

# Combined inhibition of EZH2 and histone deacetylases as a potential epigenetic therapy for non-small-cell lung cancer cells

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## Key words

3-Deazaneplanocin A, EZH2, lung cancer, polycomb-group protein, vorinostat (suberoylanilide hydroxamic acid)

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Recent discoveries have revealed that human cancer involves aberrant epigenetic alterations. We and others have previously shown that the histone methyltransferase EZH2, the catalytic subunit of polycomb repressive complex 2 (PRC2), is frequently overexpressed in non-small-cell lung cancer (NSCLC) and that an EZH2 inhibitor, 3-deazaneplanocin A, inhibits the proliferation of NSCLC cells. Transcriptional silencing by EZH2 was recently shown to be required for the activity of histone deacetylases (HDACs) that interact with another PRC2 protein, EED. To develop a more effective epigenetic therapy for NSCLC, we determined the effects of co-treatment with 3-deazaneplanocin A and the HDAC inhibitor vorinostat (SAHA) in NSCLC cells. The co-treatment synergistically suppressed the proliferation of all tested NSCLC cell lines, regardless of their epidermal growth factor receptor (EGFR) status. The synergistic effect was associated with slightly decreased histone H3 lysine 27 trimethylation, modestly increased histone acetylation, and the depletion of EZH2 and other PRC2 proteins. The co-treatment resulted in an accumulation of p27Kip1, decrease in cyclin A, and increased apoptotic fraction in an additive/synergistic manner. Interestingly, the co-treatment strongly suppressed EGFR signaling, not only in *EGFR*-wild-type NSCLC cells, but also in *EGFR*-mutant cells, mainly through dephosphorylation of EGFR. Furthermore, the co-treatment suppressed the *in vivo* tumor growth of *EGFR*-mutant, *EGFR*-tyrosine kinase-resistant H1975 cells more effectively than did each agent alone, without visible toxicity. These results suggest that the combined pharmacological targeting of EZH2 and HDACs may provide more effective epigenetic therapeutics for NSCLC.

Lung cancer is the leading cause of cancer-related deaths worldwide, with a poor prognosis for patients with advanced-stage disease who are treated with traditional cytotoxic chemotherapeutics.<sup>(1)</sup> Although the recent development of oncogene-directed drugs including *EGFR*-TKIs has significantly improved NSCLC treatment,<sup>(2,3)</sup> it has been limited to a minority of patients with a targetable mutation and almost inevitably results in drug resistance, relapse, and mortality.<sup>(4,5)</sup> Recent discoveries have shown that human cancer involves not only genetic changes, but also aberrant epigenetic alterations, leading to the successful development of epigenetic therapies in some hematologic malignancies as single drugs.<sup>(6,7)</sup> In NSCLC, however, several preclinical and clinical studies have indicated that the effects of single epigenetic drugs are modest and that the development of new approaches such as combination therapies, will be necessary.<sup>(8)</sup>

Enhancer of zeste homolog 2 (EZH2), the catalytic subunit of polycomb repressive complex 2 (PRC2), is among the potential epigenetic therapeutic targets for NSCLC.<sup>(8)</sup> EZH2, which acts as a histone lysine methyltransferase, mediates trimethylation of lysine 27 on histone H3 (H3K27me3) to

silence PRC2 target genes involved in lineage differentiation.<sup>(9)</sup> Accumulating evidence shows that EZH2 has a role in regulating the malignant transformation and biological aggressiveness of several human malignancies.<sup>(10–14)</sup> We and others have found that NSCLCs frequently overexpress EZH2 and that the high EZH2 expression is correlated with poor prognosis.<sup>(15–17)</sup> 3-Deazaneplanocin A, originally identified as an *S*-adenosyl-L-homocysteine hydrolase inhibitor,<sup>(18)</sup> downregulates PRC2 proteins including EZH2 and inhibits PRC2 activity.<sup>(19)</sup> We have reported that DZNep inhibits NSCLC cell proliferation through inhibition of PRC2,<sup>(20)</sup> as has also been shown in other types of cancer cells.<sup>(21–26)</sup>

Histone deacetylases are promising epigenetic targets that catalyze the removal of acetyl groups from lysine residues in histones, leading to chromatin condensation and the transcriptional repression of target genes, including tumor suppressor genes.<sup>(27)</sup> Overexpression of class I HDACs, especially HDAC1, is noted in several human cancers, including NSCLC,<sup>(28,29)</sup> indicating that their aberrant epigenetic activity is associated with cancer development. Vorinostat (SAHA), which inhibits class I and II HDACs, is the

first HDAC inhibitor approved for use in patients with cancer, specifically for the treatment of cutaneous T-cell lymphoma.<sup>(30)</sup> In lung cancer, SAHA has significant antitumor activity *in vitro*,<sup>(8,31)</sup> whereas a phase II clinical trial of SAHA for patients with relapsed NSCLC found that over half of the patients experienced stable disease, but no objective antitumor response was observed.<sup>(32)</sup>

Recent studies have indicated that EZH2 interacts with class I HDACs, HDAC 1 and 2, through another PRC2 protein, EED,<sup>(10,33,34)</sup> and that transcriptional repression by EZH2 requires the activity of the HDACs.<sup>(10,33)</sup> Moreover, HDAC inhibitors have also been shown to downregulate PRC2 proteins.<sup>(35)</sup> These findings suggest that the concurrent inhibition of these epigenetic silencing enzymes, using agents like DZNep and SAHA, have synergistic antitumor effects, which has recently been shown in hematological malignancies.<sup>(22,36)</sup> However, no studies have investigated the effects of such combination therapy in lung cancer.

