Centre, University of Sussex, Brighton, East Sussex, UK
¹⁰Department of Haematology, Brighton and Sussex Medical School, University of Sussex, Brighton, East Sussex, UK
¹¹Department of Leukemia, The University of Texas MD Anderson Cancer Center, Houston, TX

Running Title: DOT1L in DNMT3A-Mutant AML

Co-corresponding Authors:

Margaret A. Goodell, Ph.D.
Department of Pediatrics and Stem Cells and Regenerative Medicine Center
Baylor College of Medicine
One Baylor Plaza, N1030
Houston, TX 77030
goodell@bcm.edu

Rachel E. Rau, MD
Department of Pediatrics
Baylor College of Medicine
1102 Bates St. Suite 1025
Houston, TX 77030
rerau@bcm.edu

Text Word Count: 4,272
Abstract Word Count: 249
Number of Figures: 7
Number of Supplemental Figures: 7
Tables: 0
Number of References: 39
Key Points

- Data from $Dnmt3a^{-/-}$ 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 hematopoietic stem cells (HSCs) in which Dnmt3a had been conditionally deleted markedly overexpress the histone 3, lysine 79 (H3K79) methyltransferase, Dot1l. We demonstrate that Dnmt3a−/− 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 resulted in decreased 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-mutant 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 DNMT3A-mutant AML samples, 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. *DNMT3A* mutations in AML are almost exclusively heterozygous, and approximately 60% 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. 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. These data suggest the pathologic changes in DNA methylation are mediated by additional, unknown factors.

Conditional ablation of *Dnmt3a* in the murine hematopoietic system results in a dramatic expansion of hematopoietic stem cells (HSCs), a progressive block in differentiation, and priming for malignant transformation. 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. Canyons that expand with *Dnmt3a* deletion are highly enriched for genes dysregulated in human leukemia, including *HOX* genes, 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, and many canyon-associated genes, including *HOX* genes, are significantly changed in *DNMT3A*-mutant AML.

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\textsuperscript{13-16}. 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 \textit{MLL}-rearrangements\textsuperscript{17-20}. Pharmacologic inhibition of DOT1L has shown promising pre-clinical activity in \textit{MLL}-rearranged leukemia and is now being tested in adult and pediatric clinical trials\textsuperscript{21-25}. \textit{MLL} rearrangements rarely co-occur with \textit{DNMT3A} mutations in AML\textsuperscript{3,4,7}. The essential mutual exclusivity\textsuperscript{7} of these lesions and the overexpression of Dot1l in our murine model led us to hypothesize that \textit{MLL}-rearrangements and \textit{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 \textit{DNMT3A}-mediated leukemia and evaluated DOT1L as a therapeutic target for the treatment of this poor prognosis disease.

\textbf{METHODS}

\textbf{Murine model}

Animal procedures were approved by the Animal Care and Use Committee of Baylor College of Medicine. For ChIP-seq experiments, C57Bl/6 CD45.2 \textit{Dnmt3a}\textsuperscript{fl/fl} and \textit{Dnmt3a}\textsuperscript{WT/WT} were crossed to Rosa26-Cre \textit{ERT2}\textsuperscript{26}. \textit{Dnmt3a}\textsuperscript{fl/fl}-Rosa26-Cre \textit{ERT2} and \textit{Dnmt3a}\textsuperscript{WT/WT}-Rosa26-Cre \textit{ERT2} mice were treated with 5 daily intraperitoneal injections of tamoxifen (1mg/0.1mL corn oil/mouse/day) to induce deletion of the floxed Dnmt3a allele. This typically results in >80% bilallelic deletion as determined by genotyping of individual methocult colonies from the bone marrow of \textit{Dnmt3a}\textsuperscript{fl/fl}-Rosa26-Cre \textit{ERT2} after tamoxifen treatment. Eight weeks later, bone marrow was harvested and transplanted (1\times10^6 per mouse) into lethally irradiated syngeneic CD45.1 recipients. Additional details of murine experiments are provided in the Supplemental Methods.
**ChIP-seq**

Four months after transplantation, recipient mice were sacrificed, and pooled bone marrow hematopoietic stem cells (HSCs) from *Dnmt3a−/−* 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 (FACSria; 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). See Supplemental Methods for details of analyses performed.

