Selective inhibition of Ezh2 by a small molecule inhibitor blocks tumor cells proliferation

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Ezh2 (Enhancer of zeste homolog 2 protein) is the enzymatic component of the Polycomb repressive complex 2 (PRC2), which represses gene expression by methylating lysine 27 of histone H3 (H3K27) and regulates cell proliferation and differentiation during embryonic development. Recently, hot-spot mutations of Ezh2 were identified in diffused large B-cell lymphomas and follicular lymphomas. To investigate if tumor growth is dependent on the enzymatic activity of Ezh2, we developed a potent and selective small molecule inhibitor, EI1, which inhibits the enzymatic activity of Ezh2 through direct binding to the enzyme and competing with the methyl group donor S-Adenosyl methionine. EI1-treated cells exhibited genome-wide loss of H3K27 methylation and activation of PRC2 target genes. Furthermore, inhibition of Ezh2 by EI1 in diffused large B-cell lymphomas carrying the Y641 mutations results in decreased proliferation, cell cycle arrest, and apoptosis. These results provide strong validation of Ezh2 as a potential therapeutic target for the treatment of cancer.

Ezh2 is the mammalian homolog of Enhancer of Zeste, the key component of Polycomb repressive complex 2 (PRC2), which represses gene expression in development (1). Ezh2 and the other two proteins, Su(z)2 and EED, form the core PRC2 complex, which possesses histone methyltransferase (HMT) activity and methylates H3K27 (2, 3). H3K27 methylation has been correlated with transcriptional repression and heterochromatin formation (4). The Drosophila ortholog, E(z), was initially identified as a regulator of homeotic gene expression and body segmentation (5). In mammals, Ezh2 is highly expressed in stem cells and actively proliferating cells, and down-regulated in differentiated cells (6). Ezh2 knockout in mice leads to developmental abnormality and embryonic lethality (7, 8). Ezh2-null ES cells are viable (8) and Ezh2 is involved in maintaining pluripotency and repression of lineage-differentiation genes (8–10).

Ezh2 is overexpressed in a broad spectrum of tumors, including prostate cancer, breast cancer, myeloma, hepatocellular carcinoma, gastric cancer, and so forth (1, 6). Overexpression of Ezh2 in mouse mammary gland leads to epithelial hyperplasia (1). Multiple studies using si/shRNA show that reduction of Ezh2 expression in tumor cell lines inhibits cell proliferation (12), migration, and invasion (13) or angiogenesis (14), and leads to apoptosis (15). Ezh2 contains the characteristic SET domain [Suppressor of variegation 3–9 (Suv 3–9), Enhancer of zeste (E(z)) and Trithorax] (16) present in most HMTs. Recent somatic mutations in the SET domain of Ezh2 were identified in diffused large B-cell lymphomas (DLBCL) patients (17–22). These mutations lead to the change of tyrosine (Y) 641 to phenylalanine (F), serine (S), asparagine (N), histidine (H), cysteine (C), or alanine (A) 677 to glycine (G). The Y641 mutant and A677G proteins show enhanced activity on H3K27me2 peptide (20, 23). Cancer cell lines heterozygous for these Ezh2 mutants show increased H3K27me3 and decreased H3K27me2 level (17, 20, 21, 23). Wild-type and mutant Ezh2 proteins may work cooperatively in cells to maintain a high level of H3K27me3 (23). It is therefore hypothesized that the Ezh2 hot-spot mutations and their enhanced activity toward H3K27me2 promotes DLBCL cell proliferation.

In this study, we developed an S-Adenosyl methionine (SAM) competitive inhibitor of Ezh2, EI1, which inhibits the methyltransferase activity of the Ezh2/PRC2 with high selectivity across an HMT panel. EI1-treated cells exhibited increased H3K27 methylation without changes of other histone H3 methylation marks. Using this tool inhibitor, we showed that inhibition of Ezh2 in DLBCL cells carrying hot-spot mutations and some Ezh2 overexpressed tumor cell lines results in decreased proliferation, cell cycle arrest, and apoptosis. Thus, inhibition of Ezh2 enzymatic activity may provide a therapeutic option for the treatment of DLBCL and other cancers.

