Superior Efficacy of a Combined Epigenetic Therapy against Human Mantle Cell Lymphoma Cells

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Abstract

Purpose: A deregulated epigenome contributes to the transformed phenotype of mantle cell lymphoma (MCL). This involves activity of the polycomb repressive complex (PRC) 2, containing three core proteins, EZH2, SUZ12, and EED, in which the SET domain of EZH2 mediates the histone methyltransferase activity. We determined the effects of 3-deazaneplanocin A (DZNep), an S-adenosylhomocysteine hydrolase inhibitor, and/or pan-histone deacetylase inhibitor panobinostat (PS) on cultured and primary MCL cells.

Experimental Design: Following treatment with DZNep and/or PS, apoptosis and the levels and activity of EZH2 and PRC2 proteins in cultured and primary MCL cells were determined.

Results: Treatment with DZNep depleted EZH2, SUZ12, and 3MeK27H3 in the cultured human MCL cells. DZNep also increased expression of p21, p27, and FBXO32, whereas it depleted Cyclin D1 and Cyclin E1 levels in MCL cells. In addition, DZNep treatment induced cell-cycle arrest and apoptosis in cultured and primary MCL cells. Furthermore, as compared with treatment with each agent alone, cotreatment with DZNep and PS caused greater depletion of EZH2, SUZ12, 3MeK27H3, and Cyclin D1 levels, whereas it induced greater expression of FBXO32, p16, p21, and p27. Combined treatment with DZNep and PS synergistically induced apoptosis of cultured and primary MCL cells while relatively sparing normal CD34+ cells. Cotreatment with DZNep and PS also caused significantly greater inhibition of tumor growth of JeKo-1 xenografts in NOD/SCID mice.

Conclusions: These preclinical in vitro and in vivo findings show that cotreatment with DZNep and PS is an active combined epigenetic therapy worthy of further in vivo testing against MCL.

Introduction

Mantle cell lymphoma (MCL) is an aggressive and distinct type of B-cell malignancy that comprises up to 10% of non-Hodgkin lymphomas (1). A characteristic feature of MCL is the chromosomal rearrangement caused by the translocation t(11;14)(q13;q32), which results in overexpression of Cyclin D1 (2, 3). Copy number variations and associated gene expression changes involving CDKN2A (p16) and CDKN2B (p15), as well as altered expression of CDK4, and p27 has also been noted and may play a role in the pathogenesis of MCL (3–5). In addition, increased expression of MYC, Cyclin D1, and EZH2 has been associated with poor prognosis and correlate with poorer clinical outcome in MCL (3, 6–8). EZH2 is a core member and the catalytic subunit of the polycomb protein complex, polycomb repressive complex (PRC) 2, that also contains SUZ12 and EED, in which the SET domain of EZH2 mediates the histone lysine (K) methyltransferase (KMTase) activity of PRC2 (9). EZH2 was shown to be preferentially overexpressed in proliferating but not resting MCL cells (15). Recently, in lymphomagenesis, EZH2-mediated gene silencing in germline center B cells was shown to contribute to cell proliferation (16). Somatic gain of function mutations in tyrosine 641 (Y641C) in EZH2 have also been noted to selectively
Translational Relevance

Overexpression of EZH2 and the polycomb repressive complex (PRC) 2 proteins has been associated with poor prognosis and correlates with poorer clinical outcome in mantle cell lymphoma (MCL). PRC2 mediates trimethylation of lysine 27 on histone H3 (3MeK27H3) and epigenetically represses genes such as the tumor suppressor p16. The effects of pharmacologic inhibition of EZH2 or EZH2 knockdown have not been tested in MCL cells. Here, we show that treatment with the EZH2 antagonist DZNep or the pan-histone deacetylase inhibitor panobinostat (PS) depletes EZH2, SUZ12, and 3MeK27H3. Cotreatment with PS-augmented DZNep mediated attenuation of PRC2, which was associated with synergistic activity of the combination against human MCL cells in vitro and in vivo. These observations support the rationale to further test the in vivo anti-MCL efficacy of the combination of PS with an EZH2 antagonist or combination therapies that target the deregulated epigenome in human MCL cells.