**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 (Epizyme, Inc.) was used.

**Cell proliferation, viability, and colony formation assays**

Exponentially growing cells were plated in triplicate in 24-well plates (2x10⁵/mL; final volume 1mL). For dose-dependent assays, cells were incubated in increasing concentrations of SYC52221 or EPZ00477723 or DMSO control. For time-dependent assays, cells were incubated in 3 μM EPZ00477723 or DMSO control. Every 2-3 days media and compound were replaced and cells were split to 2x10⁵/mL. At each replating, the viable cell 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 cytopsins (Cytopro).

**Nude Rat xenografts**

*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-*Foxn1*nu, 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 RT-qPCR 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−/− HSCs**

Re-analysis of previously performed RNA-seq of Dnmt3a−/− HSCs27 (Hoechst side population-KSL CD150+ after Mx1-Cre-mediated deletion and serial transplantation) revealed that Dot1l was overexpressed in the Dnmt3a−/− relative to wild-type (WT) HSCs isolated from mice of various ages (Fig. 1A and 1B). Dot1l overexpression was confirmed by quantitative real-time PCR of two biologic replicates of purified Dnmt3a−/− and WT HSCs (Fig. 1C). In addition, modest reduction of DNA methylation and increased H3K79me2 density at the Dot1l promoter suggest that increased expression of Dot1l in this model may be attributable to altered epigenetic regulation (Suppl. Fig. S1A).

Given the aberrant expression of this histone methyltransferase, we examined whether Dot1L-induced H3K79 methylation was also altered in Dnmt3a−/− 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−/− 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 identified12. Expanding canyons are characterized by the presence of the activating H3K4 tri-methyl (me3) mark and absence of the repressive histone mark H3K27me312, 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−/− HSCs, we performed ChIP-seq for H3K79me2 on Dnmt3aFl/fl-Rosa26-Cre ER T2 and Dnmt3awt/wt-Rosa26-Cre ER T2 HSCs isolated from transplanted mice after tamoxifen-
induced deletion. The H3K79me2 data were aligned with existing whole genome DNA methylation data from \textit{Dnmt3a}⁻/⁻ HSCs\textsuperscript{12}, revealing that levels of H3K79me2 were markedly increased at transcription start sites, protein coding start sites, and at undermethylated regions (UMRs) (Fig. 1D). Importantly, a substantial increase in signal intensity was noted, particularly at DNA methylation canyons (Fig. 1D; Suppl. Fig. S1B-D). We then looked at the association between H3K79me2 and canyon dynamics with \textit{Dnmt3a} loss. We found that H3K79me2 is found at very low levels in canyons that show little change or increased methylation after \textit{Dnmt3a} loss. However, H3K79me2 densely coats canyons that expand when \textit{Dnmt3a} is ablated, such as the canyon associated with the \textit{Gata2} gene (Fig. 1E and 1F). 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 role in human \textit{DNMT3A}-mutant AML. To explore this hypothesis, we examined the relative methylation of H3K79 of the \textit{DNMT3A}-mutant human cell line OCI AML3, which harbor the most common and well-characterized type of \textit{DNMT3A} mutation, the dominant-negative acting R882 mutation\textsuperscript{5,6} compared to the \textit{MLLr} cell line, THP1, and KG-1 cells that have wild-type \textit{DNMT3A} and \textit{MLL}. Mass spectrometry demonstrated that OCI AML3 \textit{DNMT3A}-mutant cells had decreased unmethylated H3K79 and increased H3K79me2 in the compared to \textit{DNMT3A} wild-type cells (Fig. 2A-C and Suppl. Fig. S2). These results are consistent with the increased H3K79me density observed in our murine \textit{Dnmt3a}⁻/⁻ model, despite the fact that DOT1L expression at the mRNA level was not increased in this cell line relative to \textit{DNMT3A} wild-type AML cell lines (not shown).

