Clonal evolution mediates Menin-inhibitor resistance in *KMT2A*-rearranged leukemias.

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Abstract

Inhibitors of the Menin-KMT2A interaction are emerging as promising agents for the treatment of *KMT2A*-rearranged (*KMT2A*-r) acute myeloid leukemia (AML) and acute

lymphoblastic leukemia (ALL). Menin is required for the recruitment of the *KMT2A*-fusion to a subset of its target genes. We evaluated the efficacy of Menin-inhibition in *KMT2A-r* leukemias with high-risk features: high-risk *KMT2A* fusion partners in AML (*MLLT10*, *MLLT4*), infant ALL, and serial samples from patients with multiply relapsed ALL. We find that AML with high-risk fusion partners is sensitive to Menin-inhibition in xenografts. In contrast, highly pretreated samples from patients with *KMT2A-AFF1* ALL are largely resistant. Menin inhibitor resistance in these patients is acquired, as Menin inhibition shows in vivo efficacy against leukemia samples obtained earlier in the same patients' disease course. Transcriptomic analysis documents sustained on-target efficacy of the Menin inhibitor on a transcriptional level in resistant cells. However, genomic analysis documented the emergence of multiple co-mutations that may be sufficient to drive AML, and may mediate independence from the KMT2A-fusion induced transcription program. Our data suggest that it will be critical to use Menin-inhibitors upfront or in early lines of therapy before substantial clonal evolution has occurred.

Introduction

10% of acute leukemias carry rearrangements of the *KMT2A* gene (Mixed-Lineage Leukemia-1, *MLL-1*). More than 80 fusion partners have been identified, and differences in fusion partner have been linked to outcomes[1-3]. Regardless of fusion partner, *KMT2A*-r leukemias have a unique gene expression profile characterized by high expression of HOXA cluster genes, Meis1 and FLT3[4,5].

Despite advances in our molecular understanding of *KMT2A*-rearranged leukemias, little progress has been made to improve clinical outcomes. The 5-year event free and overall survival of pediatric *KMT2A*-r AML remains suboptimal at 38% and 58%[3]. The survival for infants and adults with *KMT2A*-r ALL remains dismal[6-8].

Recently, inhibition of the KMT2A-interacting protein Menin was identified as a promising therapeutic strategy[9-14]. Menin is essential for the oncogenic activity of KMT2A fusions[15]. Preclinical and early studies of the Menin inhibitor VTP-60469 demonstrated impressive single agent activity in *KMT2A*-r ALL and AML[9,10,14]. Molecularly, Menin inhibitors suppress the expression of a subset of KMT2A-fusion target genes by interfering with the recruitment of the fusion to DNA[9-11,13].

Preclinical studies published to date mostly used patient samples with the common KMT2A fusion partners MLLT3/AF9, MLLT1/ENL and AFF1/AF4 banked at initial diagnosis[9,10,12]. We sought to evaluate the efficacy of Menin inhibition in well-defined high-risk subpopulations: AML with high-risk fusion partners (*MLLT10 (AF10)* and *MLLT4 (AF6)*[2,3]), infant-ALL[6,7], and multiply relapsed *KMT2A*-r ALL including a samples with lineage switch, which is near universally lethal[16]. We found that highly pretreated ALL samples were less sensitive to Menin inhibition than samples from the same patients earlier in their course. We also found that the Menin-inhibitor maintained on-target effects on transcription in resistant samples, but this failed to translate into meaningful in vivo efficacy. Our data supports moving Menin-inhibitors into early lines of therapy to maximize their potential clinical impact.

Materials and Methods

Human samples: Samples were obtained from patients at the Children's Hospital of Philadelphia with informed consent according to the Declaration of Helsinki and Institutional Review Board (IRB) approval.