Materials and Methods

Reagents

PS was kindly provided by Novartis Pharmaceuticals, Inc. DZNep was acquired from the National Cancer Institute (Rockville, MD). Monoclonal Bmi1, polyclonal trimethylated K9 histone H3, polyclonal trimethylated K79 histone H3, acetylated K16 histone H4, acetyl K56 histone H3, and polyclonal trimethylated K4 histone H3 antibodies were purchased from Millipore. All other validated antibodies were purchased from vendors, as previously described (29, 30, 33, 34).

Cell lines and cell culture

MCL cell line MO2058 was obtained and maintained as previously described (29, 30). JeKo-1 and Z-138 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). Both cell lines were banked after receipt, and passaged for less than 6 months before use in these studies. The ATCC characterizes cell lines using short tandem repeat polymorphism analysis. MCL cells were maintained in culture, as previously described (29, 30). Logarithmically growing cells were exposed to the designated concentrations of DZNep and/or PS. Following these treatments, cells were washed free of the drug(s) before the performance of the studies.

Primary MCL cells

Primary MCL samples and normal CD34 + mononuclear cells were obtained with informed consent as part of a clinical protocol approved by the Institutional Review Board of University of Kansas (IRB approval # 12392). Peripheral blood or bone marrow aspirate samples were collected and separated for mononuclear cells, as previously described (29, 30, 33, 34).
Cell-cycle analysis
Following DZNep treatment, MCL cells were washed twice with 1X PBS and fixed in 70% ethanol at −20°C overnight. Fixed cells were washed twice with 1X PBS, resuspended in 1X PBS with 0.1% Triton X-100, RNase A, and propidium iodide and incubated in the dark at 37°C for 15 minutes. Cell-cycle data were collected on a flow cytometer with a 488 nmol/L laser and analyzed with ModFit 3.0, as previously described (34).

Assessment of percentage of nonviable cells
Following treatment with DZNep and/or PS, nonviable cells were determined by trypan blue dye uptake on a hemocytometer, as previously described (29, 30, 34). Alternatively, cells were stained with propidium iodide and the percentages of nonviable cells were determined by flow cytometry.

RNA interference
Small interfering RNAs against EZH2 were obtained from Dharmacon. JeKo-1 cells were transfected with siRNA to a final concentration of 100 nmol/L using an Anaxa Nucleofector device. Cells were incubated for 48–72 hours for detection of EZH2 knockdown. Lentiviral shRNAs against EZH2 were obtained from Sigma-Aldrich. JeKo-1 cells were transduced with lentivirus and incubated for 48 hours. Following this, cells were washed with complete media and incubated an additional 24 hours for immunoblot analyses for 72 hours to observe effects on cell proliferation, as previously described (34, 36).

RNA isolation and reverse transcription-PCR
RNA was extracted from the cultured MCL cells using an RNAqueous-4PCR kit (Applied Biosystems). Purified total RNA was quantified, reverse transcribed, and quantitative real-time PCR analyses for EZH2, SUZ12, EED, Cyclin D2, SMARCA2, TCF4 and ELF3A were done on the resulting cDNA utilizing TaqMan probes from Applied Biosystems, as previously described (29, 34). SDS-PAGE and immunoblot analyses
Seventy-five micrograms of total cell lysate was used for SDS-PAGE. Western blot analyses of DNMT1, EZH2, SUZ12, EED, 3MeK27H3, acetyl K27H3, PARP, FBXO32, Cyclin E, p16, p21, and p27 were conducted on total cell lysates as previously described (29, 30, 34). Immunoblot analyses were conducted 3 times, and the same immunoblot was probed with specific antisera or monoclonal antibodies. Representative blots were subjected to densitometric analysis where expression levels of β-actin were used as the control. Densitometry was conducted using ImageQuant 5.2 (GE Healthcare).

