Epigenetic circumvention of EGFR-TKI resistance due to \textit{BIM} deletion polymorphism by HDAC inhibitor and EGFR-TKI

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Abbreviations:

NSCLC, non-small cell lung cancer; EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; HDAC, histone deacetylase; ERK, extracellular signal-regulated kinase

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Abstract

*BIM*, also called *BCL2L11*, is a member of the Bcl-2 family encoding a pro-apoptotic protein. *BIM* up-regulation is required for apoptosis induction by epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitors (TKIs) in *EGFR* mutant non-small cell lung cancer (NSCLC). A *BIM* deletion polymorphism, present in 12.9% of East Asian individuals, impairs the generation of a pro-apoptotic isoform with BCL2-homolog domain 3 (BH3) and confers intrinsic resistance to the EGFR-TKIs gefitinib and erlotinib. NSCLC patients with mutated *EGFR* harboring this polymorphism showed significantly inferior responses to EGFR-TKIs than did individuals without the polymorphism.

We investigated whether vorinostat, a histone deacetylase (HDAC) inhibitor, could circumvent EGFR-TKI resistance in the *EGFR* mutant NSCLC cell lines, PC-3 and HCC2279, which harbor the *BIM* polymorphism. We found that both cell lines were much less sensitive to gefitinib-induced apoptosis than the *EGFR* mutant PC-9 and HCC827 cell lines, which do not harbor this polymorphism. Vorinostat dose-dependently increased the expression of BIM with a pro-apoptotic BH3 domain and, together with gefitinib, induced apoptosis in PC-3 and HCC2279 cells *in vitro*. In xenograft models, gefitinib induced marked regression, via apoptosis, of tumors without, but not with, the *BIM* polymorphism, whereas the combination of vorinostat and gefitinib induced marked...
regression of tumors with the BIM polymorphism, accompanied by tumor-cell apoptosis.

These results indicate that vorinostat induces the epigenetic restoration of BIM and that the combination of an HDAC inhibitor and an EGFR-TKI may circumvent the resistance associated with the BIM polymorphism in EGFR mutant NSCLC.

Precis

EGFR-TKI-resistance associated with the BIM polymorphism may be overcome by the HDAC inhibitor vorinostat via the epigenetic restoration of BIM, suggesting that the combination of an HDAC inhibitor and an EGFR-TKI may be effective in TKI-refractory EGFR mutant lung cancer selected by BIM status.
Introduction

The epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) gefitinib and erlotinib have shown marked therapeutic effects against non-small cell lung cancer (NSCLC) with EGFR activating mutations, such as exon19 deletions and L858R point mutations (1). About 20~30% of patients, however, show intrinsic resistance to EGFR-TKIs despite having tumors harboring these EGFR mutations. In addition, patients who respond initially later develop acquired resistance to EGFR-TKIs after varying periods of time (2). Among the molecular mechanisms associated with acquired resistance to EGFR-TKIs are 1) gatekeeper mutations in EGFR (i.e. a T790M second mutation), 2) activation of bypass signaling caused by Met amplification or hepatocyte growth factor (HGF) overexpression, 3) transformation to small-cell lung cancer, and 4) epithelial-to-mesenchymal transition (3, 4). Several therapeutic strategies, including new generation EGFR-TKIs and the combination of an EGFR-TKI and a Met-TKI, have been evaluated clinically in patients with EGFR mutant NSCLC who acquired resistance to EGFR-TKIs (2). The mechanisms of intrinsic resistance, however, remain poorly understood.