Herein, we showed for the first time that the combined inhibition of EZH2 and HDAC has a synergistic antiproliferative effect in NSCLC cells. The effect was associated with depletion of EZH2 and other PRC2 proteins, accumulation of p27<sup>Kip1</sup>, reduction of cyclin A, and induction of apoptosis. Surprisingly, a strong suppression of EGFR signaling was observed not only in *EGFR*-wild-type cells, but also in *EGFR*-mutant cells, mediated by dephosphorylation of EGFR. Furthermore, the co-treatment suppressed the *in vivo* tumor growth of *EGFR*-mutant, *EGFR*-TKI-resistant H1975 cells more effectively than did each agent alone.

## Materials and Methods

**Cell lines and reagents.** Four human NSCLC cell lines, NCI-H1299 (H1299), NCI-H1975 (H1975), A549 (ATCC, Manassas, VA, USA), and PC-3 (Japan Cancer Research Resources Bank, Tokyo, Japan), were cultured in RPMI-1640 medium (Invitrogen, Life Technologies, Carlsbad, CA, USA) supplemented with 10% (v/v) FBS and 0.03% (w/v) glutamine at 37°C in an atmosphere of 5% CO<sub>2</sub>. The PC-3 cell line used in the study is not a prostate cancer cell line, but an NSCLC cell line with an *EGFR* mutation (a deletion of exon 19).<sup>(37)</sup>

3-Deazaneplanocin A and SAHA were purchased from Funakoshi (Tokyo, Japan), and Cayman Chemical Company (Ann Arbor, MI, USA), respectively.

**Cell proliferation assay.** Cells were seeded at 500–3000 cells/well in 96-well plates in normal growth medium and kept at 37°C for 24 h. Then the cells were treated for 72 h with DZNep (0.05–0.8 μM) and SAHA (0.5–8 μM) at a fixed ratio (1:10), and with DZNep (0.025–0.4 μM) and SAHA (0.5–8 μM) at a fixed ratio (1:20). Cell growth was measured using an MTT-based assay (CellTiter 96 non-radioactive cell proliferation assay; Promega, Madison, WI, USA). The nature of the drug interaction was analyzed using the CI according to Chou and Talalay's method.<sup>(38)</sup> A CI <0.90 indicates synergism, a CI between 0.90 and 1.10 indicates an additive effect, and a CI >1.10 indicates antagonism. Data analysis was carried out using the commercially available software, Calcsyn (Biosoft, Oxford, UK).

**Analysis of apoptosis.** Cells were stained with a FITC-conjugated annexin V and PI, using the Annexin V-FITC Apoptosis Detection kit (Calbiochem, Darmstadt, Germany) according to the manufacturer's instructions. Briefly, cells were treated with trypsin, subjected to centrifugation at 1000g for 5 min, washed once with ice-cold PBS, and then resuspended in 500 μL

binding buffer. Thereafter, 1.1 μL annexin V-FITC and 10 μL PI were added to the cell suspensions, and the components were mixed for 15 min in the dark. The percentage of apoptotic cells was measured using a FACScan flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Data analysis was carried out using CellQuest version 3.1 (BD Biosciences).

**Western blot analysis.** Cell lysates derived from each NSCLC cell line were prepared by disrupting the cells in radioimmune precipitation assay buffer (150 mM NaCl, 1% [v/v] Triton X-100, 1% [w/v] deoxycholate, 0.1% [w/v] SDS, and 10 mM Tris [pH 7.4]), supplemented with 100 μg/mL leupeptin, 100 μg/mL aprotinin, and 10 mM PMSF. The cell lysates were subjected to sonication and then centrifugation to remove debris. The concentration of protein in each lysate sample was determined using the Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Samples containing equal amounts of protein were loaded onto gels, and the proteins in each sample were separated in 12% or 15% SDS gels; separated proteins were transferred to nitrocellulose membranes (Amersham Biosciences, St. Albans, UK), and the membranes were incubated with the following antibodies: anti-EZH2 (clone 11; BD Transduction Laboratories, BD Biosciences, San Jose, CA, USA), anti-SUZ12 (clone 3C1.2; Millipore, Billerica, MA, USA), trimethyl-histone H3 Lys 27 (07-449; Millipore), anti-EED (09-774; Millipore), cyclin A (H-432; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-p27<sup>Kip1</sup> (clone 57; BD Transduction Laboratories), acetyl-lysine histone H3 antibody sampler kit (acetyl-histone H3 [Lys 9, 14, 18, 27, and 56] and total histone H3; Cell Signaling Technology, Danvers, MA, USA), anti-cleaved PARP (Asp214; Cell Signaling Technology), anti-cleaved caspase-3 (Asp175; Cell Signaling Technology), anti-EGFR (clone D38B1; Cell Signaling Technology), anti-phospho-EGFR (Tyr1068) (clone D7A5; Cell Signaling Technology), anti-AKT (clone C67E7; Cell Signaling Technology), anti-phospho AKT (Ser473) (clone D9E; Cell Signaling Technology), anti-ERK1/2 (clone 137F5; Cell Signaling Technology), anti-phospho ERK1/2 (Thr202/Thr204) (clone D13.14.4E; Cell Signaling Technology), anti-NKD-1 (A-21; Santa Cruz Biotechnology), anti-PPP2R2B (Aviva Systems Biology, San Diego, CA, USA), anti-β-catenin (clone 14; BD Transduction Laboratories), anti-cyclin D1 (C-20; Santa Cruz Biotechnology), and anti-actin (A-2066; Sigma-Aldrich Co., St. Louis, MO, USA) antibodies. Primary antibodies were detected using anti-rabbit or anti-mouse secondary antibodies conjugated with HRP (NA934V and NA931V, respectively; Amersham Biosciences, Amersham, UK). Membranes were washed with TBST six times (5 min each wash) and secondary antibodies were visualized using enhanced chemiluminescence reagent (Amersham).