**Pharmacologic DOT1L inhibition reduces cellular H3K79me and decreases the proliferation of DNMT3A-mutant AML cells in a dose- and time-dependent fashion**

Rau et al.
To explore H3K79 methylation as a potential therapeutic target in \textit{DNMT3A}-mutant AML, we tested the efficacy of pharmacologic DOT1L inhibition \textit{in vitro} using two specific DOT1L inhibitors with comparable potency and specificity; SYC-522\textsuperscript{21}, and EPZ004777\textsuperscript{23}. We treated the only known human cell lines with \textit{DNMT3A} mutations, R882 mutant OCI AML3, and OCI AML2 cells which have a functionally uncharacterized non-R882 mutation (and a possible cryptic \textit{MLL} rearrangement\textsuperscript{28}), with SYC-522 or EPZ004777. DOT1L inhibitor treatment resulted in a dose- and time-dependent reduction in H3K79me2 in both cell lines (\textbf{Suppl. Fig. S3}, and \textbf{Fig. 3A}, respectively).

Both compounds inhibited growth in a dose-dependent fashion, with more pronounced effects in the OCI AML3 cells compared to the OCI AML2 cells (\textbf{Suppl. Figs. S4 and S5A}). To fully analyze the time-dependent impact of DOT1L inhibitor treatment on \textit{DNMT3A}-mutant AML cells, we performed proliferation assays for 14 days treating the \textit{DNMT3A}-mutant cell lines with 3 \mu M EPZ004777 or vehicle control. We also included the DOT1L inhibitor-sensitive \textit{MLLr} cell line, MV411, as a positive control, and the \textit{MLL}- and \textit{DNMT3A}-wild-type cell line, HL60, as a negative control. There was no impact on the growth of the HL60 cells, and modest slowing of growth of the OCI AML2 cells, whereas the proliferation of the OCI AML3 cells was profoundly inhibited from around 7 days of treatment, comparable to effects in the MV411 cell line (\textbf{Fig. 3B}). These results indicate that inhibition of DOT1L effectively suppresses the growth of \textit{DNMT3A}-mutant cells.

\textbf{Treatment with pharmacologic inhibitors of DOT1L induces apoptosis, cell cycle arrest and terminal differentiation of \textit{DNMT3A}-mutant AML cell lines}

We next sought to determine the specific mechanism of DOT1L inhibitor-induced cytotoxicity in \textit{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 cells.
to achieve significant apoptosis (Suppl. Figs. S5B and S6). 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, earlier than observed in the MV411 cell line (Fig. 4A). The HL60 cells had no induction of apoptosis and the OCI AML2 cells experienced minimal induction of apoptosis (Fig. 4A). 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 phase in a dose- and time-dependent fashion, greatest in the OCI AML3 cells (Suppl. Fig. S6B and Fig. 4B, respectively). Additionally, both OCI AML2 and OCI AML3 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. 4C).