Mantle cell lymphoma xenograft
JeKo-1 cells (10 million) were subcutaneously implanted into the flank of NOD/SCID mice. When the average tumor volume reached approximately 150 mm³, the following treatments were administered in cohorts of 5 mice for each treatment: vehicle alone (5% DMSO), 1 mg/kg DZNep, 10 mg/kg PS, and DZNep plus PS. DZNep was administered twice per week intraperitoneally for 2
weeks. PS was administered 3 times per week for 2 weeks. Tumor growth was monitored every other day by calipers and tumor volume was calculated using the equation: \( \frac{1}{2} (\text{length} \times \text{width}^2) \). We selected the dose of DZNep that had been determined to be safe in previously reported studies and combined it with a dose of PS that we had previously reported to be safe and biologically effective (29, 38). This study was conducted in duplicate with similar results.

**Statistical analysis**

Significant differences between values obtained in a population of MCL cells treated with different experimental conditions were determined using the Student’s \( t \) test. For the in vivo mouse models, the final tumor volumes at the end of treatment were used to calculate the difference between mice treated with DZNep or PS alone versus mice treated with the combination using a 2-tailed \( t \) test. \( P \) values of less than 0.05 were assigned significance.

**Results**

**Treatment with DZNep depletes PRC2 proteins EZH2 and SUZ12 levels and disrupts the PRC2 complex in MCL cells**

Treatment with DZNep has been previously shown to deplete EZH2 and SUZ12 levels in breast cancer, colon cancer and leukemia cells (28, 34, 37). Here, we determined the effect of DZNep on PRC2 proteins in the cultured MCL JeKo-1, MO2058, and Z-138 and primary MCL cells. Exposure to DZNep for 24 hours dose dependently reduced the protein expression of EZH2, SUZ12, and BMI1 in cultured and primary MCL cells (Fig. 1A and B and Supplementary Fig. S1A). Similar to our previous findings in AML cells, exposure to DZNep up to 1.0 \( \mu \text{mol/L} \) did not significantly affect the mRNA expression of EZH2 and SUZ12, as determined by quantitative RT-PCR (Fig. 1C). As shown in Supplementary Fig. 1B, cotreatment with the proteasome inhibitor bortezomib restored the DZNep-mediated depletion of EZH2 and SUZ12 levels, indicating that depletion of the PRC2 proteins by
DZNep was mediated by proteasomal degradation. In MCL cells, depletion of the PRC2 complex proteins by DZNep was associated with attenuation of the repressive histone mark 3MeK27H3 and upregulation of 3MeK4H3 (Fig. 1D and data not shown). DZNep treatment did not alter the expression levels of 3MeK9H3 and 3MeK79H3, but induced acetylation of H3K27, H3K56, and H4K16 (Fig. 1D). Treatment with DZNep for as short as 4 hours markedly depleted the binding of EZH2 with other PRC2 proteins, SUZ12 and EED, as well as diminished binding of EZH2 to DNMT1 in MCL cells (Fig. 1E). DZNep-mediated alterations in the levels of PRC2 proteins and in the chromatin marks led to decline in the binding of EZH2 to its known target promoters, for example, HOXA9, WNT1, and RUNX3, as discerned by chromatin immunoprecipitation analysis of the promoters of these genes (Fig. 2A; refs. 28, 34, 37). Both in cultured and primary MCL cells, treatment with DZNep also reduced the levels of the PRC1 protein BMI1 (Fig. 1A and B). The effects of DZNep on PRC2 and PRC1 proteins and chromatin marks were associated with decline in the levels of Cyclin D1 and Cyclin E, but increase in the levels of p27, p21, and the prodeath F-Box protein FBXO32, an E3 ubiquitin ligase (28, 34), in the cultured MCL cells (Fig. 2B and Supplementary Fig. S1A). DZNep treatment significantly increased the mRNA levels of the PRC2 target genes SMARCA2, EIF3A, and TCF4 (Supplemental Fig. S2A-C). Recently, the expression of EZH2 was shown to be inhibited by microRNA-101 in cancer cells (39, 40). Although MO2058 and JeKo-1 cells possess the pre-microRNA for hsa-miR-101 (Supplementary Fig. S3A), treatment with DZNep did not significantly alter the expression of the mature hsa-miR-101 in MO2058 and JeKo-1 cells (Supplementary Fig. S3B). Treatment with DZNep (1.0–2.0 μmol/L) also had no effect on the methylation status of the p16 promoter in MO2058 cells, whereas treatment with the DNMT1 inhibitor decitabine caused significant promoter hypomethylation (Supplementary Fig. S3C).

