Anti-AML activity of combined epigenetic therapy with novel DNMT1 inhibitors SGI-110 and SGI-1036 and histone deacetylase inhibitor panobinostat.

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INTRODUCTION

In the pathogenesis of leukemia and cancer, epigenetic mechanisms collaborate with genetic mutations and alterations to mediate silencing and loss of function of tumor suppressor genes (TSGs). Histone (H) deacetylation, H3 lysine (K) 27 trimethylation (3Me) and DNA hypermethylation are important known mechanisms involved in the epigenetic silencing of TSG, e.g., p15 and p16. Polycomb group proteins are multiprotein complexes that epigenetically silence gene expression, including TSGs. EZH2 is the the catalytic subunit of the polycomb repressive complex 2 (PRC2), which also includes SUZ12, EED and YY1. EZH2 acts as a histone lysine methyltransferase, which mediates tri-methylation (3Me) of K27 on H3 to silence expression of PRC2 target genes involved in lineage differentiation. EZH2 has been shown to be abundantly expressed in purified hematopoietic stem cell (HSCs), where it preserves HSC potential and prevents HSC exhaustion. EZH2 regulates cell proliferation by promoting S phase entry and G2-M transition, and it is highly expressed in tumor versus normal tissues. EZH2 mediated cell cycle progression promoted by gene repression also involves histone deacetylation by HDAC1, with which EZH2 interacts through its PRC2 binding partner EED. EZH2 is overexpressed in a variety of malignancies, including prostate, breast and bladder cancers with poor prognosis. Knockdown of EZH2 by siRNA has been demonstrated to inhibit breast cancer cell proliferation, while pharmacologic inhibition of EZH2 resulted in apoptosis of breast cancer but not normal cells. EZH2 was shown to directly interact with and regulate the activity of the DNA methyltransferases DNMT1, DNMT3a and DNMT3b. DNMTs function to transfer a methyl group from S-adenosyl-methionine to the 5' position of cytosine in the CpG dinucleotides in the promoters of genes, thereby maintaining a consistent pattern of epigenetic gene silencing of TSGs in cancer cells. DNA methylation by DNMTs also recruits HDAC activity to the promoters of silenced genes. Similar to the PRC2 complex, DNMT1 has a direct interaction with histone deacetylases HDAC1 and HDAC2. Treatment with the antisense oligonucleotides against DNMT1 was demonstrated to cause cell cycle arrest in the S-phase of the cell cycle and inhibited DNA replication with loss of DNMT1 at the replication fork. Although genes methylated in cancer cells are packaged with nucleosomes containing the 3Me H3K27 mark, genes silenced in cancer by 3Me H3K27 have been shown to be independent of promoter DNA methylation, thus highlighting that 3Me H3K27 could potentially be an independent mechanism for silencing TSGs. Consistent with this, DNA methylation and transcriptional silencing of cancer genes has been shown to persist despite the depletion of EZH2, suggesting that simultaneously inhibiting DNMT1 and EZH2 would be more effective in reversing 3Me H3K27 and DNA methylation.

In myeloid leukemia, all three DNMTs are overexpressed, which is associated with promoter hypermethylation of a number of genes, including JunB, E-cadherin and ERα. Moreover, in AML, hypermethylation of TSGs or DNA repair-related genes may play a significant role in the pathogenesis of leukemia. DNMT1 inhibitors, azacytidine and decitabine, inhibit global DNA methylation, as well as demethylate and de-repress TSGs. Co-treatment with a DNMT1 and HDAC inhibitor has been shown to synergistically demethylate the promoters of TSGs and inhibit leukemia growth. In a previous report, we had demonstrated that the pan-HDAC inhibitor panobinostat (LBH589) inhibits HDAC6 and induces hyperacetylation of the molecular chaperones heat shock protein (hsp) 90, associated with depletion of EZH2 and other PRC2 components. This occurred with the concomitant depletion of the transcription factors HOXA9 and MEIS 1 levels, resulting in loss of clonogenic survival of human leukemia cells. SGI-110 (S110) (Cancer Res 2007; 67: 6400) and SGI-1036 (SuperGen, Inc) are novel DNMT inhibitors, which also deplete DNMT1 levels. SGI-110 is dinucleotide containing decitabine, while SGI-1036 is a non-nucleoside hetro-cyclic compound. In the present studies, we determined the effects of treatment with SGI-110 or SGI-1036 alone, and in combination with panobinostat, on the levels of PRC2 proteins and DNMT1, as well as on the derepression of JunB. Additionally, we determined the growth inhibitory and apoptotic effects of SGI-110 or SGI-1036 and/or Panobinostat in cultured and primary AML cells.

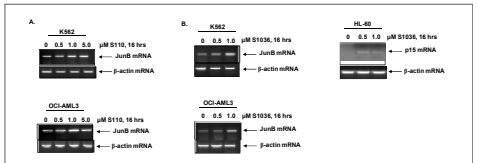

Figure 1. The DNMT-1 inhibitor S110 and S1036 induce JunB expression in AML and CML cells. A. K562 and OCI-AML3 cells were treated with the indicated concentrations of S110 for 16 hours. Then, total RNA was isolated and RT-PCR was done for JunB. A β-actin specific PCR and expression levels served as the loading control. **B.** K562, OCI-AML3 and HL-60 cells were treated with the indicated concentrations of S1036 for 16 hours. Total RNA was extracted and RT-PCR was done for JunB or p16 (HL-60). A β-actin specific PCR reaction and expression levels served to control for equal loading.