Results

Identification and Biochemical Characterization of EI1 as a Potent and Selective Inhibitor of PRC2. To identify inhibitors of Ezh2/PRC2, we performed a high-throughput screen using recombinant PRC2 protein complex containing Ezh2, Su(z)2, EED, RbAP48, and AEBP2 (24). EI1 (Fig. L4) was designed based on one chemical scaffold identified from the high-throughput screen. This compound demonstrated potent, concentration-dependent inhibition of the enzymatic activity against both Ezh2 wild-type and Y641F mutant enzymes with IC50 of 15 ± 2 nM and 13 ± 3 nM, respectively (Fig. 1B). To understand the mode of inhibition for EI1, SAM competition experiments were carried out under the conditions of saturated substrate peptide. In accordance with the Cheng–Prusoff relationship for a competitive binding mode, the IC50 value of EI1 increased linearly with increasing concentration of SAM. The fitting of the data into the Cheng–Prusoff equation for competitive inhibition using linear regression analysis gave a Ki value of 13 ± 3 nM (Fig. 1C).

Although SAM is the common cofactor for all HMTs, EI1 showed remarkable selectivity against Ezh2 over other HMTs (Table 1). All biochemical reactions were carefully characterized with enzymology studies and the SAM and substrate concentrations were kept at their respective Km for most of the HMTs. Strikingly, EI1 displayed ~90-fold selectivity for Ezh2 over Ezh1, and >10,000-fold selectivity over other HMTs (Table 1). In


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Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE41915).

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that in DLBCL cells with wild-type Ezh2 (Fig. S1B), the H3K27me3 level was much higher than levels. For example, in WSU-DLCL2, SU-DHL6, and Karpas422 cell lines, including the DLBCL cells carrying Ezh2 mutations and rhabdoid tumor cells were highly dependent on PRC2 for proliferation. Ezh2 or other components of PRC2 showed that DLBCL and Fig. 2C, the other marks were not changed. p16 is a well characterized Ezh2 target gene (26–28) and its promoter is directly bound by Ezh2 and enriched for H3K27me3 (8, 27). Depletion of Ezh2 in Ga401 cells leads to up-regulation of p16 expression and senescence (26). We therefore examined the effect of E11 on p16 expression in Ga401 and found E11 could activate p16 expression in a dose-dependent manner (Fig. 2D, Left). Consistently, E11 also caused growth inhibition of Ga401 (Fig. S3). In a time-course study, p16 expression was activated 2 d after E11 treatment and the expression level increased by 20-fold at day 5 (Fig. 2D, Right). The decrease of H3K27me3 was readily observable 24 h after E11 treatment (Fig. S1D). These results indicate that the decrease of H3K27me3 precedes the activation of p16 expression.

ChIP experiments were performed to examine the change of H3K27me3 and Ezh2 status on p16 promoter (Fig. 2E). Both Ezh2 and H3K27me3 were enriched at the p16 promoter (Fig. 2F), as previously reported (8, 27). Specific and significant enrichment of H3K27me3 and Ezh2 were observed at region A and B. and the enrichment decreased toward region C (Fig. 2F). Following EI1 treatment, H3K27me3 signals were significantly reduced across the p16 promoter (Fig. 2F). Interestingly, Ezh2 remained bound at the p16 promoter (Fig. 2F). Therefore, EI1 activated p16 expression by suppressing H3K27me3, but not Ezh2 occupancy at the promoter.