Recently, a BIM deletion polymorphism was reported to be a novel mechanism of intrinsic resistance to EGFR-TKIs (5). BIM, also called BCL2L11, is a pro-apoptotic
protein and a member of the Bcl-2 family. Gene products (such as BIMEL, BIML, and BIM3) with a BH3 domain, which is essential for apoptosis induction, antagonize anti-apoptotic proteins (such as Bcl-2, Bcl-XL, and Mcl-1) and activate pro-apoptotic proteins (such as BAX and BAK), thereby inducing apoptosis (6, 7). Activation of BAX and BAK induce cytochrome c release into the cytoplasm and result in activation of the caspase cascade (8). BIM is pivotal in apoptosis induced by EGFR-TKIs in EGFR mutant NSCLC cells (9). The expression and degradation of BIM is regulated mainly by the Mek-Erk kinase pathway (10). The BIM deletion polymorphism is relatively common in East Asian populations (12.9%), with 0.5% of individuals being homozygous for this deletion. During the transcription of BIM, either exon3 or exon4, the latter of which encodes the BH3 domain, is spliced out due to the presence of a stop codon and a polyadenylation signal within exon3 (11). The BIM deletion polymorphism involves the deletion of a 2903 bp fragment in intron2 and results in the preferential splicing of exon3 over exon4, generating a BIM isoform that lacks the BH3 domain (5). A retrospective analysis in patients with EGFR mutant NSCLC demonstrated that progression free survival (PFS) following EGFR-TKI treatment was significantly shorter in patients with the BIM polymorphism (6.6 months) than with wild-type BIM (11.9 months) (5). Another study in patients with EGFR mutant NSCLC treated with EGFR-TKIs also reported that
PFS was significantly shorter in patients with BIM-low (4.3 months) than BIM-high (11.3 months) expressing tumors (12), suggesting that reduced expression of BIM with a BH3 domain is associated with an unfavorable response to EGFR-TKIs. To date, however, no therapeutic strategy has yet been developed for EGFR mutant NSCLC patients with low BIM expression.

Histone deacetylase (HDAC) is an enzyme that regulates chromatin remodeling and is crucial in the epigenetic regulation of various genes (13). Many compounds targeting HDAC have been developed, including vorinostat, an HDAC inhibitor approved by the United States Food and Drug Administration (FDA) for the treatment of patients with cutaneous T-cell lymphoma (14). In mantle cell lymphoma (MCL) cell lines and in cells from patients with MCL, vorinostat induced histone hyperacetylation on promoter regions and consequent transcriptional activation of pro-apoptotic BH3-only genes, including BIM (15). Using in vitro and in vivo models, we assessed whether the combination of vorinostat and gefitinib restored the expression of BIM protein with a BH3 domain in EGFR mutant NSCLC cells with the BIM polymorphism and overcame EGFR-TKI resistance associated with this polymorphism.
Materials and Methods

Cell lines and reagents

The NSCLC cell lines, PC-9, HCC827, and HCC2279, all of which have \( EGFR \) mutations, were obtained from Immuno-Biological Laboratories Co., Ltd. (Fujioka, Gunma, Japan), the ATCC (Manassas, VA), and Dr. John Minna (University of Texas Southwestern Medical Center, Dallas, TX), respectively. PC-3 cells, established from a Japanese female patient with NSCLC and with an exon 19 deletion in \( EGFR \), and differing from the prostate cancer cell line PC-3 (ATCC CRL1435), were purchased from Human Science Research Resource Bank (JCRB0077: http://cellbank.nibio.go.jp/~cellbank/en/search_res_det.cgi?DB_NUM=1&ID=252) (Osaka, Japan). PC-3 and the other three cell lines were maintained in D-MEM and RPMI1640 media, respectively, each supplemented with 10% fetal bovine serum (FBS) and antibiotics. All cells were passaged for less than 3 months before renewal from frozen, early-passage stocks. Cells were regularly screened for mycoplasma using a MycoAlert Mycoplasma Detection Kit (Lonza, Rockland, ME). Vorinostat and gefitinib were obtained from Selleck Chemicals (Houston, TX) and AstraZeneca (London, UK), respectively.
Genotype and expression analysis of *BIM*

Genomic DNA was extracted from cells using DNeasy Blood & Tissue kits (Qiagen, Hilden, Germany), according to the manufacturer’s protocol. Total RNA was extracted from cells using RNeasy PLUS Mini kits (Qiagen). PCR methods were used to detect the *BIM* deletion polymorphism in the samples and the level of expression of *BIM* isoforms (5).