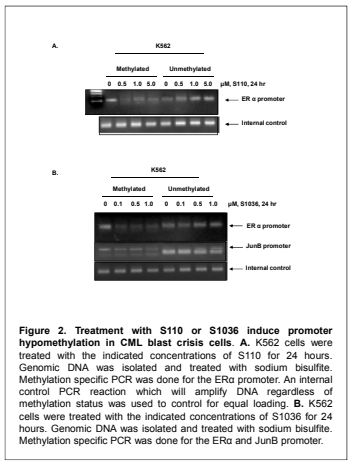

Figure 2. Treatment with S110 or S1036 induce promoter hypomethylation in CML blast crisis cells. A. K562 cells were treated with the indicated concentrations of S110 for 24 hours. Genomic DNA was isolated and treated with sodium bisulfite. Methylation specific PCR was done for the ERα promoter. An internal control PCR reaction which will amplify DNA regardless of methylation status was used to control for equal loading. **B.** K562 cells were treated with the indicated concentrations of S1036 for 24 hours. Genomic DNA was isolated and treated with sodium bisulfite. Methylation specific PCR was done for the ERα and JunB promoter.

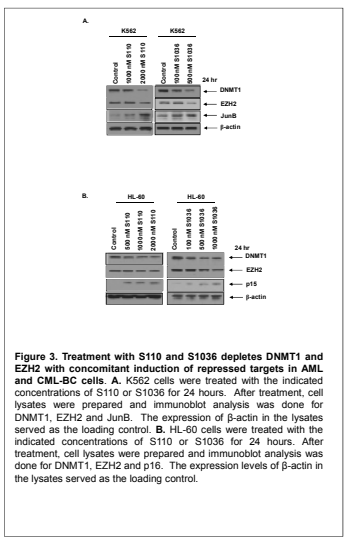

Figure 3. Treatment with S110 and S1036 depletes DNMT1 and EZH2 with concomitant induction of repressed targets in AML and CML-BC cells. A. K562 cells were treated with the indicated concentrations of S110 or S1036 for 24 hours. After treatment, cell lysates were prepared and immunoblot analysis was done for DNMT1, EZH2 and JunB. The expression of β-actin in the lysates served as the loading control. **B.** HL-60 cells were treated with the indicated concentrations of S110 or S1036 for 24 hours. After treatment, cell lysates were prepared and immunoblot analysis was done for DNMT1, EZH2 and p16. The expression levels of β-actin in the lysates served as the loading control.

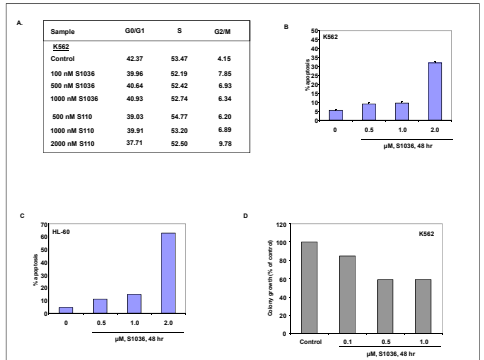

Figure 4. Treatment with S1036 induces apoptosis of AML and CML-BC cells and reduces clonogenic survival. A. K562 cells were treated with the indicated concentrations of S1036 or S110 for 24 hours. **B.-C.** K562 and HL-60 cells were treated with the indicated concentrations of S1036 for 48 hours. Following this, the percentages of apoptotic cells were determined by flow cytometry. **D.** K562 cells were treated with the indicated concentrations of S1036 for 48 hours. Then cells were washed free of the drug and plated in methylcellulose for 7 days. Colonies were counted and percentage of colony growth for each treatment was compared to untreated control cells.

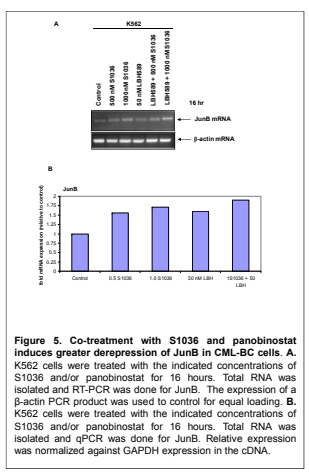

Figure 5. Co-treatment with S1036 and panobinostat induces greater derepression of JunB in CML-BC cells. A. K562 cells were treated with the indicated concentrations of S1036 and/or panobinostat for 16 hours. Total RNA was isolated and RT-PCR was done for JunB. The expression of a β-actin PCR product was used to control for equal loading. **B.** K562 cells were treated with the indicated concentrations of S1036 and/or panobinostat for 16 hours. Total RNA was isolated and qPCR was done for JunB. Relative expression was normalized against GAPH expression in the cDNA.