**DZNep treatment induces cell-cycle arrest and apoptosis of MCL cells**

We next determined the effects of DZNep treatment on the cell cycle of MO2058 and JeKo-1 cells. As shown in Table 1, treatment of MO2058 and JeKo-1 cells resulted in a dose-dependent accumulation of cells in the G0–G1 phase, with a concomitant decline in the number of cells in S-phase of the cell cycle \( (P < 0.05) \). Next, we determined the effects of DZNep treatment on induction of apoptosis in MO2058, JeKo-1, and Z-138 cells. Treatment with DZNep dose dependently induced apoptosis in all 3 MCL cell lines, although Z-138 cells were more sensitive to the lower concentrations of DZNep compared with MO2058 and JeKo-1 cells (Fig. 3A and Supplementary Fig. S4A). The apoptotic effects in MO2058 and JeKo-1 cells were further enhanced following a 72-hour exposure to DZNep (data not shown). DZNep-induced caspase 3 activity was also shown by induction of PARP cleavage, a hallmark of apoptosis (Fig. 3B and Supplementary Fig. S4B).

**DZNep-mediated induction of p16, p21, p27, and FBXO32 is mechanistically linked to depletion of EZH2 in MCL cells**

To determine whether the DZNep-mediated decline in EZH2 was mechanistically linked to increase in the levels of p21, p27, and FBXO32, we also determined the effect of siRNA to EZH2 in JeKo-1 cells. Figure 4A shows that, as compared with the control siRNA, treatment with siRNA to EZH2 for 48 hours depleted EZH2 mRNA, but modestly increased the levels of EED mRNA. SUZ12 mRNA was unaffected (data not shown). Treatment with siRNA to EZH2 was associated with decline in protein levels of EZH2 and SUZ12 but not of EED (Fig. 4B). In addition, there was no effect of EZH2 siRNA on the protein levels of
DNMT1 and HDAC2, whereas HDAC1 levels were slightly increased (Fig. 4B). Attenuation of PRC2 proteins by EZH2 siRNA and the impairment of the PRC2 complex activity were associated with decline in the levels of 3MeK27H3 but induction of acetylated K27H3 and 3Me H3K4 levels in MCL cells (Fig. 4C). This was accompanied by increase in the levels of p16, p21, and p27, with modest decline in Cyclin D1 levels (Fig. 4C). EZH2 siRNA treatment also induced the F-Box protein FBXO32 levels in MCL cells (Fig. 4C). We also determined the effects of transduction of shRNA to EZH2 on the proliferation and apoptosis of MCL cells. Depletion of EZH2 and SUZ12 by EZH2 shRNA significantly inhibited cell proliferation without inducing apoptosis of MCL cells (Fig. 4D and data not shown).

Treatment with the pan-HDAC inhibitor panobinostat depletes PRC2 complex proteins with concomitant decline in 3MeK27H3 in MCL cells

We next determined whether treatment with the pan-HDAC inhibitor PS would deplete the levels of EZH2 and SUZ12 with concomitant decline in the levels of 3MeK27H3 and 3Me H3K4 levels in MCL cells (Fig. 4C). This was accompanied by increase in the levels of p16, p21, and p27, with modest decline in Cyclin D1 levels (Fig. 4C). EZH2 siRNA treatment also induced the F-Box protein FBXO32 levels in MCL cells (Fig. 4C). We also determined the effects of transduction of shRNA to EZH2 on the proliferation and apoptosis of MCL cells. Depletion of EZH2 and SUZ12 by EZH2 shRNA significantly inhibited cell proliferation without inducing apoptosis of MCL cells (Fig. 4D and data not shown).