**Subcutaneous xenograft models.** Female BALB/cAJcl-nu/nu mice, aged 5–6 weeks, were obtained from CLEA Japan (Tokyo, Japan). H1975 cells (5 × 10<sup>6</sup> cells/mouse) were s.c. implanted into the flanks of mice. When the average tumor volume reached approximately 50–100 mm<sup>3</sup>, the following treatments were given to cohorts of five mice for each treatment: vehicle alone (5% [v/v] DMSO); 4 mg/kg DZNep; 40 mg/kg SAHA; or 4 mg/kg DZNep plus 40 mg/kg SAHA. These drugs were given twice per week i.p. for 6 weeks. Tumor volume was calculated using the equation 1/2 (length × width<sup>2</sup>). All animal experiments complied with the Hokkaido University (Sapporo, Japan) Regulations on Animal Experimentation (approval no. 19-46).

**Statistical analysis.** Statistical significance between two groups was determined by unpaired, two-sided Student's *t*-test.

For comparisons among multiple groups, statistical significance was determined by one-way ANOVA with Tukey's multiple comparison test. The level of significance was set at  $P < 0.05$ . All tests were carried out using spss software (version 18.0; IBM, Chicago, IL, USA).

## Results

**Combined treatment synergistically inhibited NSCLC cell proliferation.** We first investigated whether SAHA alone inhibited cell growth in four NSCLC cell lines differing in their *EGFR* gene status: H1299 (wild-type *EGFR*), H1975 (L858R and T790M substitutions), A549 (wild-type), and PC-3 (exon 19 deletion). The MTT assays showed that SAHA caused dose-dependent inhibition of NSCLC cell proliferation with  $IC_{50}$  ranging from 1.50 to 2.61  $\mu$ M (Fig. 1). Comparison with the results of our previous study, in which we reported that the  $IC_{50}$  values of DZNep were 0.08–0.24  $\mu$ M in these cells,<sup>(20)</sup> shows that the  $IC_{50}$  value of SAHA was almost 10 times as large as that of DZNep.

Next, we examined the combined effects of DZNep and SAHA on the four NSCLC cell lines. Combination index plot analysis using DZNep and SAHA at fixed concentration ratios of 1:10 and 1:20 indicated that co-treatment with DZNep and SAHA inhibited cell proliferation synergistically at most concentrations, especially those around the  $IC_{50}$  concentration of each drug, in all four of the cell lines, as indicated by the combination indices of  $< 0.9$  (Fig. 2). The combined treatment inhibited H1975 cell proliferation more effectively than the other cell lines.

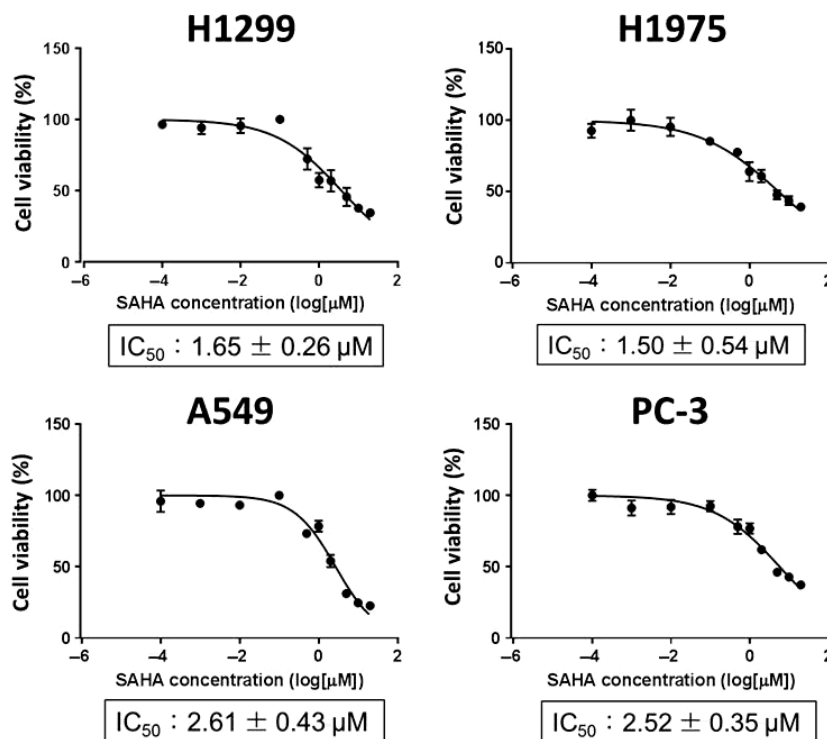
**Combined treatment depleted PRC2 proteins and decreased histone methylation and acetylation more effectively than single treatment.** We examined the effects of combined treatment with DZNep and SAHA on PRC2 proteins (EZH2, SUZ12, and EED) and other associated proteins by Western blot analysis. Both DZNep and HDAC inhibitors are known to deplete

the expression of PRC2 proteins.<sup>(19,35)</sup> The combination of DZNep and SAHA reduced the expression of EZH2 and SUZ12, whereas EED was mostly regulated by SAHA alone (Fig. 3a). The co-treatment also resulted in a slight decrease of H3K27me3, accumulation of p27<sup>Kip1</sup>, and decrease in cyclin A expression. Depending on sites of acetylation and cells, the co-treatment resulted in modest acetylation of lysine residues of histone H3 (Fig. 3b).