Patient derived xenografts: NSG or NSGS (NOD-scid IL2Rgnull or NOD-scid IL2Rgnull-3/GM/SF, Jackson laboratories®) were conditioned with Busulfan and patient cells were injected into the tail vein. Menin inhibitor (VTP-50460 or equipotent SNDX-5613) 0.1% chow, abbreviated "VTP" or control chow was given as indicated. Human leukemia cells were detected in peripheral blood, bone marrow and spleen using anti-human CD45. For leukemia free survival mice were censored when mice died without signs of CNS leukemia or detectable disease in bone marrow, blood and spleen.

RNA-Seq analysis: Raw Fastq files were aligned using STAR against reference Mus musculus GRCm38, read-counts were quantified by Kallisto and directly imported into DESeq2. Gene Set Enrichment Analysis (GSEA) was carried out using GSEA software. RNA-Seq data has been submitted to GEO and access will be provided upon request.

Targeted DNA sequencing: Targeted sequencing of patient leukemia samples was performed using the in-house clinical platform to the Division of Genomic Diagnostics (DGD) at the Children's Hospital of Philadelphia.

Results and Discussion

We first evaluated the efficacy of VTP in three AML patient samples with high-risk fusion partners. Baseline patient characteristics are shown in **Figure 1A**. VTP treated mice lived significantly longer than untreated mice; two out of three cohorts died with undetectable disease by flow cytometry in blood, spleen and bone marrow **(Patients 1- 3, Figure 1B-D, and**

supplementary Figure 1A). VTP was also effective in xenografts of infant ALL at initial diagnosis, with no detectable disease in all animals for patient 4 (**Figure 1E, F and supplementary Figure 1B**), and substantially prolonged latency for patient 21 (**Figure 1G, solid lines and supplementary Figure 2A left panel**).

We next interrogated the efficacy of VTP in paired samples from patients with *KMT2A*-r ALL, obtained early and late in their disease course (relapse after several lines of therapy including high dose chemotherapy, immunotherapy and stem cell transplant, with lineage switch to AML) (Figure 1A, lower panel). We found that leukemia samples obtained after multiple lines of therapy were substantially less sensitive to Menin inhibition than samples from the same patients earlier in their disease course (**Figure 1F+G and supplementary Figure 2**). Although relapsed samples had not been previously exposed to Menin inhibitors, they became less sensitive to VTP inhibition over the course of the preceding chemo/immunotherapy.

In order to understand the mechanism of decreased sensitivity we performed RNA-Seq of in vivo leukemia from patient 8, comparing the transcriptional response to Menin inhibition vs control treatment. Principal component analysis demonstrated that VTP treatment induced a stronger transcriptional response in the sample obtained earlier in the disease course (**Figure 2A**). However, when focusing on the primary Menin targets, we found that Menin-inhibition resulted in a profound downregulation of the canonical Menin-inhibitor responsive gene signature in both sensitive and resistant AML (**Figure 2B, Supplementary Table 1**). Enriched signatures included genes downregulated upon Menin inhibitor treatment (top row)[13], sites that lose Menin binding and are downregulated upon Menin inhibitor treatment (middle row)[10] and genes downregulated upon Menin deletion (bottom row)[10]. Therefore, VTP maintained on-target transcriptional activity in resistant cells. This is consistent with the recently reported phase I clinical trial of revumenib (SNDX-5613), which saw downregulation of the key KMT2A target genes HOXA9, Meis1, FLT3, PBX3 and RUNX1 in nearly all patients, while the overall response and CR rates were much lower at 50% and 20%[14].

We next interrogated the co-mutational landscape of our serial samples (**Figure 2C and Supplementary Material**). We found substantial clonal evolution, including the emergence of RAS pathway mutations in both patients. In patient 21, a KMT2D mutation also became dominant, while patient 8 showed increasing variant allelic frequencies (VAFs) of *PRPF8* and *TP53* mutations. Analysis of the RNA-Seq data obtained in the xenografts demonstrated that in patient sample 8-2 nearly all reads for TP53 carried the R282P mutation, and VTP treatment was associated with a trend towards higher expression of the mutant NRASQ61K allele (**Figure 2D, Supplementary Figure 3**). Of note, we did not observe any of the mutations in the *MEN1* gene that were recently reported in patients and PDX models after prolonged exposure to SNDX-5613[17].