Table 1. Treatment with DZNep induces cell-cycle arrest of MCL cells in a dose-dependent manner

<table>
<thead>
<tr>
<th>Cells and treatment</th>
<th>G0-G1</th>
<th>S</th>
<th>G2/M</th>
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<tbody>
<tr>
<td>MO2058</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>35.51 ± 1.20</td>
<td>60.98 ± 0.47</td>
<td>3.51 ± 0.77</td>
</tr>
<tr>
<td>0.5 µmol/L DZNep</td>
<td>36.75 ± 0.56</td>
<td>58.26 ± 0.52</td>
<td>4.99 ± 0.26</td>
</tr>
<tr>
<td>1.0 µmol/L DZNep</td>
<td>46.63 ± 1.87</td>
<td>46.67 ± 1.90</td>
<td>6.70 ± 0.11</td>
</tr>
<tr>
<td>2.0 µmol/L DZNep</td>
<td>52.89 ± 1.23</td>
<td>39.42 ± 0.86</td>
<td>7.69 ± 0.47</td>
</tr>
<tr>
<td>JeKo-1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>49.08 ± 1.12</td>
<td>48.70 ± 1.02</td>
<td>2.22 ± 0.12</td>
</tr>
<tr>
<td>0.5 µmol/L DZNep</td>
<td>60.22 ± 0.10</td>
<td>36.46 ± 0.23</td>
<td>3.32 ± 0.32</td>
</tr>
<tr>
<td>1.0 µmol/L DZNep</td>
<td>67.77 ± 0.98</td>
<td>28.76 ± 1.21</td>
<td>3.47 ± 0.28</td>
</tr>
<tr>
<td>2.0 µmol/L DZNep</td>
<td>72.06 ± 0.54</td>
<td>24.18 ± 0.70</td>
<td>3.76 ± 0.20</td>
</tr>
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</table>

NOTE: Cell-cycle status of MO2058 and JeKo-1 cells treated with the indicated concentrations of DZNep for 24 hours. Values represent the mean of 3 independent experiments ± SEM.

Cotreatment with DZNep and PS shows superior anti-EZH2 activity, induces more p21 and p27, and exerts synergistic apoptotic effects against MCL cells

We next determined the effects of combined treatment with DZNep and PS in MCL cells. Figure 6A and B show that, as compared with treatment with either agent alone, cotreatment with DZNep (250–2,000 nmol/L) and PS synergistically induced apoptosis of MO2058 and JeKo-1 cells, as highlighted by the combination indices of less than 1.0.

Figure 3. Treatment with DZNep induces dose-dependent apoptosis of MCL cells. A, MO2058 and JeKo-1 cells were treated with DZNep as indicated for 48 hours. The percentages of annexin-V–positive cells were determined by flow cytometry. Columns, mean of 3 experiments; bars, SEM. B, immunoblot analyses of PARP and β-actin in MO2058 and JeKo-1 cells after treatment with DZNep for 24 hours.
Figure 4. RNA interference–mediated depletion of EZH2 induces p16, p27, p21, and FBXO32 expression and inhibits cell proliferation of MCL cells. A, quantitative RT-PCR for EZH2 and EED following 48-hour transfection with scrambled or EZH2 siRNA. The relative expression was normalized against GAPDH. B and C, relative expression was normalized with scrambled or EZH2 siRNA. The EED following 48-hour transfection quantitative RT-PCR for EZH2 and proliferation of MCL cells. A, expression and inhibits cell p16, p27, p21, and FBXO32 mediated depletion of EZH2 induces

then, 1 × 10⁵ cells were plated in triplicate and cell growth was measured for an additional 72 hours. Graph shows cell growth of 2 independent experiments conducted in triplicate. The inset shows the level of EZH2 and SUZ12 knockdown in the cells.