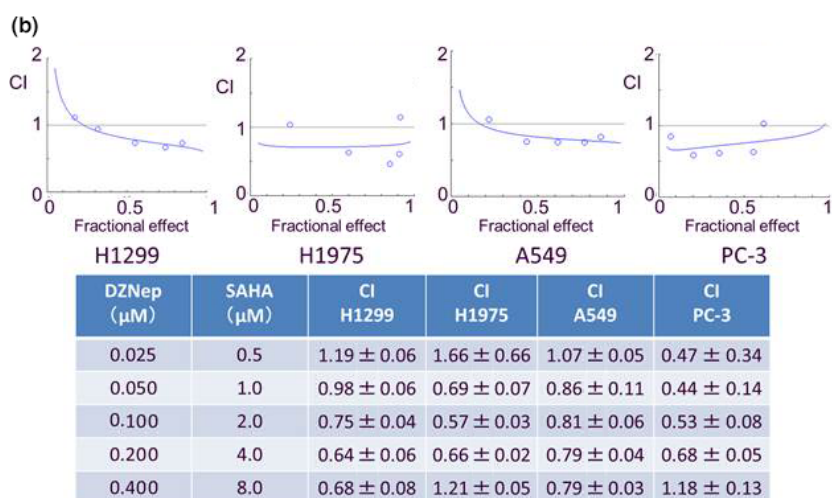
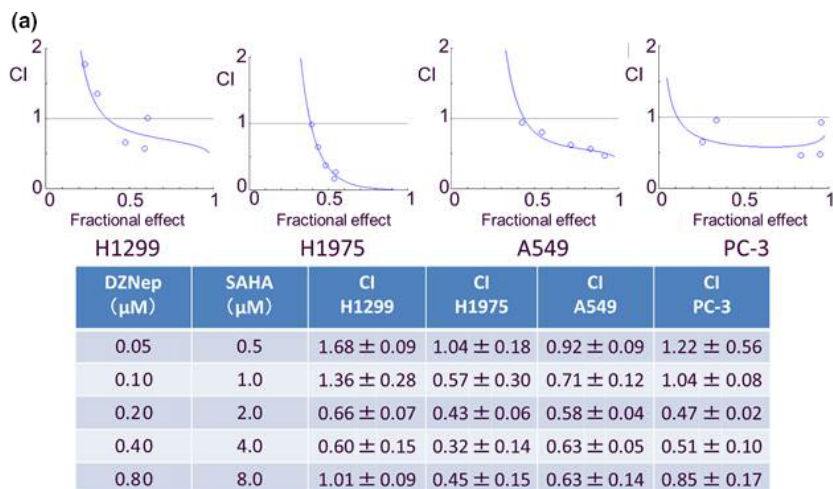
**Combined treatment induced more apoptosis than single treatment.** We determined the effects of combined treatment on apoptosis in the four NSCLC cell lines. Flow cytometry analysis using annexin V and PI showed that the apoptotic fraction was induced more by DZNep and SAHA co-treatment than by each agent alone (Fig. 4a). These effects were remarkable in *EGFR*-mutant H1975 and PC-3 cells, whose apoptotic fraction exceeded 20%. The combined treatment increased cleaved PARP and cleaved caspase-3 in all four cell lines, compared with treatment with each agent alone (Fig. 4b).

**Combined treatment suppressed the EGFR signaling pathway in both *EGFR*-wild-type and *EGFR*-mutant NSCLC cells.** The remarkable induction of apoptosis in *EGFR*-mutant H1975 and PC-3 cells prompted us to investigate the effects of co-treatment on EGFR signaling. Western blot analysis revealed that the co-treatment slightly reduced EGFR expression. Interestingly, co-treatment strongly suppressed EGFR phosphorylation, not only in *EGFR*-wild-type H1299 and A549 cells, but also in *EGFR*-mutant H1975 and PC-3 cells, which had abundant basal phosphorylated EGFR protein (Fig. 5). The phosphorylation of AKT and ERK1/2, which are downstream molecules of EGFR, was also reduced by the combined treatment.