**Gene expression analysis after pharmacologic DOT1L inhibition**

To assess the effects of DOT1L inhibition on gene expression, RNA-seq was performed on OCI AML2 and OCI AML3 cells after treatment with EPZ004777. We specifically probed canyon-associated genes and found that almost all differentially expressed genes had significantly reduced expression with treatment (Fig. 5A). While a small subset of canyon-associated genes increased in expression, these canyons characteristically lacked H3K79me2 in our murine model. To validate the RNA-seq results, RTqPCR was performed for specific HOX genes and MEIS1, confirming that expression of MEIS1 and HOX cluster genes was suppressed in both the OCI AML2 and OCI AML3 cells after treatment (Fig. 5B). HOX B cluster genes were preferentially suppressed in the OCI AML3 cells, consistent with the data from patient cohorts showing specific overexpression of HOX B cluster genes in DNMT3A-mutant leukemia4,8 (Fig. 5B). Additionally, Ingenuity Pathway Analysis (IPA) of differentially expressed genes showed enrichment of genes involved in cell death, cell cycle arrest and differentiation (Fig. 5C), consistent with the phenotypic consequences of DOT1L inhibitor treatment reported above.
Gene Set Enrichment Analysis (GSEA) of genes up-regulated after EPZ treatment demonstrated significant enrichment for genes associated with G1-S transition, including up-regulation of cyclin-dependent kinase inhibitor CDKN1A. Strong positive enrichment was further observed for genes involved in myeloid differentiation including increased expression of lysozyme and myeloperoxidase (Fig. 5D and 5E). There was also significant overlap between up-regulated genes in both cell lines after EPZ treatment and genes up-regulated by knockdown of HOXA9 in the MLLr AML cell line, MOLM1429. Given the known correlation between suppression of HOXA9 expression and therapeutic effect of DOT1L inhibition in MLLr cells, this overlap highlights the likely important role of programs downstream of HOXA9 in both MLLr leukemia and DNMT3A-mutant leukemia (Fig. 5E). This is confounded by a possible MLL fusion in the OCI AML2 cells, however the overlap between this data set and the gene expression changes in the OCI AML3 cells point towards mechanistic overlap between MLLr leukemia and AML harboring R882 DNMT3A mutations.

**DOT1L inhibition suppresses 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 decreased 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 at suppressing tumor growth in an *in vivo* nude rate xenograft model of DNMT3A-mutant AML.
Ideally, treatment of patient derived xenografts models would be performed to confirm these results, however given the need for administration of the drug by continuous infusion for prolonged periods of time, such studies are currently not feasible.

DOT1L inhibitor treatment selectively reduces 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 potential functional differences of R882 vs non-R882 mutations, we examined DNMT3A-mutant samples by mutation type. The average CFC of R882-mutant samples 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 evidenced by increased expression of the mature monocyte marker CD14 compared to vehicle-treated controls, including in one sample that did not have a significant reduction in CFC (Pt 743509) (Fig. 7C, Suppl. Fig. S7A). Histologic evaluation of the isolated cells also showed evidence of differentiation with reduced nuclear to cytoplasmic ratio, increased granules in the
cytoplasm and condensation of the nuclei (Suppl. Fig. S7B). 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 certain $MLL$-rearrangements$^{17,20,30}$ and it is now being explored as a therapeutic target for patients with these genetic alterations$^{21-25}$. 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$^{1,3,4,31-33}$, 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 $MLL$-r leukemias, DOT1L is believed to contribute to leukemogenesis via the fusion partners, including AF9, AF10 and ENL, which normally interact with DOT1L$^{17,18,20}$. 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$^{18,30,34,35}$. We have identified $DNMT3A$-mutant AML as an additional distinct subset of leukemia in which DOT1L may contribute to leukemogenesis via MLL fusion-independent mechanisms.

