ORIGINAL

Resminostat, a histone deacetylase inhibitor, circumvents tolerance to EGFR inhibitors in *EGFR*-mutated lung cancer cells with *BIM* deletion polymorphism

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Abstract : Drug-tolerant cells are mediators of acquired resistance. *BIM*-intron2 deletion polymorphism (*BIM*-del) is one of the mechanisms underlying the resistance to epidermal growth factor tyrosine kinase inhibitor (EGFR-TKI)-mediated apoptosis that induces drug tolerance. Here, we investigated whether resminostat, a histone deacetylase inhibitor, circumvents *BIM*-del-associated apoptosis resistance. The human *EGFR*-mutated non-small cell lung cancer (NSCLC) cell line PC-9 and its homozygous *BIM*-del-positive variant (PC-9 *BIM*$^{i2-/-}$), established by editing with zinc finger nuclease, were used. In comparison with PC-9 cells, PC-9 *BIM*$^{i2-/-}$ cells were less sensitive to apoptosis mediated by EGFR-TKIs such as gefitinib and osimertinib. The combined use of resminostat and an EGFR-TKI preferentially induced the expression of the pro-apoptotic BIM transcript containing exon 4 rather than that containing exon 3, increased the level of pro-apoptotic BIM protein (BIM$_{EL}$), and stimulated apoptosis *in vitro*. In a subcutaneous tumor model derived from PC-9 *BIM*$^{i2-/-}$ cells, gefitinib monotherapy decreased tumor size but retained residual lesions, indicative of the presence of tolerant cells in tumors. The combined use of resminostat and gefitinib increased BIM$_{EL}$ protein level and induced apoptosis, subsequently leading to the remarkable shrinkage of tumor. These findings suggest the potential of resminostat to circumvent tolerance to EGFR-TKIs associated with *BIM* deletion polymorphism. J. Med. Invest. 67 : 343-350, August, 2020

Keywords **:** *drug tolerance, BIM polymorphism, EGFR tyrosine kinase inhibitor, lung cancer*

INTRODUCTION

Non-small cell lung cancer (NSCLC) with classical epidermal growth factor receptor (*EGFR*) mutations such as exon 19 deletion and L858R point mutation is highly sensitive to EGFR tyrosine kinase inhibitors (EGFR-TKIs), including the first-generation inhibitor gefitinib and the third-generation inhibitor osimertinib (1, 2). However, almost all patients experience disease recurrence, owing to the acquired resistance to EGFR-TKIs. Recent studies have revealed the presence of a small population of cells that adapts to the initial treatment with EGFR-TKIs as persisters, which form the basis for acquired resistant lesions (3). Elucidation of the adaptation mechanism following initial treatment with EGFR-TKIs may allow development of novel initiation therapies to eradicate tumor cells and improve the therapeutic outcome in advanced *EGFR*-mutated NSCLC by preventing the development of acquired resistance.

The decrease in the activity of BIM, also known as Bcl-2-like protein 11, a pro-apoptotic molecule that belongs to the Bcl-2 family, has been recognized as one of mechanisms underlying the intrinsic resistance or tolerance to EGFR-TKIs. BIM expression upregulation is essential for the induction of apoptosis in NSCLC cells carrying *EGFR* mutations following treatment with first- to third-generation EGFR-TKIs. On the contrary, low BIM protein level is associated with resistance or tolerance to EGFR-TKIs (4, 5). A functional *BIM* deletion polymorphism, specifically a 2,903 bp deletion in intron 2, was reported in East Asian individuals (13%–18%) (6) and South American patients with NSCLC (15.7%) (7) and was associated with poor response to EGFR-TKIs (6, 8). Mechanistically, *BIM* deletion results in the mutually exclusive splicing of exon 3 over the BH3-encoding (pro-apoptotic) exon 4 of the *BIM* pre-mRNA, leading to the production of an inactive BIM protein isoform (BIMγ). This protein lacks the BH3 domain, and its production results in the reduction in the expression of the pro-apoptotic BIM protein isoform (BIM$_{EL}$) in *EGFR*-mutant lung cancer cell lines upon TKI exposure, thereby inducing TKI resistance (6). Several meta-analyses have reported an association between *BIM* deletion polymorphism and shorter progression-free survival (PFS) in patients with NSCLC carrying *EGFR* mutations who received either gefitinib or erlotinib (9-14). Therefore, the restoration of BIM activity may be an important strategy to overcome the intrinsic resistance or tolerance to EGFR-TKI in patients with *EGFR*-mutated NSCLC with *BIM* deletion.