E11 Inhibits Cellular H3K27 Methylation and Activates Ezh2 Target Gene Expression. Previous studies using si/shRNA to knock down Ezh2 or other components of PRC2 showed that DLBCL and rhabdoid tumor cells were highly dependent on PRC2 for proliferation (25, 26). Therefore, we tested the effect of E11 in these cell lines, including the DLBCL cells carrying Ezh2 mutants [WSU-DLCL2 (Ezh2Y641F) and SU-DHL6 (Ezh2Y641W)] could not fully inhibit Ezh2 protein level but caused similar loss of Ezh2 occupancy at the promoter.

A time-course study was performed in WSU-DLCL2 cells to understand the kinetics of H3K27 methylation inhibition. The H3K27me3 level was decreased 24 h after E11 treatment, and reached the lowest level after 4–5 d (Fig. 2B). To test the specificity of E11, we examined other histone H3 lysine methylation marks. As shown in Fig. 2C, the other marks were not changed.

The effect was similar in these cell lines, although they have different basal H3K27me3 and H3K27me2 levels. For example, in WSU-DLCL2, SU-DHL6, and Karpas422 cells with Ezh2 mutations, the H3K27me3 level was much higher than that in DLBCL cells with wild-type Ezh2 (Fig. S1C).

Table 1. HMT Profiling of E11 with a panel of HMT enzymes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>E11 IC50 (nM)</th>
<th>E11 fold-selectivity*</th>
<th>Sinefungin IC50 (nM)</th>
<th>Sinefungin fold-selectivity*</th>
<th>Assay with enzyme complex</th>
<th>Substrate</th>
</tr>
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<tbody>
<tr>
<td>Ezh2</td>
<td>9.4</td>
<td>1</td>
<td>20</td>
<td>1</td>
<td>Ezh2/SUZ12/EED/AEBP2/RbAP48</td>
<td>H3[21–44]</td>
</tr>
<tr>
<td>Ezh1</td>
<td>1,340</td>
<td>142</td>
<td>33</td>
<td>1.65</td>
<td>Ezh1/SUZ12/EED/AEBP2/RbAP48</td>
<td>H3[21–44]</td>
</tr>
<tr>
<td>G9a</td>
<td>&gt;100,000</td>
<td>&gt;10,000</td>
<td>18</td>
<td>0.9</td>
<td>H3[1–21]</td>
<td></td>
</tr>
<tr>
<td>Suv39H2</td>
<td>&gt;100,000</td>
<td>&gt;10,000</td>
<td>25</td>
<td>1.25</td>
<td>H3[1–21]</td>
<td></td>
</tr>
<tr>
<td>Set7/9</td>
<td>&gt;100,000</td>
<td>&gt;10,000</td>
<td>1.5</td>
<td>0.075</td>
<td>H3[1–21]</td>
<td></td>
</tr>
<tr>
<td>CARM 1</td>
<td>&gt;100,000</td>
<td>&gt;10,000</td>
<td>0.5</td>
<td>0.025</td>
<td>H3[1–21]</td>
<td></td>
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<tr>
<td>SmyD2</td>
<td>&gt;100,000</td>
<td>&gt;10,000</td>
<td>0.15</td>
<td>0.0075</td>
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<tr>
<td>SETD8</td>
<td>&gt;100,000</td>
<td>&gt;10,000</td>
<td>&gt;60</td>
<td>&gt;3</td>
<td>H4[11–33]</td>
<td></td>
</tr>
<tr>
<td>NSD3</td>
<td>&gt;100,000</td>
<td>&gt;10,000</td>
<td>45</td>
<td>2.25</td>
<td>H3[1–45]</td>
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</tr>
<tr>
<td>SETD2</td>
<td>&gt;100,000</td>
<td>&gt;10,000</td>
<td>16</td>
<td>0.8</td>
<td>H3[1–21]</td>
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<tr>
<td>MLL</td>
<td>&gt;100,000</td>
<td>&gt;10,000</td>
<td>12</td>
<td>0.6</td>
<td>MLL/WD5S/RBBP5/ASH2L</td>
<td>H3[1–21]</td>
</tr>
<tr>
<td>Dot1L</td>
<td>&gt;100,000</td>
<td>&gt;10,000</td>
<td>&gt;60</td>
<td>&gt;3</td>
<td>Nucleosome</td>
<td></td>
</tr>
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*Fold selectivity is calculated as the ratio of the IC50 of the enzyme tested over the IC50 of Ezh2.
siEzh2-2, knockdown Ezh2 mRNA by ∼80% (Fig. S2). Both siRNAs inhibited the proliferation of WSU-DLCL2 but had little effect on OCI-LY19 (Fig. 4A), suggesting the proliferation of DLBCL cells with Ezh2 mutations is dependent on Ezh2.