Cell apoptosis

Cells (3×10³) were seeded into each well of 96-well, white-walled plates, incubated overnight, and treated with the indicated compounds or vehicle (DMSO) for 48h. Cellular apoptosis was analyzed with Caspase-Glo 3/7 assay kits (Promega, Madison, WI), which measure caspase-3/7 activity, and PE-Annexin V Apoptosis Detection Kits (BD Biosciences, San Jose, CA), in accordance with the manufacturers’ directions.

Apoptotic cells in tumor xenografts were detected by terminal deoxynucleotidyl transferase–mediated nick end labeling (TUNEL) staining, using the DeadEnd™ Fluorometric TUNEL system (Promega, Madison, WI), according to the manufacturer’s protocol.
RNA interference

Duplexed Stealth RNAi (Invitrogen, Carlsbad, CA) against *BIM* and Stealth RNAi-negative control low GC Duplex #3 (Invitrogen) were used for RNA interference (RNAi) assays as described (4). The siRNA target sequences were

5’-CAUGAGUUGUGACAAAUCAACAA-3’ and 5’-UUGUGUUGAUUUGUCACAACUAG-3’ for BIM #1, and

5’-UGAGUGUGACCGAGAAGGUAGACAA-3’ and 5’-UUGUCUACCUCUGGUCACACUCA-3’ for BIM #2.

Western blot analysis

Western blotting was performed with antibodies against phospho-EGFR (Tyr1068), Akt, phospho-Akt (Ser473), cleaved PARP, cleaved caspase-3, histone H3, acetylated histone H3 (Lys27), BIM, and β-actin (Cell Signaling Technology, Beverly, MA); and against phospho-Erk1/2 (Thr202/Tyr204), Erk1/2 and EGFR (R&D Systems, Minneapolis, MN). Blots were subsequently incubated with HRP-conjugated secondary antibodies specific to mouse or rabbit IgG, with signals detected by enhanced chemiluminescence (Pierce Biotechnology).
Subcutaneous xenograft models

Male BALB/cAJcl-nu/nu mice, aged 5–6 weeks, were obtained from CLEA Japan Inc (Tokyo, Japan) and injected subcutaneously into their flanks with cultured tumor cells (5x10^6 cells/0.1mL/mouse). When tumor volumes reached 100-200 mm³, the mice were randomized and treated once daily with gefitinib and/or vorinostat. Each tumor was measured in two dimensions, and the volume was calculated using the formula: tumor volume (mm³) = 1/2 × length (mm) × width (mm)². All animal experiments complied with the Guidelines for the Institute for Experimental Animals, Kanazawa University Advanced Science Research Center (approval No. AP-081088).

Statistical analysis

Between group differences were analyzed by one-way ANOVA. All statistical analyses were performed using GraphPad Prism Ver. 4.01 (GraphPad Software, Inc., San Diego, CA), with P < 0.05 considered statistically significant.
Results

**EGFR** mutant NSCLC cell lines harboring the **BIM** deletion polymorphism have low susceptibility to gefitinib-induced apoptosis.

We first examined the **BIM** deletion polymorphism in **EGFR** mutant NSCLC cell lines by PCR. PC-9 and HCC827 had wild-type alleles, with a PCR product 4.2kb in size.

Consistent with a previous report (5), HCC2279 cells were heterozygous for the **BIM** deletion polymorphism, with PCR products 4.2kb (wild-type) and 1.3kb (2.9kb deletion polymorphism) in size. Among the 7 additional cell lines with **EGFR** mutations (Supplementary Table 1), one, PC-3, was heterozygous for the **BIM** deletion polymorphism (Fig 1A). Western blot analyses reveal that the expression of the pro-apoptotic BIM protein was markedly lower in PC-3 and HCC2279 than in PC-9 and HCC827 cells. Analysis of **BIM** isoform transcripts showed that cells with the **BIM** polymorphism expressed more exon3- than exon4-containing transcripts (Supplementary Fig 1A, B). Treatment with gefitinib enhanced BIM expression, caspase-3/7 activities, and apoptosis in PC-9 and HCC827 cells much more than in PC-3 and HCC2279 cells (Fig 1B; Supplementary Fig 1C, D, and 2). Moreover, gefitinib did not increase caspase-3/7 activity in PC-9 and HCC827 cells treated with **BIM** siRNA (Fig 1C), indicating the crucial role of BIM in apoptosis induction in **EGFR** mutant cancer.
NSCLC cells treated with EGFR-TKI. These observations clearly showed that EGFR mutant NSCLC cells with the BIM deletion polymorphism are much less sensitive to gefitinib, as shown by induction of apoptosis, than cells with wild-type BIM.