Histone deacetylase (HDAC) is one of the targets for cancer treatment. For instance, vorinostat (suberoylanilide hydroxamic acid [SAHA]) is a small molecule inhibitor of pan HDACs that targets both class I HDACs (HDAC-1, 2, 3, and 8) and class IIb HDACs (HDAC-6 and 10), causes acetylation of histone proteins, and induces cell differentiation, cell cycle arrest, and apoptosis in several types of tumor cells (15). Vorinostat monotherapy has been approved for cutaneous T-cell lymphoma. We have previously reported that the combination of vorinostat and gefitinib

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could preferentially induce the transcription of the pro-apoptotic exon 4-containing *BIM* isoform over that of the inactive exon 3-containing isoform, thereby resensitizing the *BIM* deletion-containing *EGFR*-mutated NSCLC cell lines to TKIs *in vitro* and *in vivo* (16). Based on our preclinical findings, we performed a phase I study, VICTORY-J, in patients with *EGFR*-mutated NSCLC harboring *BIM* deletion polymorphism to evaluate the safety of the combined therapy with vorinostat and gefitinib. Vorinostat at 400 mg/day biweekly combined with gefitinib 250 mg/day was the recommended dose in phase II studies (17). As panobinostat, another pan-HDAC inhibitor, has been recently approved for multiple myeloma (18), newer generations of HDAC inhibitors are currently under development.

Resminostat is a potent inhibitor of class I, IIb, and IV HDACs. It induces hyperacetylation of histone H4 in multiple myeloma cells (19). Safety and efficacy of resminostat were evaluated in various clinical studies for hepatocellular carcinoma, non-small cell lung cancer, biliary tract cancer and Hodgkin's lymphoma (20-24). Its activity is being evaluated in a clinical trial (25). In the present study, we examined the effect of resminostat on the susceptibility of *EGFR*-mutated NSCLC cell lines with *BIM* deletion polymorphism to an EGFR-TKI *in vitro* and *in vivo*.

MATERIALS AND METHODS

Cell lines and reagents

PC-3 cells, derived from a Japanese female patient with NSCLC carrying an exon 19 deletion in *EGFR* and differing from the prostate cancer cell line PC-3 (ATCC CRL1435), were purchased from the Human Science Research Resource Bank. The NSCLC cell line, PC-9 carrying *EGFR* mutations, was obtained from the RIKEN Cell Bank (Ibaraki, Japan). PC-9 cells with a homozygous *BIM*-intron 2 deletion polymorphism (PC-9 *BIM*$^{i2\text{-}/\text{-}}$) were established by editing with zinc finger nuclease, as previously reported (26). PC-9 GXR cells carrying deletions in the *EGFR* exon 19 and T790M mutation were established at Kanazawa University (Kanazawa, Japan) from PC-9 cell xenograft tumors in nude mice that had acquired resistance to gefitinib (27). PC-3 and the other three cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) and Roswell Park Memorial Institute (RPMI)-1640 medium, respectively, both supplemented with 10% fetal bovine serum (FBS) and antibiotics. Resminostat was produced by Yakult Honsha Co., Ltd (Tokyo, Japan). Gefitinib, osimertinib, and vorinostat were obtained from Selleck Chemicals (Houston, TX).

Genotype and expression analysis of BIM

Genomic DNA was extracted from cells using a Maxwell 16 Tissue DNA Purification Kit on a Maxwell 16 Instrument (Promega) according to manufacturer's instructions. To recognize the presence of the wild-type and deletion polymorphism alleles, we conducted polymerase chain reactions (PCR) using the discriminating primers (forward 5′-AATACCACAGAGGCCCACAG-3′ and reverse 5′-GCCTGAAGGTGCTGAGAAAG-3′). Genomic DNAs were amplified using a Veriti Thermal Cycler (Applied Biosystems) with REDAccuTaq LA DNA Polymerase (Sigma). The PCR amplicons for the wild-type (4226 bp) and deletion (1323 bp) alleles were separated by agarose gel electrophoresis.

RNA interference

The cells (2×10^5 cells/well) cultured in a medium containing 10% FBS (antibiotic-free) for 24 h were treated with a Stealth RNAi small-interfering RNA (siRNA) against BIM and Stealth RNAi siRNA Negative Control Lo GC (Invitrogen) using Lipofectamine RNAiMAX (Invitrogen) for 48 h.

Western blot analysis

Western blotting was conducted using antibodies against phospho-EGFR (Tyr1068), protein kinase B (Akt), phospho-Akt (Ser473), cleaved poly (ADP-ribose) polymerase (PARP), cleaved caspase-3, acetylated histone H3 (Lys27), BIM, and β-actin (Cell Signaling Technology) ; phospho-extracellular signal-regulated kinase 1/2 (ERK1/2 ; Thr202/Tyr204), ERK1/2, and EGFR (R&D Systems). Blots were subsequently incubated with horseradish peroxidase-conjugated secondary antibodies specific to goat or rabbit immunoglobulin G, and the signals were detected by enhanced chemiluminescence using SuperSignal West Dura Extended Duration Substrate (Thermo Fisher Scientific).