KMT2A-fusion leukemias carrying *TP53* mutations or activating Ras mutations were previously reported as highly sensitive to Menin inhibition[18-20], therefore either mutation alone does not explain the decrease in sensitivity we observed. We confirmed that MV4;11 cells engineered to carry inactivating mutations of *TP53* showed no difference in the response to VTP (**Figure 2E+F, Supplementary Figure 4**). However, the combination of *TP53* inactivation and RAS pathway mutation together was sufficient to initiate leukemogenesis in a murine genetic model of AML[21]. We suspect that the combination of multiple co-mutations in highly pretreated samples eventually renders the leukemia independently of the initiating genomic event (*KMT2A* fusion), resulting in Menin-inhibitor resistance despite maintained on-target activity of the inhibitor on a transcriptional level (**Figure 2G**). *KMT2A*-fusion independence due to clonal evolution is one potential mechanism explaining resistance in the nearly 50% of patients who show a transcriptional response to revumenib without a clinical response[14]. Careful subset analysis in clinical trials will be required to precisely determine the influence of prior lines of therapy and the co-mutational landscape. Regardless of the extent of this effect, our data supports treating patient with Menin-inhibitors early in their disease course before significant clonal evolution has occurred.

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Author contributions:

KMB designed the study. LM, AL, SRR, FA and CL performed experiments. GMG provided VTP50469 compound and chow, as well as critical expertise. SKT provided a critical PDX sample as well as helpful discussion. SC provided provided critical expertise and assistance with patient mutational analysis. BM, GW, SS and MPK designed, engineered and validated the *TP53* mutant MV4;11 cells. HMX conducted data analysis and statistical analysis. LM and KMB wrote the manuscript.

Conflict of Interest disclosures:

GMM is an employee and shareholder of Syndax Pharmaceuticals Inc.

KMB has previously consulted for Agios and Novartis and has received research funding from Syndax Pharmaceuticals Inc.

CL received a research training grant from Institut Servier.

SKT has served on scientific advisory boards for Kura Oncology and Syndax Pharmaceuticals for pediatric clinical development of Menin inhibitors and receives research funding from Kura Oncology. She also serves on the scientific advisory board for Aleta Biotherapeutics, receives

research funding from Beam Therapeutics and Incyte Corporation, received travel support from Amgen, and has consulted for bluebird bio for unrelated studies.

MPC has been involved as a consultant for Janssen Pharmaceuticals and on advisory committee for Cartography Biosciences.

Figure 1

A

#	Diagnosis	Age at diagnosis	Sex	Day from diagnosis	Prior lines of therapy	Fusion	Other mutations
1-1	AML	18 months	M	0	0	KMT2A-MLLT10 (MLL-AF10)	NRAS, NSD2, CSF1R, CREBBP, TERT
2-1	AML	6.8 years	F	0	0	KMT2A-MLLT10 (MLL-AF10)	NRAS, SETD2m KMT2Am SETD2
3-1	AUL	12 years	M	0	0	KMT2A-MLLT4 (MLL-AF6)	FLT3, SUZ12, ASXL1, RUNX1, BORL1, PHF6, USH2A
4-1	Infant ALL	10 months	F	626	2	KMT2A-MLLT6 (MLL-AF17)	SETBP1
8-1	B-ALL	14 years	F	217	3	KMT2A-AFF1 (MLL-AF4)	NRAS, TP53, PRPF8, NOTCH1, BCORL1
8-2	Lineage switch			282	7		NRAS, TP53, PRPF8, NOTCH1, BCORL1
21-1	Infant ALL	1 month	F	0	0	KMT2A-AFF1 (MLL-AF4)	KMT2D, KRAS, NT5C2, SMC3
21-2				270	6		KMT2D, KRAS, NT5C2, SMC3