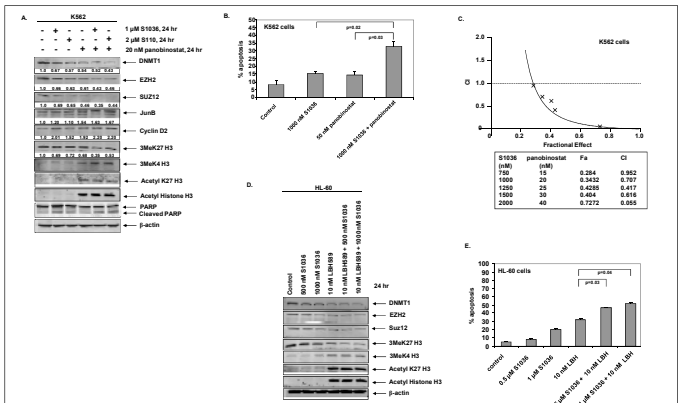

Figure 6. Combined treatment with S1036 and panobinostat induces greater depletion of DNMT1 and EZH2 and synergistic anti-leukemia effects in CML and AML cells. A. K562 cells were treated with the indicated concentrations of S110 or s1036 and panobinostat for 24 hours. Cell lysates were prepared and immunoblot analysis was done for DNMT1, EZH2 and PARP. The expression levels of β actin in the lysates served as the loading control. **B.** K562 cells were treated with the indicated concentrations of S1036 and/or panobinostat for 48 hours. At the end of treatment, cells were harvested and percentage of apoptotic cells were determined by flow cytometry. Columns, mean of three experiments; bars, standard error of the mean. **C.** K562 cells were treated with S1036 and Panobinostat at a constant ratio for 48 hours. The percentages of apoptotic cells were determined by flow cytometry and median dose effect analysis was performed using CALCUSYN. Combination indices under 1.0 represent synergism of the two agents in the CML-BC cells. **D.** HL-60 cells were treated with the indicated concentrations of S1036 and/or panobinostat for 24 hours. Cell lysates were prepared and immunoblot analysis was done for DNMT1, EZH2 and SUZ12. The expression levels of β actin in the lysates served as the loading control. **E.** HL-60 cells were treated with the indicated concentrations of S1036 and/ panobinostat for 48 hours. At the end of treatment, cells were harvested and percentage of apoptotic cells were determined by flow cytometry. Columns, mean of three experiments; bars, standard error of the mean.

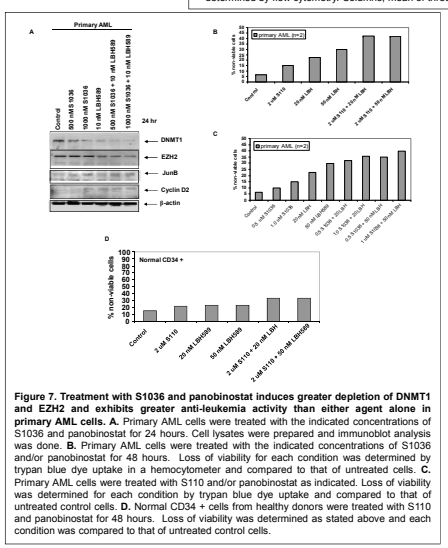

Figure 7. Treatment with S1036 and panobinostat induces greater depletion of DNMT1 and EZH2 and exhibits greater anti-leukemia activity than either agent alone in primary AML cells. A. Primary AML cells were treated with the indicated concentrations of S1036 and panobinostat for 24 hours. Cell lysates were prepared and immunoblot analysis was done. **B.** Primary AML cells were treated with the indicated concentrations of S1036 and/or panobinostat for 48 hours. Loss of viability for each condition was determined by trypan blue dye uptake in a hemocytometer and compared to that of untreated cells. **C.** Primary AML cells were treated with S110 and/or panobinostat as indicated. Loss of viability was determined for each condition by trypan blue dye uptake and compared to that of untreated control cells. **D.** Normal CD34 + cells from healthy donors were treated with S110 and panobinostat for 48 hours. Loss of viability was determined as stated above and each condition was compared to that of untreated control cells.

CONCLUSIONS

1. Novel DNMT1 inhibitors SGI-110 and SGI-1036 attenuate DNMT1 levels and activity, de-repressing silenced genes, e.g., JunB, p15 and ERα, associated with colony growth inhibition and apoptosis of human AML and CML-BC cells.

2. Importantly, SGI-110 and SGI-1036 also deplete EZH2 and 3Me K27H3 in AML cells.

3. Co-treatment with SGI-1036 and panobinostat cause greater depletion of DNMT1 and EZH2 levels, de-repress epigenetically silenced genes and synergistically induces apoptosis of AML and CML-BC cells.

4. Notably, co-treatment with SGI-1036 and panobinostat relatively spares normal, human, CD34+ bone marrow progenitor cells.

5. These findings support the rationale to test the in vivo efficacy of the combination of SGI-1036 and panobinostat human AML and CML-BC cells.

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