Real-time reverse-transcriptase quantitative PCR (RT-qPCR)

Total cellular RNA was extracted from cells using ISOGEN (NIPPON GENE) in accordance with the manufacturer's instructions. Reverse transcription was performed using SuperScript VILO cDNA synthesis Kit and Master Mix (Invitrogen). Expression of *BIM* mRNA was quantitatively measured by ViiA 7 Real-Time PCR System (Applied Biosystems) using the following primers : BIM exon 2A (forward 5′-ATGGCAAAGCAACCTTCTGATG-3′ and reverse 5′-GGCTCTGTCTGTAGGGAGGT-3′) ; BIM exon 3 (forward 5′-CAATGGTAGTCATCCTAGAGG-3′ and reverse 5′-GACAAAATGCTCAAGGAAGAGG-3′) ; BIM exon 4 (forward 5′-TTCCATGAGGCAGGCTGAAC-3′ and reverse 5′-CCTCCTTGCATAGTAAGCGTT-3′) ; and b-actin (forward 5′-GGACTTCGAGCAAGAGATGG-3′ and reverse 5′-AGCACTGTGTTGGCGTACAG-3′).

Staining for live cells and dead cells

Cell death induced by the drugs was determined through the use of Fluoroskan Ascent™ FL (Thermo Fisher Scientific) using the Live or Dead cell viability Assay Kit (AAT Bioquest Inc, Sunnyvale, CA), which detected and quantified living cells with Cellbrite™ Orange at 630 nm and apoptotic cells with Blue™ DCS1 at 420 nm using the microplate reader.

Subcutaneous xenograft models

Male BALB/cAJcl-nu/nu mice, aged 5 to 6 weeks, were obtained from CLEA Japan Inc., and subcutaneously injected with cultured tumor cells (5×10^6 cells/0.1 mL) into their flanks. Once the tumor volume reached 100 to 200 mm^3, the mice were randomized and treated once daily with gefitinib and/or resminostat. Each tumor was two-dimensionally measured, and the volume was calculated using the following formula : tumor volume (mm^3) = 1/2 × length (mm) × width $(mm)^2$. The study protocol was approved by the Ethics Committee on the Use of Laboratory Animals and the Advanced Science Research Center, Kanazawa University, Kanazawa, Japan.

RESULTS

Resminostat upregulates BIM expression in EGFR-mutant NSCLC cell lines harboring BIM deletion polymorphism

We first examined the *BIM*-intron2 deletion polymorphism in *EGFR*-mutant NSCLC cell lines by PCR. PC-9 cells had wild-type alleles with a PCR product of 4.2 kb. Consistent with a previous report (16, 26), PC-3 cells were heterozygous for *BIM* deletion polymorphism, as evident from the PCR products of 4.2 kb (wild-type) and 1.3 kb (2.9 kb deletion polymorphism). PC-9*BIM*$^{i2\text{-}/\text{-}}$ cells had only deletion allele with a PCR product size of 1.3 kb. Western blot analysis revealed the markedly lower

expression of the major proapoptotic BIM protein (BIM_{EL}) in PC-3 and PC-9*BIM*$^{i2-/-}$ cells than in PC-9 cells (Fig. 1A). We have previously reported that the HDAC inhibitor vorinostat upregulates the level of acetylated histone H3 and BIM with the BH3 domain in *EGFR*-mutated NSCLC cells heterozygous for *BIM* deletion (16). In line with this observation, we found that vorinostat dose-dependently increased the expression of acetylated histone H3 and BIM with the BH3 domain in PC-9*BIM*$^{i2-/-}$ cells (Fig. 1B) homozygous for *BIM* deletion polymorphism as well as in PC-3 cells (Fig. 1C). Under same experimental conditions, resminostat increased the expression of acetylated histone H3 and BIM with the BH3 domain (BIM_{EL}, BIM_L, and BIM_S) in PC-9*BIM*$^{i2-/-}$ (Fig. 1D) and PC-3 (Fig. 1E) cells in a dose-dependent manner. These results indicate that resminostat could upregulate the expression of the pro-apoptotic BIM protein in *EGFR*-mutated NSCLC cells that are either heterozygous or homozygous for *BIM* deletion polymorphism.

Resminostat upregulates BIM expression and efficiently induces apoptotic proteins in combination with EGFR-TKIs

We investigated whether the addition of resminostat to EGFR-TKIs results in the induction of apoptosis of *EGFR*-mutant NSCLC cells homozygous for *BIM* deletion polymorphism. In parental PC-9 cells, gefitinib inhibited the phosphorylation of EGFR and the downstream molecules (AKT and ERK), increased the expression of the major pro-apoptotic BIM isoform (BIM_{EL}), and induced the expression of apoptotic markers (cleaved caspase-3 and cleaved PARP) (Fig. 2A). On the contrary, while gefitinib inhibited the phosphorylation of EGFR and downstream molecules (AKT and ERK), it had minimal effects on the expression of BIM_{EL} and apoptotic markers in PC-9*BIM*$^{i2-/-}$ cells (Fig. 2A). However, the combination of resminostat and gefitinib markedly increased the expression of BIM_{EL} as well as the apoptosis markers cleaved caspase-3 and cleaved PARP (Fig. 2A).