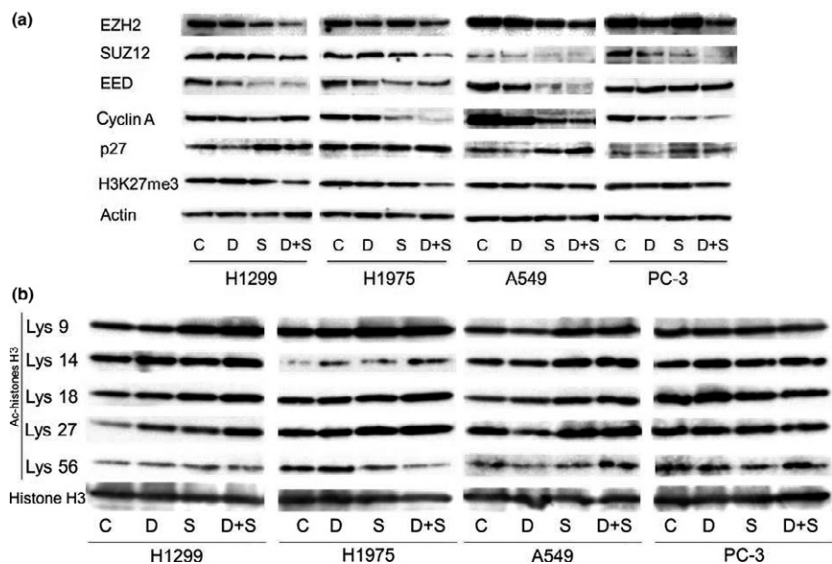
Inhibition of EZH2, in cooperation with HDAC inhibition, has been shown to decrease EGFR expression by suppressing  $\beta$ -catenin, a transcriptional activator of EGFR, by inducing multiple endogenous Wnt/ $\beta$ -catenin signaling antagonists, including NKD1 and PPP2R2B, which are direct epigenetic targets of EZH2.<sup>(39)</sup> The combined treatment increased NKD1



**Fig. 1.** Four human non-small-cell lung cancer cell lines (H1299, H1975, A549, and PC-3) were treated with suberoylanilide hydroxamic acid (SAHA) for 72 h and subjected to an MTT-based assay. Data are representative of three independent experiments. Data represent mean  $\pm$  SD of triplicate samples. Similar results were obtained from all three independent experiments.



**Fig. 2.** Combined therapy with 3-deazaneplanocin A (DZNep) and suberoylanilide hydroxamic acid (SAHA) synergistically inhibited non-small-cell lung cancer cell (NSCLC) proliferation. (a) Four human NSCLC cell lines were treated with DZNep (dose range, 0.05–0.8 μM) and SAHA (dose range, 0.5–8 μM) at a fixed ratio of 1:10 for 72 h and subjected to MTT-based assay. (b) Four human NSCLC cell lines were treated with DZNep (dose range, 0.025–0.4 μM) and SAHA (dose range, 0.5–8 μM) at a fixed ratio of 1:20 for 72 h and subjected to MTT-based assay. Combination index (CI) values were determined using the commercially available software, Calcsyn. Data are representative of three independent experiments. Similar results were obtained in all three independent experiments.

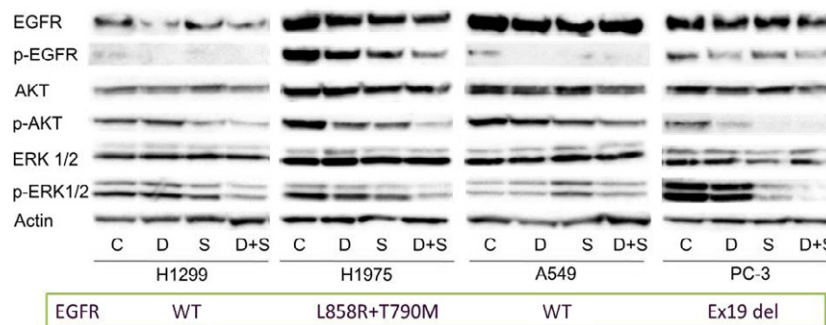
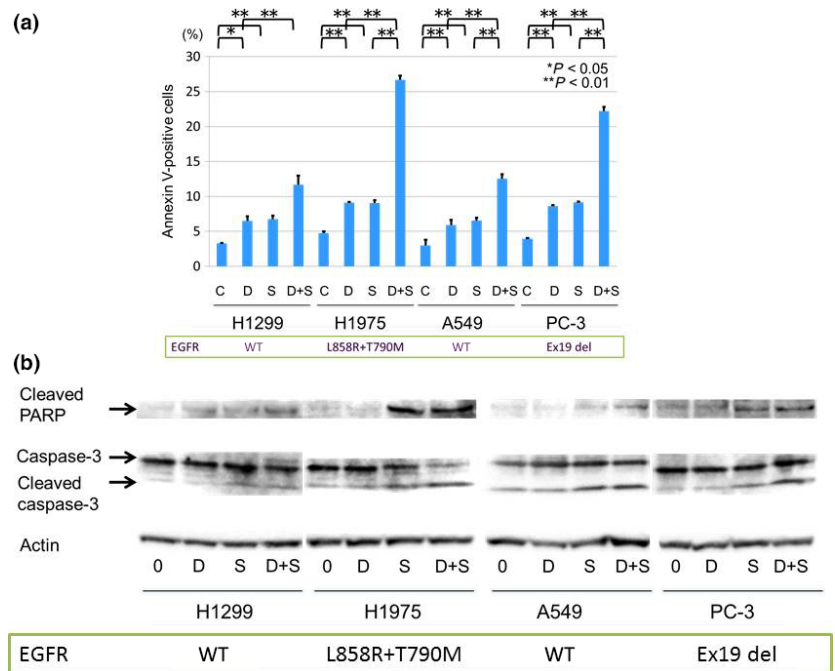