To test whether EI1 treatment has a similar effect as Ezh2 knockdown, we measured the proliferation after EI1 treatment. In contrast, the colony formation of OCI-LY19 was not affected. Taken together, these results indicate that EI1 is not generally toxic to cells, but selectively inhibits the neoplastic properties of DLBCL cells with Ezh2 mutation.

EI1 Causes Cell Cycle Arrest and Apoptosis of DLBCL Cells with Y641 Mutations. To understand the role of Ezh2 in the regulation of the proliferation of DLBCL lines carrying Ezh2 Y641 mutations, we examined the DNA synthesis and cell cycle progression by BrdU incorporation and FACS analysis. In WSU-DLCL2 cells treated with EI1 for 7 d, significant decrease of BrdU+ cells (reduced from 41.82% to 28.72%) was detected (Fig. 5A). The percentage of cells in G2/M also decreased, whereas the percentage of cells in G1 was increased (Fig. 5A and B). Similar results were observed in two other Ezh2 mutant DLBCL cell lines (Fig. 5B).

In contrast, the Ezh2 wild-type cells OCI-LY19, GA10, and Toledo did not show significant changes of DNA synthesis or cell cycle distribution (Fig. 5A and B).

Cell cycle progression is regulated by the activity of cyclin-dependent kinases and levels of various cyclins (31). We therefore surveyed the expression of cyclins in EI1-treated WSU-DLCL2 cells. Cyclin A and B1 protein levels were diminished after 6 d of EI1 treatment (Fig. 5C). Similar effects were observed in other DLBCL cells with Ezh2 mutations (Fig. S4). This finding is consistent with the reduction of the G2/M population from FACS analysis. A mild increase of sub-G1 population was observed in EI1-treated SU-DHL6 and Karpas422, suggesting an increase of apoptosis. To further examine this phenomenon, we carried out the Western blotting of procaspase 3 and cleaved (c)-caspase 3 in EI1-treated SU-DHL6 cells. Indeed, c-caspase 3 level was gradually elevated with increasing treatment time (Fig. 5D).

These results show that EI1 can significantly block cell cycle progression and induce apoptosis in Ezh2 mutant DLBCL cells.

Inhibition of Ezh2 Causes Down-Regulation of a Proliferation Gene Signature and Up-Regulation of a Memory B-Cell Signature in DLBCL. We examined the gene-expression changes in Karpas422 (Ezh2Y641N) after EI1 treatment in a time-course experiment. EI1-treated cells were compared with the corresponding controls to obtain the significantly differentially expressed gene lists using a two-tailed Student’s t test at each time point. A cutoff of P value < 0.05 and fold-change > 1.5 was used to select the EI1 regulated genes. The number of genes that showed statistically significant change increased with increasing treatment time (Fig. 6A). More genes were up-regulated than down-regulated between days 1 and 3 after EI1 treatment (Fig. 6A), suggesting the early response to Ezh2 inhibition is primarily gene up-regulation, which is consistent with Ezh2’s role in transcriptional repression. By day 6, the number of WSU-DLCL2 colonies were decreased after EI1 treatment. In contrast, the colony formation of OCI-LY19 was not affected. Taken together, these results indicate that EI1 is not generally toxic to cells, but selectively inhibits the neoplastic properties of DLBCL cells with Ezh2 mutation.
of down-regulated genes dramatically increased, which could be because of the secondary response (Fig. 6A).