Vorinostat up-regulates BIM and efficiently induces apoptosis when combined with gefitinib.

Since HDAC inhibition modulates the expression of various genes, including pro-apoptotic molecules (13), we hypothesized that the HDAC inhibitor, vorinostat, may sensitize EGFR mutant NSCLC cells with the BIM polymorphism to gefitinib. In EGFR mutated NSCLC cell lines, including PC-3 and HCC2279 cells, vorinostat dose-dependently increased the expression of acetylated histone H3 and BIM with the BH3 domain (Fig 2A, Supplementary Fig 3A). We further explored whether the addition of vorinostat to gefitinib induced apoptosis in EGFR mutant NSCLC cells with the BIM polymorphism (Fig 2B, D). In HCC827 and PC-9 cells, which contain only wild-type BIM, gefitinib inhibited downstream signaling, including the phosphorylation of EGFR, Erk, and Akt, resulting in apoptosis, as shown by the expression of cleaved PARP and cleaved caspase-3. The further addition of vorinostat augmented BIM expression and caspase-3/7 activity. In PC-3 and HCC2279 cells, which contain the BIM polymorphism,
however, treatment with gefitinib alone induced minimal apoptosis, although the
phosphorylation of EGFR, Erk, and Akt was inhibited, whereas the combination of
vorinostat and gefitinib markedly increased the expression of BIM, as well as of cleaved
PARP and cleaved caspase-3 (Fig 2B, Supplementary Fig 3B). This combination also
augmented caspase-3/7 activity compared with that of gefitinib or vorinostat alone (Fig
2D, Supplementary Fig 3C), but this activation of caspase-3/7 was inhibited by
knockdown of $BIM$ (Supplementary Fig 4A, B). Conversely, overexpression of $BIM_{EL}$
itself stimulated caspase-3/7 activities in cells with the $BIM$ polymorphism, with these
activities further enhanced by gefitinib treatment (Supplementary Fig 4C, D). These
results indicate that BIM mediates the activation of caspase-3/7 induced by gefitinib and
vorinostat. Analysis of $BIM$ transcripts revealed that vorinostat alone induced $BIM$
mRNA, which was enhanced by the inclusion of gefitinib. Moreover, vorinostat treatment
preferentially induced transcripts containing exon4 over those containing exon3 (Fig 2C).
These results indicate that the combination of vorinostat and gefitinib inhibits HDAC and
increases the expression of BIM protein with the BH3 domain, thereby sensitizing $EGFR$
mutant NSCLC cells with the $BIM$ polymorphism to apoptosis $in$ $vitro$.

**Combined treatment with vorinostat with gefitinib shrinks tumors produced by**
**EGFR mutant NSCLC cells with the BIM polymorphism.**

We next determined the *in vivo* efficacy of vorinostat and gefitinib. Gefitinib alone almost completely shrunk xenograft tumors induced by HCC827 cells (Fig 3A). Although gefitinib monotherapy prevented the enlargement of tumors produced by PC-3 cells, which harbor the BIM polymorphism, it did not induce their complete regression, indicating that PC-3 cells remained less susceptible to gefitinib *in vivo*. Under these experimental conditions, vorinostat monotherapy inhibited tumor growth slightly, whereas the combination of vorinostat with gefitinib resulted in marked tumor shrinkage (Fig 3B). None of the mice treated with these agents showed any macroscopic adverse effects, including loss of body weight (data not shown).