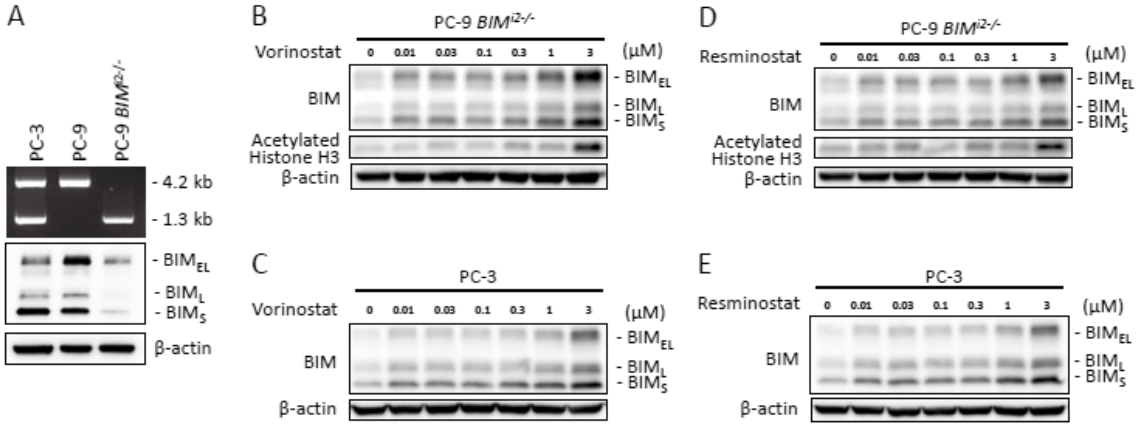

Figure 1. Resminostat upregulates BIM expression in *EGFR*-mutant NSCLC cell lines harboring *BIM* deletion polymorphism
A, top, PCR products from three *EGFR*-mutated NSCLC cell lines generated by primers flanking the deletion regions. The PCR products of 4.2 and 1.3 kb correspond to the alleles without and with deletion, respectively, in the presence of both products, indicating the heterozygosity of the deletion polymorphism. Bottom, the level of the expression of the products BIM_{EL}, BIM_L, and BIM_S in each line. B-E, PC-9*BIM*$^{i2-/-}$ cells and PC-3 cells were incubated with serial dilutions of vorinostat (B, C) or resminostat (D, E) for 24 h. The cell lysates were harvested and the indicated proteins were analyzed by western blotting.

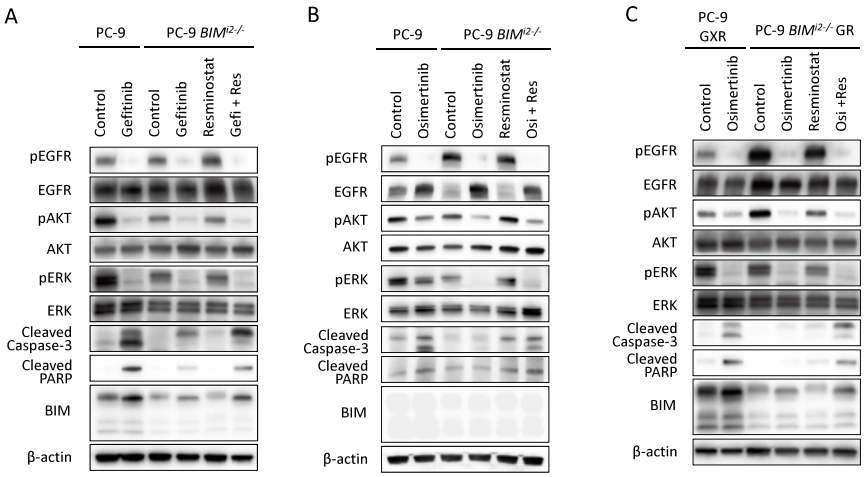

Figure 2. Resminostat upregulates BIM expression and efficiently induces pro-apoptotic markers in combination with EGFR-TKIs
A, PC-9 and PC-9*BIM*$^{i2-/-}$ cells were incubated with gefitinib (1 μM) and/or resminostat (5 μM) for 12 h. The cell lysates were harvested and the indicated proteins were analyzed by western blotting. B, PC-9 GXR and PC-9*BIM*$^{i2-/-}$ GR cells were incubated with osimertinib (1 μM) and/or resminostat (5 μM) for 12 h. The cell lysates were harvested and the indicated proteins were determined by western blotting.

Similar results were obtained when resminostat was combined with the third-generation EGFR-TKI osimertinib (Fig. 2B).