**Fig. 3.** Combined therapy with 3-deazaneplanocin A (DZNep) (D) and suberoylanilide hydroxamic acid (SAHA) (S) depleted PRC2 proteins and decreased the histone methylation and acetylation levels more effectively than did individual treatment, in non-small-cell lung cancer cells. Cells were treated with 0.2 μM DZNep and/or 2 μM SAHA for 72 h. Total cell lysates were then harvested and subjected to Western blot analysis. (a) Representative Western blots of EZH2, SUZ12, EED, trimethylation of lysine 27 on histone H3 (H3K27me3), cyclin A, p27<sup>Kip1</sup>, and actin from three independent experiments are shown. Actin levels in the lysates served as the loading control (C). Similar results were obtained in all three independent experiments. (b) Representative Western blots of acetylation of lysine (Lys) 9, 14, 18, 27, and 56 of histone H3 and total histone H3 from three independent experiments are shown. Total histone H3 levels in the lysates served as the loading control. Similar results were obtained in all three independent experiments.

protein expression in all NSCLC cell lines and increased the expression of PPP2R2B in H1975 cells only (Fig. 6a). The co-treatment reduced the expression of β-catenin only in H1975 cells, and reduced the expression of cyclin D1, a downstream target of β-catenin, in H1975, A549, and PC-3 cells (Fig. 6b).

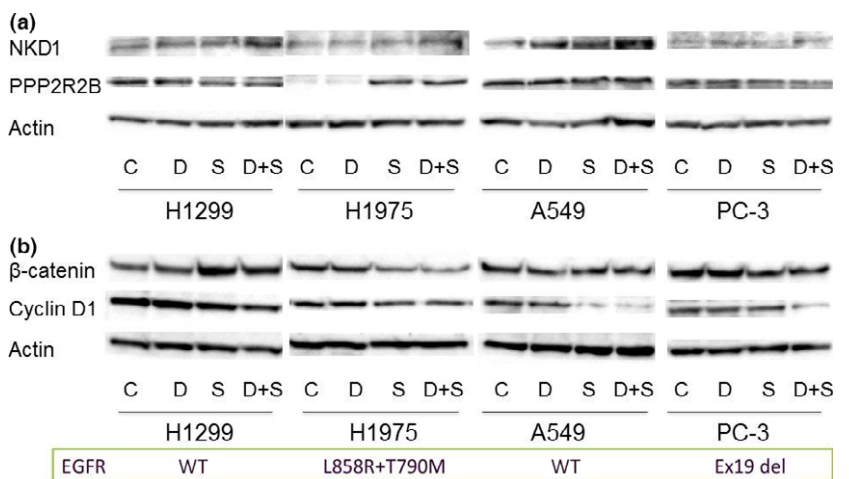
**Combined treatment inhibited *in vivo* tumor growth of H1975 xenografts.** We examined the therapeutic effect of combined treatment with DZNep and SAHA on xenografts of H1975 cells transplanted into nude mice. As shown in Figure 7(a), both DZNep and SAHA significantly suppressed the *in vivo*

**Fig. 4.** Combined therapy with 3-deazaneplanocin A (DZNep) (D) and suberoylanilide hydroxamic acid (SAHA) (S) induced more apoptosis than individual treatments, in non-small-cell lung cancer cells. (a) Flow cytometric analysis of apoptosis with annexin V-FITC and propidium iodide staining. Cells were treated with 0.2  $\mu$ M DZNep and/or 2  $\mu$ M SAHA for 72 h. The percentage of apoptotic cells was measured using a flow cytometer. Data represent mean  $\pm$  SD of triplicate samples. Similar results were obtained in all three independent experiments. \* $P$  < 0.05 and \*\* $P$  < 0.01 between indicated groups by one-way ANOVA with Tukey's multiple comparison test. (b) Cells were treated with 0.2  $\mu$ M DZNep and/or 2  $\mu$ M SAHA for 72 h. Total cell lysates were then harvested and subjected to Western blot analysis. Representative Western blots of cleaved poly(ADP-ribose) polymerase (PARP), cleaved caspase-3, and actin are shown. Actin levels in the lysates served as the loading control (C). Similar results were obtained in all three independent experiments. EGFR, epidermal growth factor receptor; Ex19 del, exon 19 deletion.