determined through median dose effect isobologram analyses. Superior activity of the combination was also noted in Z-138 cells (Supplementary Fig. 5C). This activity of the combination was also associated with greater attenuation of EZH2, SUZ12 and the 3MeK27H3 chromatin mark, with a concomitant, greater induction of 3MeK4H3 mark in the cultured MCL cells (Fig. 6C and Supplemental Fig. 5A and B). Cotreatment with DZNep and PS also caused greater decline in DNMT1 levels. It is noteworthy that, as compared with each agent alone, combined treatment with DZNep and PS induced greater increase in the levels of FBXO32, p21 and p27 in the cultured MO2058 MCL Cells (Fig. 6C). Similar effects were also observed in JeKo-1 and Z-138 cells (Supplemental Fig. 5A).

Treatment with DZNep and/or PS inhibits tumor growth of JeKo-1 xenografts

We next determined the in vivo anti-MCL activity of treatment with DZNep and/or PS on JeKo-1 cell xenografts in NOD/SCID mice. In cohorts of mice, JeKo-1 cells were implanted in the flanks, and the treatment of each agent or vehicle alone was begun after the flank tumors achieved a size of approximately 150 mm³. As shown in Fig. 6D, although treatment with DZNep or PS alone also caused inhibition of tumor growth, combined treatment with DZNep and PS exerted superior growth inhibitory, antitu-

Table 1. Effects of DZNep and/or PS on cell viability of patient-derived primary MCL cells versus normal CD34+ bone marrow progenitor cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cell Viability (%)</th>
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<tr>
<td>DZNep</td>
<td>85 ± 5</td>
</tr>
<tr>
<td>PS</td>
<td>80 ± 7</td>
</tr>
<tr>
<td>Combined</td>
<td>70 ± 4</td>
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Cotreatment with DZNep and PS shows greater cytotoxicity against primary MCL cells than either agent alone

Finally, we determined the effects of treatment with DZNep and/or PS on the cell viability of patient-derived primary MCL cells versus normal CD34+– bone marrow progenitor cells. Figure 7A shows that exposure to either DZNep or PS for 48 hours induced more loss of viability in 6 primary MCL cell samples, as compared with normal CD34+–progenitor cells. In addition, compared with treatment with each agent alone, cotreatment with DZNep (0.5 or 2.0 µmol/L) and PS (50 nmol/L) induced significantly greater lethality of MCL versus normal progenitor cells (Fig. 7A). Similar to the effects observed in the cultured MCL cell lines, combined treatment with DZNep and PS was synergistically lethal against primary MCL cells (Fig. 7B). On 1 primary sample, where adequate numbers of MCL cells were available, immunoblot analyses showed that combined treatment with PS and DZNep, as compared with treatment with DZNep alone, caused greater decline in the levels of EZH2, SUZ12, BMI1, and Cyclin D1 expression levels in primary MCL cells (Fig. 7C).
Discussion

Deregulated epigenome plays a pathogenic role and contributes to the aggressive biology in human MCL (3, 7, 8, 14). In this, the specific role of overexpression of the PRC2 and PRC1 proteins EZH2, SUZ12, and BMI1 has also been elucidated (3, 5, 7, 14). Our present studies show the PRC2 and PRC1 proteins EZH2, SUZ12, and BMI1 has (3, 7, 8, 14). In this, the specific role of overexpression of RUNX3. In addition, and consistent with prior reports, known EZH2 target genes, including HOXA9, WNT1, and decreased binding of EZH2 to the promoters of the known EZH2 target genes, including HOXA9, WNT1, and RUNX3. In addition, and consistent with prior reports, exposure to DZNep induced the expression of FBXO32 (atrogin) and depleted Cyclin E (28, 32, 37), as well as up regulated the levels of p16, p21, and p27 in MCL cells. As SUZ12 is aberrantly overexpressed and promotes survival of MCL cells (42), DZNep-mediated depletion of SUZ12 may also impair survival of MCL cells. Collectively, these effects of DZNep treatment explain the growth inhibitory and apoptotic effects of DZNep in MCL cells.