In PC-9 GXR cells with *EGFR*-T790M mutation, osimertinib inhibited the phosphorylation of EGFR and the downstream molecules AKT and ERK, increased the level of BIM$_{EL}$, and induced the expression of cleaved caspase-3 and cleaved PARP. PC-9*BIM*$^{i2-/-}$ GR cells were positive for *EGFR*-T790M and resistant to gefitinib, and were derived following continuous exposure to gefitinib. In PC-9*BIM*$^{i2-/-}$ GR cells, osimertinib inhibited the phosphorylation of EGFR and the downstream molecules but minimally increased the expression of BIM$_{EL}$ and apoptotic markers (Fig. 2C). However, the combination of resminostat and osimertinib increased the expression of BIM$_{EL}$ as well as the apoptosis markers cleaved caspase-3 and cleaved PARP (Fig. 2C).

We further evaluated whether resminostat combined with EGFR-TKI induced apoptosis by cytochemical staining for live cells (Cellbrite™ Orange-positive) and apoptotic cells (Blue™ DCS1-positive). Gefitinib remarkably increased the number of apoptotic cells in PC-9 cells but not PC-9*BIM*$^{i2-/-}$ cells. Under these experimental conditions, gefitinib combined with resminostat discernibly increased the number of apoptotic cells in PC-9*BIM*$^{i2-/-}$ cells (Fig. 3). These results indicate that resminostat upregulates BIM expression and efficiently induces apoptotic in combination with EGFR-TKIs.

Resminostat induces apoptosis of PC-9BIM$^{i2-/-}$ cells through the upregulation of BIM expression

To investigate whether the induction of apoptosis with the combined use of resminostat and EGFR-TKIs relies on the expression of BIM protein, PC-9*BIM*$^{i2-/-}$ cells were transfected with BIM-specific siRNA and then treated with resminostat and gefitinib for 48 h. We found that the knockdown of BIM protein expression mediated by BIM-specific siRNA resulted in the abrogation of apoptosis, as evident from the absence of cleaved PARP in resminostat- and gefitinib-treated PC-9*BIM*$^{i2-/-}$ cells (Fig. 4A). Analysis of *BIM* transcripts revealed that resminostat alone induced *BIM* mRNA expression, and this effect was enhanced in the presence of gefitinib. Moreover, the combination of resminostat and gefitinib preferentially induced the expression of the transcripts containing exon 4 over those containing exon 3 (Fig. 4B, C). These findings suggest that the combined use of resminostat and EGFR-TKIs results in the upregulation of the expression of the pro-apoptotic BIM protein and induction of apoptosis of *EGFR*-mutated NSCLC cells homozygous for *BIM* deletion polymorphism *in vitro*.

Resminostat combined with gefitinib regresses the growth of the tumors derived from EGFR-mutated NSCLC cells with homozygous BIM deletion polymorphism in vivo

We next examined the effect of the combination of gefitinib and resminostat on *EGFR*-mutated NSCLC cells homozygous for *BIM* deletion polymorphism *in vivo*. PC-9 and PC-9*BIM*$^{i2-/-}$ cells were subcutaneously implanted in mice to produce tumors. The mice were then treated with gefitinib, resminostat, or the combination of both. Gefitinib alone markedly reduced the volume of the xenograft tumor induced by PC-9 cells (Fig. 5A). Although gefitinib monotherapy prevented the enlargement of the tumor produced by PC-9*BIM*$^{i2-/-}$ cells harboring homozygous *BIM*-intron2 deletion