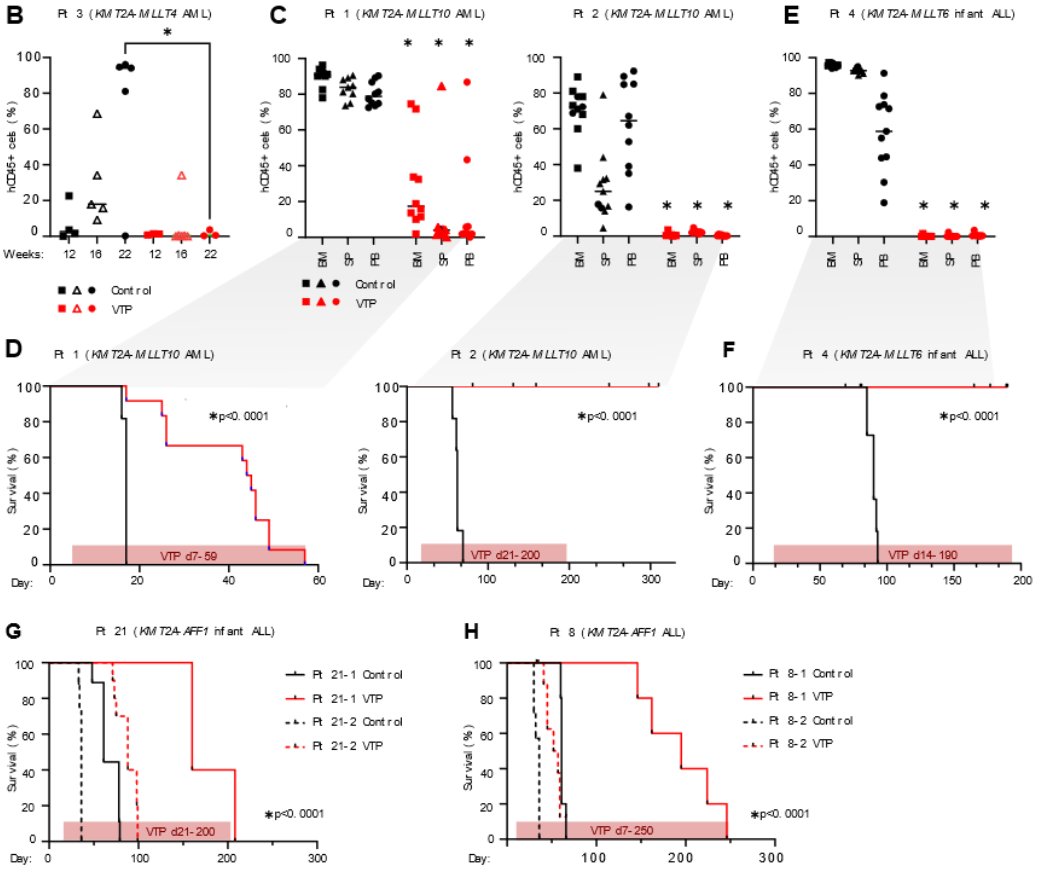

Figure 1: In vivo treatment of patient derived xenografts from *KMT2A* rearranged leukemias with the Menin inhibitor (VTP, red) or control (black).

A: Baseline characteristics of samples used in this study. Additional detail is provided in the supplementary material.

B-D: AML patients with high-risk fusions partners (*MLLT10, MLLT4*). **B***: KMT2A-MLLT4* (*MLL-AF6*) AML leukemic burden in bone marrow (measured as huCD45+/mCD45-) at the indicated time points. **C:** *KMT2A-MLLT10* (*MLL-AF10*) AML leukemic burden in bone marrow at necropsy (measured as huCD45+/mCD45-). **D:** leukemia free survival of mice shown in B.

E: infant B-ALL, leukemic burden in bone marrow (measured as huCD45+/mCD45-) at necropsy. **F**: leukemia free survival of mice shown in D.