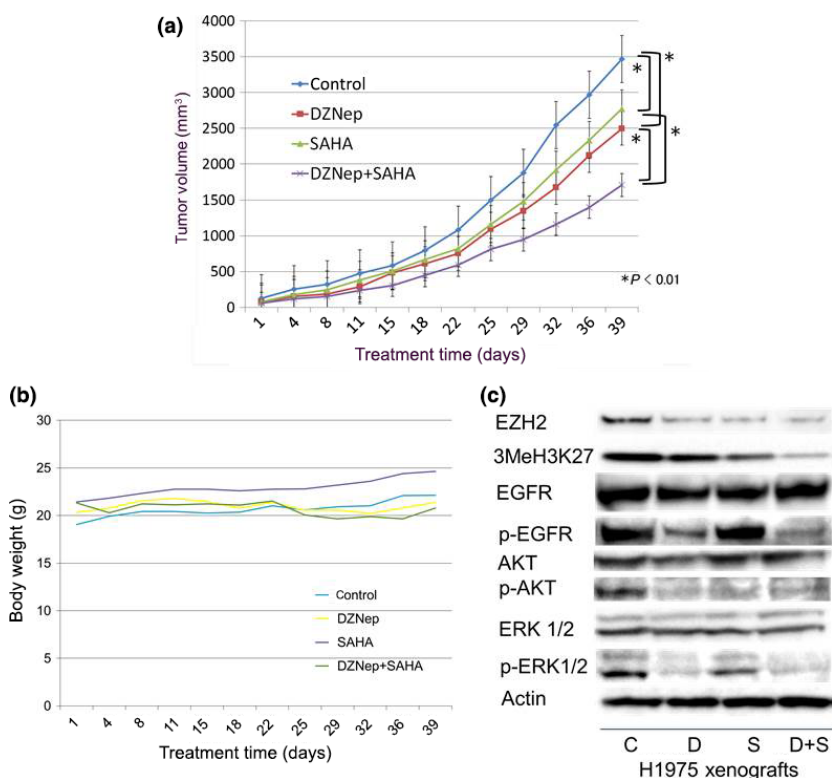
**Fig. 5.** Combined therapy with 3-deazaneplanocin A (DZNep) (D) and suberoylanilide hydroxamic acid (SAHA) (S) suppressed the epidermal growth factor receptor EGFR signaling pathway in both EGFR-wild-type and EGFR-mutant non-small-cell lung cancer cells. Cells were incubated with 0.2  $\mu$ M DZNep and/or 2  $\mu$ M SAHA for 72 h. The cell lysates were then harvested and subjected to Western blot analysis. Representative Western blots of EGFR, phosphorylated (p-)EGFR, protein kinase B (AKT), p-AKT, extracellular signal-regulated kinase (ERK) 1/2, p-ERK1/2, and actin are shown. Actin levels in the lysates served as the loading control (C). Similar results were obtained in all three independent experiments. Ex19 del, exon 19 deletion.

**Fig. 6.** Effects of combined therapy with 3-deazaneplanocin A (DZNep) (D) and suberoylanilide hydroxamic acid (SAHA) (S) on  $\beta$ -catenin, a transcriptional activator of epidermal growth factor receptor (EGFR), NKD1, and PPP2R2B, which are the direct epigenetic targets of EZH2 involved in  $\beta$ -catenin regulation. Cells were incubated with 0.2  $\mu$ M DZNep and/or 2  $\mu$ M SAHA for 72 h. Cell lysates were harvested and subjected to Western blot analysis. (a) Representative Western blots of NKD1, PPP2R2B, and actin are shown. Actin levels in the lysates served as the loading control (C). Similar results were obtained in all three independent experiments. (b) Representative Western blots of  $\beta$ -catenin, cyclin D1, and actin are shown. Actin levels in the lysates served as the loading control (C). Similar results were obtained in all independent experiments. Ex19 del, exon 19 deletion.



tumor growth of H1975 cells compared with control. Co-treatment with DZNep and SAHA showed significantly greater inhibition of tumor growth than did each agent alone. Neither

individual agent nor co-treatment caused significant weight loss or other physical signs of toxicity in the mice (Fig. 7b). Western blot analysis revealed that combined treatment



**Fig. 7.** Combined therapy with 3-deazaneplanocin A (DZNep) (D) and suberoylanilide hydroxamic acid (SAHA) (S) inhibited *in vivo* tumor growth of H1975 xenografts. (a) H1975 cells ( $5 \times 10^5$  cells/mouse) were s.c. implanted into the flanks of BALB/cAJcl-nu/nu nude mice. After the tumor volume reached approximately 50–100 mm<sup>3</sup>, the following treatments were given to cohorts of five mice for each treatment: vehicle alone (5% DMSO) (Control), 4 mg/kg DZNep, 40 mg/kg SAHA, or 4 mg/kg DZNep plus 40 mg/kg SAHA twice per week i.p. for 6 weeks. Tumor volume was calculated using the equation  $1/2 (\text{length} \times \text{width}^2)$ . Data represent means  $\pm$  SD of quintuple samples. \* $P < 0.01$  between indicated groups at day 39 by one-way ANOVA with Tukey's multiple comparison test. (b) Body weights were measured at the indicated times. Data represent mean  $\pm$  SD of quintuple samples. (c) Western blot analysis of H1975 xenograft. Proteins were extracted from tumor tissues soon after the mice were killed. Representative Western blots of EZH2, trimethylation of lysine 27 on histone H3 (H3K27me3), epidermal growth factor receptor (EGFR), phosphorylated (p)-EGFR, protein kinase B (AKT), p-AKT, extracellular signal-regulated kinase (ERK)1/2, p-ERK1/2, and actin are shown. Actin levels in the lysates served as the loading control.

reduced the expression of EZH2 and H3K27me3 in H1975 xenografts (Fig. 7c). Additionally, the co-treatment markedly reduced phosphorylation of EGFR, AKT, and ERK1/2.

## Discussion

To develop an epigenetic therapy for NSCLC with higher effectiveness than existing treatments, we determined the effect of co-treatment with an EZH2 inhibitor (DZNep) and an HDAC inhibitor (SAHA) in NSCLC cells. The present study showed, for the first time, that the combined inhibition of EZH2 and HDACs had synergistic antiproliferative effects in NSCLC, consistent with previous results investigating other types of cancer.<sup>(22,36,40,41)</sup> The effect was associated with a slight decrease of H3K27me3, modest increase of multiple sites of histone acetylation, depletion of EZH2 and other PRC2 proteins, accumulation of p27<sup>Kip1</sup>, and reduction of cyclin A. Flow cytometry analysis has indicated the additive/synergistic induction of apoptosis by DZNep and SAHA, which was accompanied by caspase-3 and PARP cleavage, consistent with studies on other types of cancer.<sup>(22,36)</sup> Interestingly, the co-treatment suppressed EGFR signaling effectively, not only in *EGFR*-wild-type cells, but also in *EGFR*-mutant cells. Furthermore, the co-treatment suppressed the *in vivo* tumor growth of *EGFR*-mutant, *EGFR*-TKI-resistant H1975 cells more effectively than did each agent alone, without visible toxicity.