To clarify the mechanisms by which vorinostat and gefitinib act *in vivo*, we assessed tumor cell apoptosis by TUNEL staining. Gefitinib treatment increased the number of apoptotic cells in HCC827 tumors but had little effect on PC-3 tumors (Fig 4A, B), indicating that *EGFR* mutant NSCLC cells with the BIM polymorphism are refractory to gefitinib-induced apoptosis *in vivo* as well as *in vitro*. Importantly, while vorinostat alone had little effect on apoptosis, the combination of vorinostat and gefitinib induced marked apoptosis in PC-3 tumors (Fig 4A, B). Western blot analyses showed that gefitinib induced cleavage of caspase-3 in HCC827, but not in PC-3, tumors. In PC-3 tumors,
treatment with gefitinib or vorinostat had little effect on caspase-3 cleavage, whereas their combination increased BIM expression and the cleavage of caspase-3 (Fig 4C, D). These findings indicate that the combination of vorinostat and gefitinib increases BIM protein expression and induces tumor cell apoptosis, thereby shrinking tumors produced by EGFR mutant NSCLC cells with the BIM polymorphism.
Discussion

*EGFR* mutant NSCLC cells with the *BIM* deletion polymorphism show impaired generation of BIM with the pro-apoptotic BH3 domain, as well as resistance to EGFR-TKI-induced apoptosis (5). We have shown here that treatment of cells with the combination of vorinostat, a HDAC inhibitor, and gefitinib, an EGFR-TKI, restored the expression of BIM protein with a BH3 domain (predominantly BIMEL), induced apoptosis, and overcame gefitinib resistance *in vitro* and *in vivo*.

Although vorinostat preferentially induced expression of BIM containing the BH3 domain, its exact mechanisms of action remain unclear. The wild-type allele may be more susceptible to the effects of HDAC inhibition than the deletion allele due to differences in the acetylation status of these alleles. Alternatively, vorinostat may affect the splicing process, resulting in the production of exon4- rather than exon3-containing transcripts from the deletion polymorphism allele since HDAC has been found to affect the splicing of RNA (16).

Vorinostat has been shown to induce the expression of several genes other than *BIM* (13). However, we found that BIM was pivotal not only for gefitinib-induced apoptosis but also when combined with vorinostat. Moreover, the combination of vorinostat and gefitinib increased BIM expression and markedly induced apoptosis in PC-3 and...
HCC2279 cells. Collectively, these findings strongly suggest that vorinostat promotes gefitinib-induced apoptosis in \textit{EGFR} mutant NSCLC cells with the \textit{BIM} polymorphism, primarily by increasing BIM expression. Several other mechanisms, including inhibition of epigenetic modifications leading to a drug-tolerant state (17) and transition of cancer cells from a resistant mesenchymal state to an E-cadherin expressing epithelial state (18), may be also involved.

Both the \textit{BIM} polymorphism and \textit{EGFR} mutations are more prevalent in East Asian than in Caucasian populations. Few East Asian patients with \textit{EGFR} mutant NSCLC show a complete response to EGFR-TKIs (1). This incomplete response, including intrinsic resistance, may be due, in part, to low BIM expression associated with the \textit{BIM} polymorphism (6). Our preclinical data indicate that vorinostat increases BIM even in \textit{BIM}-wild type \textit{EGFR} mutant NSCLC cells. However, a clinical trial with erlotinib and entinostat, an HDAC inhibitor, in unselected NSCLC patients, >65% of whom were Caucasian, failed to show therapeutic benefits (19). These findings suggest that the combination of vorinostat and an EGFR-TKI should be tested in selected NSCLC patients with \textit{EGFR} mutations and the \textit{BIM} polymorphism.

Resistance to EGFR-TKIs associated with the \textit{BIM} deletion polymorphism may be overcome by treatment with BH3 mimetics, such as ABT-737 (5). Although ABT-737
antagonized anti-apoptotic proteins, such as Bcl-2 and Bcl-X\textsubscript{L}, it did not antagonize the anti-apoptotic protein Mcl-1, which is overexpressed in NSCLC (20), suggesting that the effects of BH3 mimetics may be limited to overcoming EGFR-TKI resistance caused by the \textit{BIM} polymorphism in NSCLC. BH3 mimetics are being evaluated in early phase clinical trials but are not ready for use in clinical practice. In contrast, vorinostat has been approved by the US FDA for the treatment of patients with advanced primary cutaneous T-cell lymphoma (15). Therefore, the combination of gefitinib and vorinostat could easily be tested clinically.