Using our murine $Dnmt3a^{-/-}$ 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, 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. 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. We expect H3K79me2 is one 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-/- HSCs, hypomethylated canyons gain additional H3K79 methylation. These observed perturbations of both DNA methylation and covalent histone modifications including H3K79 methylation may contribute to the aberrant expression of the associated genes, including HOX cluster genes. Indeed, when we treated DNMT3A-mutant AML cell lines with DOT1L inhibitor, we observed a reduction in the expression of genes associated with expanding H3K79me2-coated canyons including HOX and MEIS1 genes, potentially indicating a direct inhibitory effect. Supportive of this possibility is the finding that in the R882-mutant cell line, the expression of HOX B cluster genes is impacted to a greater extent than HOX A cluster genes, contrary to what is seen in MLLr leukemia cell lines. This is consistent with a more extensive loss of DNA methylation in the Hox B cluster-associated DNA methylation canyon compared to the canyon associated with the Hox A cluster in our murine Dnmt3a-/- HSCs and more pronounced overexpression of HOX B cluster genes in DNMT3A-mutant AML compared to HOX A cluster genes. However, as recent work has demonstrated, HOX gene expression in AML might simply reflect the HOX expression pattern of the hematopoietic stem/progenitor cell from which the AML arose. Therefore, it is possible that the decreased expression of HOX genes observed in response to DOT1L inhibitor therapy, reflects differentiation of the cells rather than a direct cause of DOT1L inhibition on gene expression. Furthermore, while in our murine model, Dot1l was overexpressed in Dnmt3a-/- HSCs relative to wild-type, we did not observe overexpression of DOT1L in human DNMT3A-mutant AML cell lines relative to DNMT3A wild-
type cell lines nor in $DNMT3A$-mutant patient samples relative to wild-type in the TCGA RNAseq data. Thus, it is possible that $DNMT3A$-mutant AML may exploit DOT1L by alternative mechanisms such as aberrant recruitment. It is also possible that the therapeutic responses observed in $DNMT3A$-mutant cell lines and primary patient samples after treatment with DOT1L inhibitor could be attributable to other yet unidentified factors. 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. In particular, the effects observed in the R882 $DNMT3A$-mutant cell line and patient samples were comparable to DOT1L-inhibitor sensitive $MLL$-rearranged cell lines and patient samples. We observed much less effect in the non-R882 mutant OCI AML2 cells, potentially suggesting there may be differences in response based on biological differences between types of $DNMT3A$ mutations. However, our ability to evaluate the effect of DOT1L inhibitor therapy on non-R882 mutant AML is limited by a lack of available cell lines without additional confounding mutations and a paucity of patient samples with any given non-R882 $DNMT3A$ mutation, as no other true mutational hotspots exist.

While our data indicate that sensitivity of the cell lines to DOT1L inhibition can be conferred by mutant $DNMT3A$, we cannot exclude contributions from other genetic or epigenetic aberrations. While one study suggested that OCI AML2 cells harbor a $MLLr^{28}$, they lack cytogenetic evidence of 11q23 anomaly and exhibited only modest anti-proliferative response to EPZ treatment. The OCI AML3 cell line, in addition to the $DNMT3A$ mutation, also harbors a nucleophosmin ($NPM1$) mutation. Both $DNMT3A$ and $NPM1$ mutations have been associated with overexpression of $HOX$ cluster genes and altered DNA methylation$^{4,6,8,37-39}$, however, as two of the most commonly co-occurring mutations in human AML$^{7}$, it is difficult to ascertain if
these biologic features are attributable to the \textit{DNMT3A} mutation or the \textit{NPM1} mutation, or are secondary to the combined effect of these likely cooperative events. Therefore, while the cell line experiments provided important evidence that \textit{DNMT3A}-mutant AML might be sensitive to DOT1L inhibition, validation in human primary AML samples was essential. Utilizing a number of \textit{DNMT3A}-mutant samples specifically lacking other known confounding mutations, we observed near-universal reductions in CFC and induction of differentiation in the \textit{DNMT3A} mutant primary AML patient samples consistent with the anti-proliferative and pro-differentiation effects observed in our cell lines experiments. Furthermore, our initial data indicating a link between aberrant DNA methylation and H3K79 methylation come from our \textit{Dnmt3a}-/- murine model, which only differ from the wild-type control by lack of \textit{Dnmt3a}. Together, these observations suggest that the majority of therapeutic effect noted with DOT1L inhibitor therapy is attributable to the biology of the \textit{DNMT3A} mutation.