To investigate if genes with expression changes at early time points are more likely direct PRC2 targets, two differential expressed gene lists were generated at days 3 and 9 after EI1 treatment, respectively. These lists were compared with the PRC2 and H3K27me3 target genes in human embryonic fibroblasts and centroblasts, respectively (10, 25). The gene list at day 3 showed better enrichment to the known PRC2 target genes than the day 9 gene list (Fig. S5). Almost all these genes are up-regulated, suggesting early response genes are direct Ezh2 targets. We further selected 13 genes that were up-regulated by EI1 from microarray and revalidated their expression by quantitative PCR (qPCR) (Fig. S6A). ChIP experiments were performed to determine if these genes were labeled by H3K27me3 at the promoters. EI1 treatment significantly decreased the H3K27me3 enrichment in these 13 gene promoters, but not the negative control GAPDH promoter (Fig. S6B). These data further strengthen the notion that the depletion of H3K27me3 at target gene promoters correlates to EI1-dependent transcriptional up-regulation.

To further delineate the pathways that are responsive to Ezh2 inhibition, we performed gene set enrichment analysis (GSEA) using the microarray data (P value < 0.05 and fold-change > 1.5) after 6 d of EI1 treatment (32, 33). The analysis revealed that the genes down-regulated by EI1 showed significant enrichment of a proliferation signature in DLBCL (Fig. 6B) (Proliferation_DLBCL signature from Lymphoma/Leukemia Molecular Profiling Project), consistent with our data on cell proliferation and cell cycle (Figs. 4 and 5) (34, 35). Interestingly, genes up-regulated by EI1 showed significant enrichment of a gene set distinguishing splenic marginal zone memory B cell from the germinal center B cell (Splenic_marginal_zone_B cell_gt_GC_B cell signature from Lymphoma/Leukemia Molecular Profiling Project), indicating that Ezh2 inhibition may promote differentiation to memory B-cell (Fig. 6C) (35, 36). We compared our data with a geneset that negatively correlated with Ezh2 mRNA level in DLBCL (25), and found that this gene signature showed significant enrichment in the genes up-regulated by EI1 treatment (Fig. 6D). Intriguingly, our data also displayed significant enrichment of a geneset regulated in prostate cancer cell after siRNA-mediated knockdown of Ezh2 (Fig. 6E and F and Fig. S7) (37).

Discussion

In this study, we developed a potent small molecule inhibitor, EI1, which inhibits Ezh2 activity through competition with the cofactor SAM. EI1 is highly selective against Ezh2 over its close homolog Ezh1 and other HMTs. The SET domains of Ezh1 and Ezh2 are 90% identical (Fig. S8A). Structural modeling shows the divergent residues I626 and C663 in Ezh2 (T664 and S664 in Ezh1) are around the EI1 binding pocket, even though no direct interaction with EI1 (Fig. S8B and C), suggesting these residues may contribute to the selectivity. Future structural studies of EI1-Ezh2 cocrysal structure may help to understand how such high selectivity is achieved.
EI1 treated cells show specific reduction of H3K27me2 and H3K27me3 (Fig. 2C). Using Ezh2-flxed MEF cells, we further demonstrated that EI1 had similar effect as Ezh2 knockout on H3K27 methylation and proliferation, indicating that EI1 is a highly specific Ezh2 inhibitor suitable for functional studies. siRNA knockdown of PRC2 complex, such as Ezh2, Eed, and Suv39, could disrupt the complex and block cancer cell proliferation (1, 12–14, 37). Here, we demonstrated that inhibition of HMT activity of Ezh2 by EI1 without disrupting the chromatin binding of PRC2 is sufficient to block cancer cell proliferation and colony formation, particularly in Ezh2 mutant containing DLBCL cells. Thus, a small molecule inhibitor of Ezh2 can be efficacious in treating cancers.