G: survival of *KMT2A-AFF1I* PDX established early (solid line, initial diagnosis or three prior lines of therapy) or late (broken line, 6-7 prior lines of therapy).

n=9-10 per treatment arm, error bars = SEM, *p<0.0001, unpaired t-test (A), 2-Way Anova (B), Mantle-Cox (C+D).

Figure 2

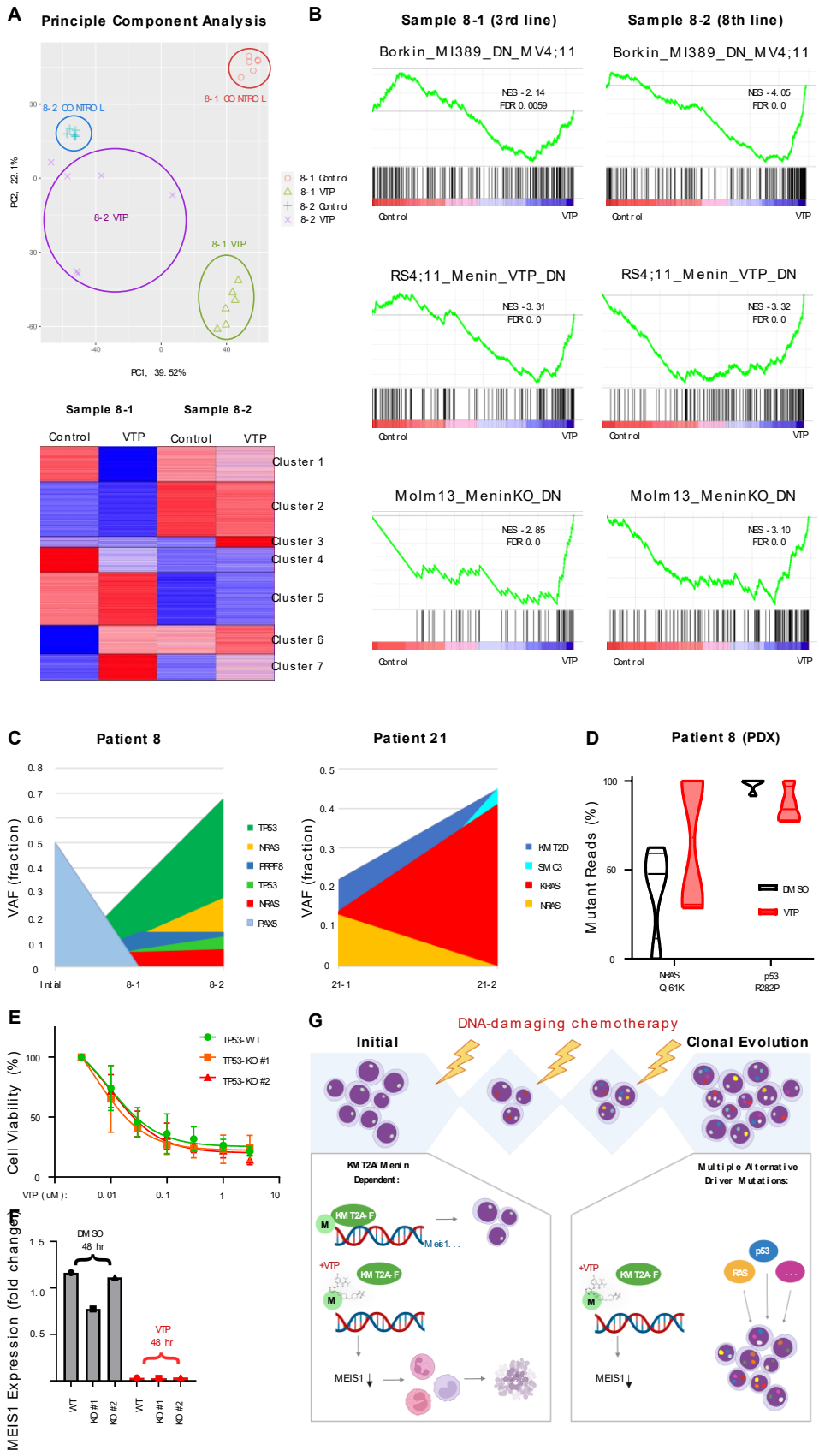
A
Principle Component Analysis
8-2 CONTROL
8-1 CONTROL
8-2 VTP
8-1 VTP
PC2, 22.1%
PC1, 39.52%
8-1 Control
8-1 VTP
8-2 Control
8-2 VTP
Sample 8-1
Sample 8-2
Control
VTP
Control
VTP
Cluster 1
Cluster 2
Cluster 3
Cluster 4
Cluster 5
Cluster 6
Cluster 7
B
Sample 8-1 (3rd line)
Sample 8-2 (8th line)
Borkin_MI389_DN_MV4;11
NES -2.14
FDR 0.0059
Borkin_MI389_DN_MV4;11
NES -4.05
FDR 0.0
RS4;11_Menin_VTP_DN
NES -3.31
FDR 0.0
RS4;11_Menin_VTP_DN
NES -3.32
FDR 0.0
Molm13_MeninKO_DN
NES -2.85
FDR 0.0
Molm13_MeninKO_DN
NES -3.10
FDR 0.0
Control
VTP
C
Patient 8
VAF (fraction)
TP53
NRAS
PRPF8
TP53
NRAS
PAX5
Initial
8-1
8-2
Patient 21
VAF (fraction)
KMT2D
SMC3
KRAS
NRAS
21-1
21-2
D
Patient 8 (PDX)
Mutant Reads (%)
DMSO
VTP
NRAS Q61K
p53 R282P
E
Cell Viability (%)
TP53-WT
TP53-KO #1
TP53-KO #2
VTP (uM):
0.01
0.1
1
10
F
MEIS1 Expression (fold changes)
DMSO 48 hr
VTP 48 hr
WT
KO #1
KO #2
WT
KO #1
KO #2
G
DNA-damaging chemotherapy
Initial
Clonal Evolution
KMT2A/Menin Dependent:
KMT2A-F
Meis1...
+VTP
MEIS1
Multiple Alternative Driver Mutations:
p53
RAS
...