In summary, based on novel observations from our \textit{Dnmt3a}-/- murine model, we hypothesized that DOT1L may play a role in \textit{DNMT3A}-mutant human leukemia, and therefore may represent a therapeutic target. Our \textit{in vitro} and \textit{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 \textit{DNMT3A}-muant leukemia. Ongoing adult and pediatric phase I clinical trials of the DOT1L inhibitor, EPZ-5676, for patients with \textit{MLL}-rearranged hematologic malignancies could greatly facilitate the translation of these findings into clinical investigations for patients with \textit{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 figures; 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. and S.M.K., 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.

Acknowledgements

The authors thank members of the Goodell Laboratory for helpful discussions; Y. Zheng, A. Guzman, and R. Gupta for technical support; C. Gillespie for critical review of the manuscript; M. Redell and M. Minden for providing cell lines; and J. Matthews and S. Piece for assistance with patient samples. This project was supported by the Cytometry and Cell Sorting Core at Baylor College of Medicine with funding from the NIH (P30 AI036211, P30 CA125123, and S10 RR024574) and the expert assistance of J. Sederstrom and A. White.

This work was supported by The Faust Foundation (R.R.); The Edward P Evans Foundation, The Samuel Waxman Cancer Research Foundation, Henry Malvin Helis Foundation, and the Lester and Sue Smith Foundation (M.A.G.); and National Institutes of Health, National Cancer Institute grant K12 CA090433-11 (R.R.), CA183252, CA183252, and CA126752, National Institute of Diabetes and Digestive and Kidney Diseases grants DK092883 and DK084259, National Human Genome Research Institute grants R01HG007538 and HG007538.

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*<sup>−/−</sup> hematopoietic stem cells (HSCs) purified as Hoechst side population-KSL and CD150+. *Dnmt3a*<sup>−/−</sup> HSCs were purified after Mx-Cre-mediated deletion and serial transplantation as we have previously reported (Sun, et al. *Cell Stem Cell* 2014;14:673-88. and Challen, et al. *Nat Genet* 2012;44:23-31.) 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. *Dnmt3a*<sup>−/−</sup> HSCs (two independently-obtained biological replicates of each cohort represented). C) Dot1L expression determined by quantitative real-time PCR relative to GAPDH expression in wild-type HSCs (12-month old) compared to two biologic replicates of *Dnmt3a*<sup>−/−</sup> HSCs purified after Mx-Cre-mediated deletion and serial transplantation (calculated by 2<sup>−ΔΔCt</sup> equation). Assay performed in triplicate. Error bars represent standard deviation. D) ChIP-seq of H3K79me2 of *Dnmt3a*<sup>fl/fl</sup>-Rosa26-Cre-ER<sup>T2</sup> and *Dnmt3a*<sup>wt/wt</sup>-Rosa26-Cre-ER<sup>T2</sup> HSCs isolated from primarily transplanted mice after tamoxifen induced deletion. 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 *Dnmt3a*<sup>−/−</sup> 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 *Dnmt3a*<sup>−/−</sup> HSCs (dark red). E) Average normalized H3K79me2 signal at DNA methylation canyons that expand with *Dnmt3a* deletion (*Dnmt3a*<sup>−/−</sup> expand, red; WT expand, gold), canyons that do not change with *Dnmt3a* deletion (*Dnmt3a*<sup>−/−</sup> no change, green; WT no change, teal), and canyons that contract with *Dnmt3a*.
deletion ($Dnmt3a^−/−$ contract, blue; WT contract, purple). P value was determined using unpaired two-way t-test. ***, P<0.001.