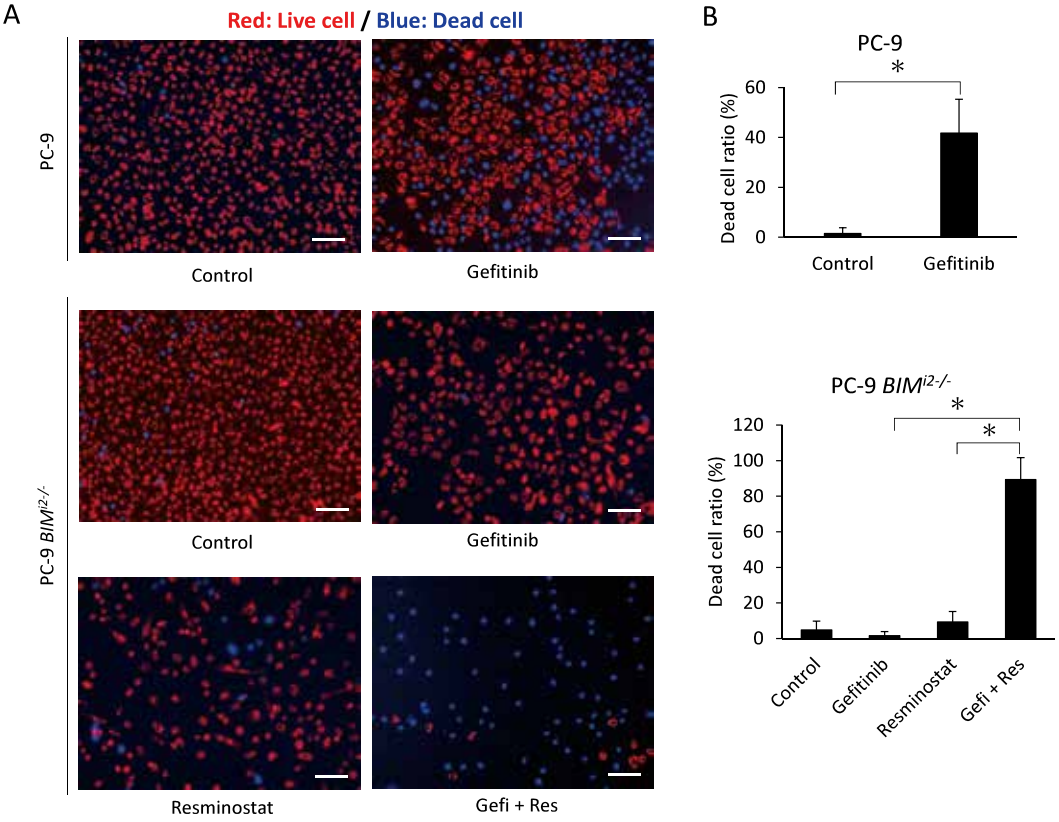

Figure 3. Resminostat induces cell death in combination with EGFR-TKIs
A, PC-9 and PC-9*BIM*$^{i2-/-}$ cells were incubated with gefitinib (1 μM) and/or resminostat (5 μM) for 48 h. The resultant cells were stained using the Live or Dead cell viability Assay Kit (AAT Bioquest Inc, Sunnyvale, CA), which detected and quantified living cells with Cellbrite™ Orange (Red) at 630 nm and apoptotic cells with Blue™ DCS1 (Blue). Bar = 100 μm B, The live cells and dead cells were counted in four representative area and the percentage of dead cells was shown. * $P < 0.05$ Student's *t*-test.

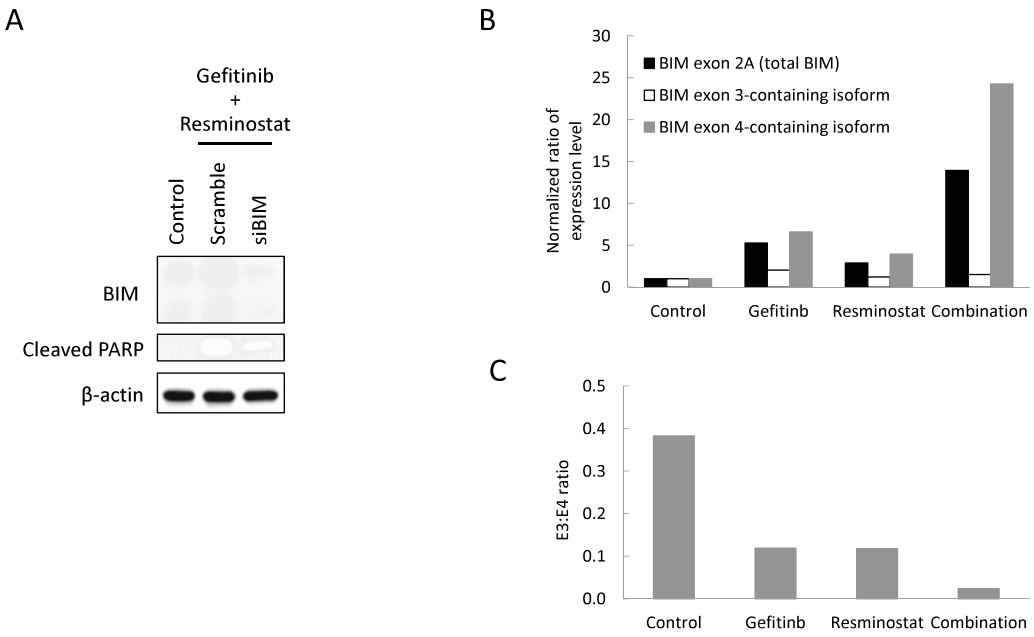

Figure 4. Resminostat induces apoptosis of PC-9*BIM*$^{i2-/-}$ cells through the upregulation of BIM expression
A, PC-9*BIM*$^{i2-/-}$ cells were transfected with *BIM* or control siRNA for 24 h before gefitinib (1 μM) and resminostat (3 μM) treatment for 48 h. B, PC-9*BIM*$^{i2-/-}$ cells were treated with gefitinib (1 μM) and/or resminostat (3 μM) for 12 h. The levels of various transcripts containing exon 2A, 3, or 4 are expressed after normalization to actin level. C, Ratio of exon 3-containing transcripts to exon 4-containing transcripts in PC-9*BIM*$^{i2-/-}$ cells after treatment with each compound.