In addition to the suppression of the catalytic activity of EZH2 and HDAC, the depletion of EZH2 and other PRC2 proteins observed *in vitro* and *in vivo* might be an important mechanism underlying the antitumor effect of the combination therapy. Consistent with this hypothesis, both DZNep<sup>(19,22,36)</sup> and HDAC inhibitors<sup>(22,35,36,40)</sup> have been shown to deplete PRC2 proteins in other types of cancer. Furthermore, a study

on gallbladder cancer indicated that the antitumor effect of SAHA was dependent on a decrease in EZH2 expression.<sup>(40)</sup> In that study, SAHA inhibited the proliferation of carcinoma cells, which express EZH2 and HDACs 1 and 2, but did not affect that of normal epithelial cells, which have almost no EZH2 expression but substantial HDAC 1 and 2 expression. Depletion of PRC2 proteins by DZNep has been shown to occur due to protein degradation.<sup>(19)</sup> The protein levels of each PRC2 member is, in part, dependent on the presence of the other subunits, and each individual protein is unstable outside of a functional PRC2 complex.<sup>(42,43)</sup> Because PRC2 also requires interaction with HDACs to execute its function,<sup>(10,33)</sup> depletion of PRC2 proteins following HDAC inhibition by SAHA might also be related to protein instability.

The suppression of EGFR signaling may be involved in the growth-suppressive effect of EZH2 and HDAC inhibition in NSCLC cells *in vitro* and *in vivo*. It is intriguing that the strong dephosphorylation of EGFR was observed even in *EGFR*-mutant cells on both conditions. Histone deacetylase inhibition has been shown to decrease phosphorylation and expression of EGFR in both *EGFR*-wild-type and *EGFR*-mutant NSCLC cells,<sup>(44)</sup> although the underlying mechanism remains unclear. EZH2 inhibition is not known to suppress phosphorylation of EGFR, but it has been shown to decrease EGFR expression by suppressing  $\beta$ -catenin, a transcriptional activator of EGFR, by inducing multiple endogenous Wnt/ $\beta$ -catenin signaling antagonists, including NKD1 and PPP2R2B in hepatocellular carcinoma.<sup>(39)</sup> Our data suggest that NKD1- and PPP2R2B-mediated suppression of  $\beta$ -catenin is not the main mechanism of the decreased EGFR expression by DZNep and SAHA in NSCLC cells, although it may have some involvement for H1975 cells. Nonetheless, these results suggest that the combined treatment with DZNep and SAHA effectively suppress EGFR signaling, mainly through

dephosphorylation of EGFR, not only in *EGFR*-wild-type but also in *EGFR*-mutant NSCLC cells. Further investigation of the underlying mechanisms is warranted.

The remarkable apoptosis in *EGFR*-mutant PC-9 and H1975 cells suggests that the combined treatment may be more effective in *EGFR*-mutant lung cancer cells than in *EGFR*-wild-type cells. In this study, the abundant basal phosphorylated EGFR was strongly reduced by the combined treatment. Because *EGFR*-mutant lung cancer cells including the T790M *EGFR*-TKI-resistant mutation are addictive to EGFR signaling,<sup>(45,46)</sup> the reduction of phosphorylated EGFR by the combined treatment may induce apoptosis in *EGFR*-mutant lung cancer cells more effectively than in *EGFR*-wild-type cells.

Owing to high basal phosphorylation of EGFR, NSCLC with *EGFR* mutations, including that with T790M *EGFR*-TKI-resistant mutation, may be a good candidate subgroup for combined epigenetic therapy by DZNep and SAHA. The importance of the strategy including EZH2 inhibition for *EGFR*-mutant NSCLC is also underscored by the recent discovery that EZH2 inhibition sensitizes *EGFR*-mutant NSCLC cells to topoisomerase II inhibitors.<sup>(47)</sup>

In conclusion, the results suggest that DZNep and SAHA have a synergistic growth-suppressive effect and induce substantial apoptosis in NSCLC cells, including *EGFR*-mutant, *EGFR*-TKI-resistant cells, through inhibition of the EGFR signaling pathway *in vitro* and *in vivo*. Combined pharmacological targeting of EZH2 and HDAC may provide more effective epigenetic therapeutics for NSCLC.

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## Disclosure Statement

The authors have no conflict of interest.

## Abbreviations

AKT	protein kinase B
CI	combination index
DZNep	3-deazaneplanocin A
EED	embryonic ectoderm development
EGFR	epidermal growth factor receptor
ERK	extracellular signal-regulated kinase
EZH2	enhancer of zeste homolog 2
H3K27me3	trimethylation of lysine 27 on histone H3
HDAC	histone deacetylase
NKD	naked cuticle
NSCLC	non-small-cell lung cancer
PARP	poly(ADP-ribose) polymerase
PI	propidium iodide
PPP2R2B	Protein phosphatase 2, regulatory subunit B, $\beta$
PRC2	polycomb repressive complex 2
SAHA	suberoylanilide hydroxamic acid (vorinostat)
SUZ12	suppressor of zeste 12
TKI	tyrosine kinase inhibitor

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