Notably, the PRC1 protein BMI1 is commonly amplified and overexpressed in MCL cells (9, 14). Our findings show that DZNep treatment also depleted BMI1, thereby affecting PRC1 activity in MCL cells. Although this was not studied here, DZNep may be exerting this effect on BMI1 levels by perturbing the recently described, EZH2-regulated miRs that regulate the expression of BMI1 (43). By inhibiting both PRC2 and PRC1 activity, DZNep treatment reduced the levels of 3MeK27H3 and ubiquitylated-K119H2A chromatin marks, but increased the acetylation of K27H3, K56H3 and K16H4. While the precise underlying mechanism for this is unknown, exposure to DZNep also increased the levels of 3MeK4H3 in MCL cells. Although its impact here is unknown, this is known to be a permissive chromatin mark mediated by trithorax (TRX) family of proteins facilitating gene expression involved in stem cell-lineage differentiation (44). Collectively, DZNep-mediated inhibition of PRC2 and PRC1, and the notable alterations in the chromatin marks: decreased 3MeK27H3 and increased 3MeK4H3, as well as increased ubiquitylation of EZH2, SUZ12 and EED in cells, which was reversed by treatment with a proteasome inhibitor (41). Treatment with DZNep also did not affect the expression levels of hsa-miR-101, which is known to down regulate the levels of EZH2 (39, 40). Consistent with DZNep-mediated attenuation of EZH2 levels, in MCL cells, we observed decreased binding of EZH2 to the promoters of the known EZH2 target genes, including HOXA9, WNT1, and RUNX3. In addition, and consistent with prior reports, attenuation of EZH2 levels, in MCL cells, we observed decreased binding of EZH2 to the promoters of the known EZH2 target genes, including HOXA9, WNT1, and RUNX3.
target gene promoters, this was not the case for p16 gene promoter where CpG methylation was found to be unaffected by DZNep treatment. However, both EZH2 and BMI1-mediated chromatin effects have been implicated in the repression of Ink4a/Arf locus encoding p16 and ARF (p19; refs. 46, 47). Therefore, notwithstanding the absence of its effects on DNA methylation, it is not surprising that treatment with DZNep led to up regulation of p16 expression in MCL cells. BMI1 has also been shown to be required to reinforce bivalent domains at key loci for maintaining lineage specification poised for activation in hematopoietic stem cells (48).

DZNep treatment in MCL cells increased the expression of the cell cycle inhibitory proteins p16, p21 and p27 in MCL cells and caused induction of the E3 ubiquitin ligase FBXO32 (28, 32, 34, 37). DZNep induced FBXO32 levels...
were associated with decline in the levels of Cyclin E, which is known to be targeted by FBXO32 (28). Induction of FBXO32 has also been shown to promote apoptosis (28, 33). Similar to DZNep treatment, depletion of EZH2 by siRNA also induced the levels of p16, p21, p27, and FBXO32, which indicates that DZNep-mediated depletion of EZH2 is responsible for modulating these protein levels and growth inhibition in MCL cells. It is notable that while treatment with DZNep induced growth inhibition and apoptosis, EZH2 shRNA treatment only induced growth inhibition in MCL cells. This may be because DZNep treatment induced significantly higher levels of FBXO32 in MCL cells than treatment with EZH2 siRNA. Other off-target effects of DZNep may also be responsible for this discrepancy. Other studies have also shown that DZNep is not a specific inhibitor of EZH2, and can affect the methylation status of chromatin marks other than H3K27 at high concentrations (49). Searching for more specific and less toxic EZH2 antagonists is a clear priority but prototype, direct EZH2 antagonists are not yet available for testing.