Figure 2: A+B: RNA-Seq of sorted leukemia cells (huCD45+, mCD45-, DAPI-) from the bone marrow of mice treated with VTP50469 at 3 weeks of treatment (Sample 8-1, responding) or at necropsy (Sample 8-2, resistant). **A:** Principal component analysis and K-means clustering, **B:** GSEA of established Menin-response signatures in sensitive (left column) and resistant (right column) samples. Gene signatures contain genes that are downregulated upon Menin inhibitor treatment in MV4;11 cells (top row), sites that lose Menin binding and are downregulated upon Menin inhibitor treatment in RS4;11 cells (middle row), and genes that are downregulated upon Menin deletion in Molm13 cells (bottom row) **C:** Variant allelic frequency (VAF) of mutations identified on targeted NGS of primary patient bone marrow cells obtained at different time points during the treatment course corresponding to time points from which PDXs were established (Pt 21, left and Pt 8, right panel). **D:** fraction of mutant reads by RNA-Seq in leukemia cells obtained from mice transplanted with sample 8-2 on control or VTP50469 chow at 3 weeks of treatment. E: in vitro treatment response of MV4;11 (KMT2A-AFF1, TP53 wild type) as well as CRISPR engineered biallelic TP53 mutant sublines (TP53-KO1 and 2) to VTP50469. Cell viability after 3 days of drug treatment was determined by trypan blue exclusion. **F:** Menin expression by qPCR in TP53WT and KO cells after 48 hours of exposure to DMSO (control) or VTP. Shown it fold change normalized to GAPDH, E+F: N=3, error bar = SEM, differences between WT and KO were not statistically significant. **G:** schematic of Menin-inhibitor resistance that evolves under pressure of multiple courses of standard ALL therapy and is likely driven by the evolution of multiple alternative “driver” mutations that are not reliant on Menin for their transforming activity.

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