During the review of our manuscript, Knutson et al. (38) and McCabe et al. (39) published two Ezh2 inhibitors, which also act in a SAM-competitive manner and are highly selective over Ezh1. Although it was not shown whether the gene up-regulation by these compounds correlated with the decrease of H3K27me3 mark, both compounds could inhibit the proliferation of several Ezh2 mutant lymphoma cell lines. This finding is consistent with our findings and independently validates Ezh2 as a potential target for cancer treatment.

All Ezh2 mutations are heterozygous in DLBCL and the mutant cells contain much higher levels of H3K27me3 than wild-type cells (Fig. S1C) (17, 20, 21). Ezh2-Y641 mutants have weak biochemical activity toward unmethylated H3K27 and H3K27me1 peptide substrates, whereas they have much higher catalytic activity converting H3K27me2 to H3K27me3 (20, 23, 40). Wild-type and mutant Ezh2 complexes may collaborate to convert unmethylated H3K27 toward high levels of H3K27me3 (40). Interestingly, although H3K27me2 and H3K27me3 are similarly depleted in DLBCL cells with or without Ezh2 mutation, Ezh2
mutant cells are specifically growth-inhibited by EHI (Figs. 2 A and 4 B and C, and Fig. S3). This finding suggests that the Ezh2 mutation in DLBCL may be an oncogenic driver. Because EHI is equally active against both the wild-type and Y641 mutant Ezh2, it will be interesting to develop a mutant-selective inhibitor in the future to evaluate if it will be equally efficacious as EHI.

In this study, we conducted a detailed cell cycle analysis of EHI-treated DLBCL cells. Ezh2 mutant cells are growth-arrested by EHI with G1 arrest (Fig. 5). Expression of cyclin A and B1 is reduced in treated DLBCL cells. Ezh2 mutant cells are growth-arrested by EHI based on the inducible knockout of Ezh2 in response to 4-OH-T (100 nM).

Materials and Methods

Cell Culture. Cells were maintained in a humidified incubator at 37 °C, 5% (vol/vol) CO2. OCI-Ly19, GA10, Toledo, WSU-DLCL2, DB, SU-DHL4, Karpos22 and SU-DHL6 were cultured in RPMI-1640 (Invitrogen, 11875) with 15% (vol/vol) FBS. Karpas22 and SU-DHL6 (11993) were cultured in RPMI-1640 (Invitrogen) with 10% (vol/vol) FBS and 0.05 mM 2-mercaptoethanol (Sigma, M7522). MEFs were from the Antoine Peters laboratory (41) and cultured in DMEM with 10% (vol/vol) FBS. The immortalized MEF was generated by retroviral transduction of pBABE-SV40 T and selected with hygromycin (Invitrogen). Single clones were selected based on the inducible knockout of Ezh2 in response to 4-OH-T (100 mM).

Biochemical Assay. PRC2 biochemical reaction was carried out in reaction buffer (20 mM Tris pH 8.0, 0.1% BSA, 0.01% Triton, 0.5 mM DTT). The 20 nM wild-type PRC2 complex, 1 μM SAM, and 1.5 μM H3K27me2 (21–44); or 10 nM Y641F mutant complex, 1 μM SAM, and 3 μM H3K27me2 (21–44) were used, respectively, because they have differential substrate preference (38).

Microarray Analysis. RNA was purified from Karpas22 after 1, 2, 3, 6, and 9 d after DMSO or EHI (5 μM) treatment. Triplicate samples were collected and hybridized to U133 Plus 2 Chips (Affymetrix). The gene-expression data were normalized using the Robust Multiarray Averaging method (42). To generate a differentially expressed gene list, EHI-treated samples were compared with the corresponding DMSO controls using a cutoff of fold-change > 1.5 and P value < 0.05 (Student's t test). GSEA was performed as previously described (33).

Additional details of biochemical and cellular reagents and protocols are described in Supporting Materials and Methods. See Tables S2 and S3 for primers used.

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