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

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-four 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 suppresses proliferation of $DNMT3A$-mutant human AML cell lines. A) Immunoblot analysis of cellular H3K79me2 in OCI AML2 and OCI AML3 cells after treatment with 3 μM EPZ004777. Fraction of H3K79me2/total H3 relative to DMSO-treated control indicated below each lane. 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 4: 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) 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) (Left Panel). Quantification of % of EPZ004777-treated cells that are AVB+ minus % AVB+ vehicle control-treated cells (Right Panel). B) Representative flow cytometry plots of PI DNA content cell
cycle analysis for OCI AML2 cells and OCI AML3 cells (Left Panel) 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 Panel) 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 5: Gene expression changes following DOT1L inhibitor treatment of DNMT3A-mutant AML cell lines.** A) Gene expression heat map (gene scaled and centered log2 counts per million) of top differentially expressed canyon-associated genes in OCI AML2 and OCI AML3 cell lines after 14 days of treatment with DOT1L inhibitor. B) Relative expression determined by quantitative RT PCR of canyon-associated leukemogenic genes, HOXA9, MEIS1, HOXB3, HOXB8 and the housekeeping gene GAPDH in OCI AML 2 and OCI AML3 cells treated with 3 μM EPZ004777 or vehicle control for 14 days. RTqPCR done in triplicate. Error bars represent standard deviation. C) Ingenuity IPA functional enrichment analysis of genes differentially expressed (FDR q-value < 0.1) in AML cells after treatment with EPZ004777. D) GSEA of AML cells treated with EPZ004777. X-axis, normalized enrichment score; color, log transformed FDR corrected q-value; size, enrichment signal strength of the leading edge subset of a gene set. E) Gene expression heat maps (gene scaled and centered log2 counts per million) of representative, core enriched gene sets from GSEA.

**Figure 6: In vivo efficacy of pharmacologic DOT1L inhibition in a rat xenograft model of DNMT3A-mutant AML.** A) H3K79me2 levels in acid-extracted histones as measured by ELISA in 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. P value was determined using unpaired two-way t-test. *, P<0.05; ns, not
statistically significant.
Figure 2. **A**, **B**, **C**
Figure 3. Rau

A

<table>
<thead>
<tr>
<th></th>
<th>OCI AML2</th>
<th>OCI AML3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>0.75</td>
<td>0.67</td>
</tr>
<tr>
<td>5</td>
<td>0.58</td>
<td>0.51</td>
</tr>
<tr>
<td>7</td>
<td>0.43</td>
<td>0.31</td>
</tr>
<tr>
<td>9</td>
<td>0.24</td>
<td>0.3</td>
</tr>
<tr>
<td>12</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

K79me2  H3

B

HL60

<table>
<thead>
<tr>
<th>Visible Cell Number</th>
<th>DMSO</th>
<th>3μM EPZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10^5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10^6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10^7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10^8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Days

OCI AML2

<table>
<thead>
<tr>
<th>Visible Cell Number</th>
<th>DMSO</th>
<th>3μM EPZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10^5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10^6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10^7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10^8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Days

MV411

<table>
<thead>
<tr>
<th>Visible Cell Number</th>
<th>DMSO</th>
<th>3μM EPZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10^5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10^6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10^7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10^8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Days

OCI AML3

<table>
<thead>
<tr>
<th>Visible Cell Number</th>
<th>DMSO</th>
<th>3μM EPZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10^5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10^6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10^7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10^8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Days
Figure 5. Rau
Figure 6. Rau

A

Tumor H3K79me2

Bone Marrow H3K79me2

Relative H3K79me2

(% Control)

Vehicle 35 70

EPZ-5676
mg/kg/day

C

EPZ-5676 Infusion

Tumor Volume (mm³)

P < 0.05

Days

B

HOXB3 expression

MEIS1 expression

Relative expression (% Control)

Vehicle 35 70

EPZ-5676
mg/kg/day
Figure 7. Rau

A

Wild-type MLL and DNMT3A

B

C

DMSO

3μM EPZ
DOT1L as a therapeutic target for the treatment of DNMT3A-mutant acute myeloid leukemia

Rachel E. Rau, Benjamin Rodriguez, Min Luo, Mira Jeong, Allison Rosen, Jason H. Rogers, Carly T. Campbell, Scott R. Daigle, Lishing Deng, Yongcheng Song, Steve Sweet, Timothy Chevassut, Michael Andreeff, Steven M. Kornblau, Wei Li and Margaret A. Goodell