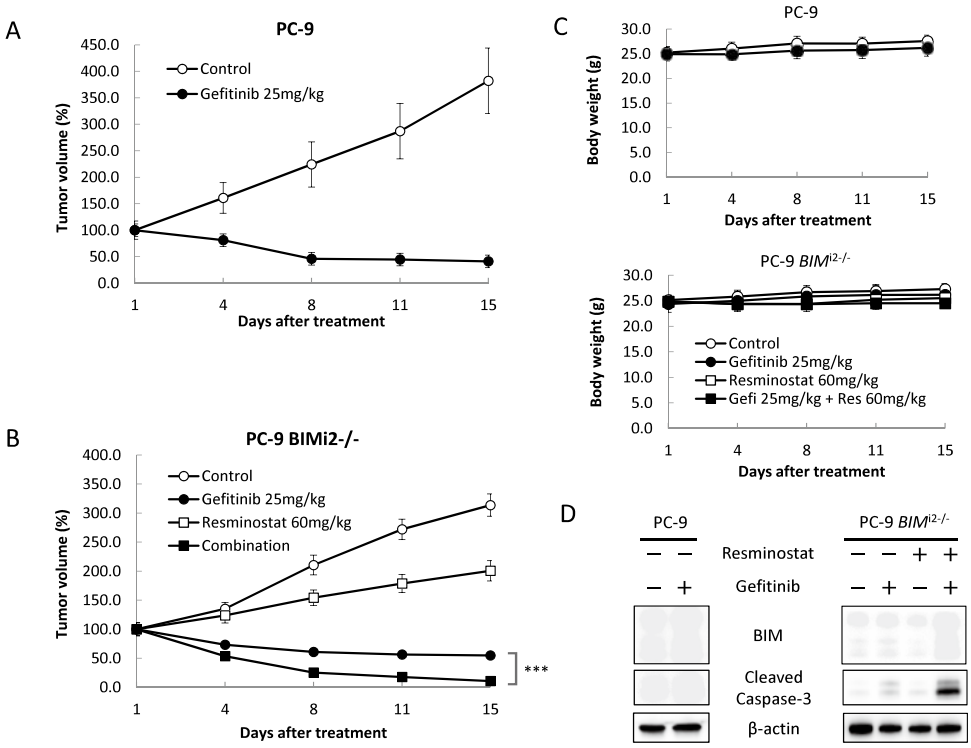

Figure 5. Resminostat combined with gefitinib regresses the growth of the tumors derived from *EGFR*-mutated NSCLC cells with homozygous *BIM* deletion polymorphism *in vivo*
The antitumor activity of gefitinib and/or resminostat in mouse xenograft models of PC-9 and PC-9*BIM*$^{i2-/-}$ tumors. Nude mice bearing established tumors with PC-9 (A) or PC-9*BIM*$^{i2-/-}$ (B) cells were treated with 25 mg/kg gefitinib and/or 60 mg/kg resminostat once daily for 15 days. Tumor volume was measured using calipers on the indicated days. Mean ± SE of tumor volumes are shown for groups of 6 to 7 mice. C, The body weight of mice examined in A and B was evaluated. D, Tumors were harvested 4 h after four consecutive treatments with each compound, and the levels of protein in tumor lysates were detected by western blotting.

polymorphism, we failed to observe complete tumor regression. This observation indicates the tolerance of PC-9*BIM*$^{i2-/-}$ cells to gefitinib *in vivo*. Resminostat monotherapy, on the contrary, slightly inhibited tumor growth but markedly reduced the tumor volume in combination with gefitinib (Fig. 5B). None of the mice treated with these agents showed any macroscopic adverse effects, including loss of body weight (Fig. 5C). To clarify the mechanisms underlying the effect of resminostat and gefitinib *in vivo*, we performed western blot analysis on tumor lysates. As a result, we found that gefitinib induced the cleavage of caspase-3 in PC-9 tumors. In PC-9*BIM*$^{i2-/-}$ tumors, treatment with gefitinib or resminostat had no effect on caspase-3 cleavage, which was increased along with BIM expression following the combination treatment with both agents (Fig. 5D). These findings indicate that the combination of resminostat and gefitinib increases BIM protein expression and induces tumor cell apoptosis, resulting in the shrinkage of the tumors produced by *EGFR*-mutated NSCLC cells carrying homozygous *BIM* deletion polymorphism.