Figure 7. Cotreatment with DZNep and PS exerts synergistic cytotoxic effects against primary MCL cells. A, primary MCL (n = 6) and normal CD34+ cells (n = 3) were treated with DZNep and/or PS for 48 hours. * loss of viability values significantly greater (P < 0.05) than those resulting from treatment with either agent alone. †, values significantly less in normal CD34+ progenitor cells than those observed in primary MCL cells. B, median dose effect and isobologram analyses of primary MCL cells treated with DZNep and PS for 48 hours, CI values less than 1.0 indicate the synergistic interaction of these 2 agents. C, immunoblot analyses of EZH2, SUZ12, BMI1, Cyclin D1, and β-actin in primary MCL cells treated with DZNep and/or PS for 24 hours. D, schematic model of the mechanism of action of DZNep and PS in MCL cells. Treatment with DZNep results in the inhibition of EZH2 expression and depletion of lysine 27 trimethylation on Histone H3 (H3K27Me3). This results in induction of p16, p21, and p27. PS inhibits the activity of Class I (HDAC1, HDAC2, and HDAC3) and Class II HDACs, particularly HDAC6, the deacetylase for hsp90. Inhibition of HDAC6 activity results in the hyperacetylation of hsp90, thereby reducing chaperone association of hsp90 with EZH2. This leads to depletion of EZH2 expression and subsequent loss of H3K27Me3. Inhibition of hsp90 function also results in depletion of CDK4 and cyclin D1 expression levels. PS treatment also induces acetylation of Histone H3 and H4 and upregulates p16, p21, and p27 in MCL cells. Combined treatment with DZNep and PS causes greater depletion of H3K27Me3 and greater induction of p16, p21, and p27 in MCL cells.
As was reported for other cancer cell-types, treatment with PS also depleted EZH2, SUZ12, and BMI1 in MCL cells. Concomitantly, this was associated with decline in the 3MeK27H3 but increase in 3MeK4H3, acetylated-K27 H3, and acetylated-K16 H4 chromatin marks. PS treatment also inhibited H2A ubiquitylation, possibly because of decline in PRC1 activity in MCL cells (9, 14). Importantly, treatment with PS also significantly depleted the levels of Cyclin D1, as well as induced apoptosis of cultured and primary MCL cells. It should be noted that we have previously documented multiple nonepigenetic mechanisms that may also contribute to the anti-MCL activity of PS (29, 30). Therefore, the overall anti-MCL activity of PS is likely to be because of multiple mechanisms. Our findings also show that, as compared with treatment with each agent alone, cotreatment with DZNep and PS resulted in greater depletion of EZH2, SUZ12, DNM1T1, Cyclin D1, and 3MeK27H3, with concomitant induction of 3MeK4H3, FBXO32, p21, and p27. These molecular perturbations resulting from cotreatment with DZNep and PS are likely to have contributed to the superior anti-MCL activity of the combination (Fig. 7D). Indeed, cotreatment with DZNep and PS synergistically induced apoptosis of cultured and primary MCL cells. Notably, combined treatment with DZNep and PS was only slightly more toxic than each agent alone against normal CD34+ progenitor cells. The combination also induced significantly more apoptosis in primary MCL versus normal CD34+ progenitor cells. These results indicate that cotreatment with DZNep and PS is selectively more toxic against MCL cells. This is further confirmed by our findings that the combination exerts greater in vivo antitumor effects against the Jeko-1 xenograft model, without inducing weight loss or other physical side effects during treatment of the NOD/SCID mice. In the current study, we used previously determined doses of DZNep and PS that improved survival of NOD/SCID mice implanted with human AML cell xenografts without resulting in toxicity to the mice (34). It is noteworthy that, so far, neither DZNep nor any of its active analogues has been administered to humans. On the other hand, Phase I/II clinical studies have highlighted the activity of PS in patients with hematologic malignancies, especially those with lymphoma (50). Findings presented here support the rationale to further test the in vivo anti-MCL efficacy of the combination of PS with an EZH2 antagonist. In addition, our findings also create the rationale to develop and test combination therapies that target the deregulated epigenome in human MCL cells.

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