DISCUSSION

Drug tolerant cells, also called as drug persisters or minimal residual lesions at dormant state, serve as a reservoir for the development of acquired resistance (3). Tolerance to EGFR-TKIs is mediated by several mechanisms, including activation of insulin-like growth factor receptor 1 (IGF-1R) (3), AXL (27), and Yes-associated protein (YAP) (28) and inactivation of BIM, in *EGFR*-mutated NSCLC cells. Treatment to circumvent drug tolerance is still under evaluation in clinical trials. Here, we show that resminostat could sensitize *BIM* deletion polymorphism-positive *EGFR*-mutated NSCLC cells to EGFR-TKI-induced apoptosis and that the combined use of resminostat and EGFR-TKI could reduce the volume of the tumor produced by *BIM* deletion polymorphism-positive *EGFR*-mutated NSCLC cells *in vivo*.

HDAC inhibitors increase the acetylation of histones and other proteins that results in the induction of chromatin remodeling, promotion of tumor suppressor gene transcription, and apoptosis, leading to antitumor effects (25). Clinically, the activity of HDAC inhibitors has been mainly demonstrated in hematologic malignancies, including cutaneous T cell lymphoma and multiple myeloma (25). The activity of resminostat, an oral drug, is higher toward HDAC-1 and 3 (class I HDACs) and HDAC-6 (class IIb HDAC) than toward HDAC8 (class I HDAC) (19). In the present study, we found that resminostat with HDAC3 inhibitory activity circumvented the tolerance to apoptosis developed by *BIM* deletion polymorphism-positive *EGFR*-mutated NSCLC cells treated with EGFR-TKIs. This observation in the line with our previous finding that HDAC3 inhibition by HDAC inhibitors plays a crucial role in apoptosis induction through the promotion of transcription and modulation of alternative splicing to upregulate active BIM protein level in *BIM* deletion polymorphism-positive *EGFR*-mutated NSCLC cells (26). A phase I trial in Japanese patients with solid tumors demonstrated the drug safety ; the recommended dose for phase II study with resminostat monotherapy was 800 mg once daily on days 1-5 biweekly (29). A phase II trial is currently ongoing to evaluate whether resminostat can serve as a maintenance treatment for patients with mycosis fungoides or Sézary syndrome after disease control with other systemic therapies (NCT02953301).

Resminostat belongs a class of HDAC inhibitor that is called hydroxamic acid-based histone deacetylase inhibitor as same as vorinostat. However, the chemical structure of resminostat differs from that of vorinostat in detail (30). Actually, it was reported that a HDAC inhibitor interacted with a variety of histone and non-histone protein, and their profile of interacting proteins tends to be different for different HDAC inhibitors, and it was not depended on a class of HDAC inhibitor (31). It was also reported that mechanisms of antitumor effect of HDAC inhibitors may be different and depend on HDAC inhibitors (32). We speculated that the difference of the profile of interacting proteins may contribute to a difference of safety and efficacy profile of HDAC inhibitors. Therefore, we examined resminostat efficacy as a new HDAC inhibitor other than vorinostat that we previously reported the efficacy.

We have previously conducted investigator initiated trials for *BIM* deletion/*EGFR* mutation double-positive NSCLC patients to determine the safety of vorinostat-gefitinib combination and evaluate the pharmacodynamic biomarkers of vorinostat activity (17). We failed to notice any dose-limiting toxicity, and proposed 400 mg vorinostat as the recommended phase II dose. Although this was a phase I study with limited number of patients, the median PFS was 5.2 months (95% confidence interval 1.4–15.7) ; the disease control rate at 6 weeks was 83.3% (10/12) in a previous heavily-treated patient population (17). Moreover, the analysis of peripheral blood mononuclear cells revealed that vorinostat preferentially induced the expression of *BIM* mRNA containing exon 4 over that containing exon 3, acetylated histone H3 protein, and pro-apoptotic BIM$_{EL}$ protein in 11/11, 10/11, and 5/11 patients, respectively (17). These data indicate that the *BIM* mRNA exon3/exon4 ratio in PBMCs may be a useful pharmacodynamic marker for treatment (17). As osimertinib is recognized as one of standard first-line treatment for *EGFR*-mutated NSCLC (2), the use of the combination of osimertinib and new-generation HDAC inhibitors, including resminostat, along with the monitoring of this pharmacodynamic marker may be worth testing in *BIM* deletion polymorphism-positive EGFR-mutated NSCLC.

In conclusion, resminostat with HDAC3 inhibitory activity could preferentially induce the expression of the pro-apoptotic BIM transcript containing exon 4 rather than the BIM transcript containing exon 3 ; it also increased pro-apoptotic BIM protein (BIM$_{EL}$) level and stimulated apoptosis *in vitro*. In addition, the combined use of resminostat and gefitinib increased BIM$_{EL}$ protein level and induced apoptosis, thereby leading to a remarkable shrinkage of tumors produced by *EGFR*-mutated NSCLC with *BIM* deletion polymorphism. These findings suggest the potential application of resminostat to circumvent tolerance to EGFR-TKIs associated with *BIM* deletion polymorphism.

DETAILED CONFLICT OF INTEREST STATEMENTS

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