The \textit{BIM} polymorphism can be detected in formalin fixed paraffin embedded tumor tissues and peripheral blood (5). Moreover, a convenient and easy access PCR screening method can detect this polymorphism in circulating DNA from serum (Supplementary Fig 5A, B). Since the \textit{BIM} polymorphism is a germ line alteration, it can be assayed in serum obtained at any time point. Collectively, our findings illustrate the importance of clinical trials testing the ability of combinations of vorinostat and EGFR-TKIs to overcome EGFR-TKI resistance associated with the \textit{BIM} polymorphism in patients with \textit{EGFR} mutant NSCLC.
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References


prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. Mol Cell 2005;17:393-403.


Figure legends

Figure 1. *EGFR* mutated NSCLC cell lines harboring the *BIM* deletion polymorphism show low susceptibility to gefitinib-induced apoptosis. (A) The upper panel shows PCR products from the four *EGFR* mutated NSCLC cell lines generated by primers flanking the deletion. PCR products 4.2 kb and 1.3 kb in size correspond to the alleles without and with the deletion, respectively, with the presence of both products indicating heterozygosity for the deletion polymorphism. The lower panel shows the levels of expression of the proteins BIM<sub>EL</sub>, BIM<sub>L</sub> and BIM<sub>S</sub> in each cell line. (B) Cell lines were treated with gefitinib (1 μM) or DMSO control for 48 h, and the activity of caspase-3/7 was measured using Caspase-Glo3/7 assay kits. Each bar represents the mean ± SD. (C) PC-9 (left) and HCC827 (right) cells were transfected with *BIM* or control siRNA for 24 h prior to gefitinib (1 μM) treatment for 48 h, and the activity of caspase-3/7 was measured as in (B). Each bar indicates the mean ± SD. Lysates were collected and proteins were analyzed by western blotting.

Figure 2. Up-regulation of BIM by vorinostat enhances induction of apoptosis in *EGFR* mutated NSCLC cell line with the *BIM* polymorphism. (A) PC-3 cells were incubated with serial dilutions of vorinostat for 24 h. The cell lysates were harvested and
the indicated proteins were analyzed by western blotting. (B) HCC827 cells (left) and PC-3 cells (right) were incubated with gefitinib (1 μM) and/or vorinostat (3 μM) for 48 h. The cell lysates were harvested and the indicated proteins were determined by western blotting. (C) PC-3 cells were treated with gefitinib (1 μM) and/or vorinostat (3 μM) for 12 h. The amounts of the various transcripts containing exon 2A, 3 or 4 are expressed as normalized ratios relative to actin (Upper). Ratio of exon 3-containing transcripts to exon 4-containing transcripts in PC-3 cells after treatment with each compound. *P < 0.05 versus control. Bar indicates the mean ± SD. (D) Apoptosis was analyzed by measurement of caspase-3/7 activity. *P < 0.05 gefitinib or vorinostat versus control; **P < 0.05 combination versus control and single agents. Bars represent the mean ± SD.

**Figure 3. Antitumor activity of gefitinib and/or vorinostat in mouse xenograft models of HCC827 and PC-3 tumors.** Nude mice bearing established tumors with HCC827 (A) or PC-3 (B) cells were treated with 25 mg/kg gefitinib and/or 40 mg/kg vorinostat once daily for 21 days. Tumor volume was measured using calipers on the indicated days. Mean ± SE tumor volumes are shown for groups of four to five mice. *P < 0.05 vs control, **P < 0.05 vs gefitinib by one-way ANOVA.
Figure 4. Vorinostat combined with gefitinib increases apoptosis in xenograft tumors with the BIM polymorphism. HCC827 and PC-3 xenograft tumors were resected from mice treated with 25 mg/kg gefitinib and/or 40 mg/kg vorinostat for 4 days.

(A) Analysis of apoptosis by TUNEL staining. Representative fluorescent images are shown. Green fluorescence indicates apoptotic cells. Bar indicates 50 μm. (B) Quantitation of number of apoptotic cells. *P < 0.05 gefitinib or vorinostat versus control; **P < 0.05 combination versus control and single agents. Bars represent mean ± SD.

(C) Tumors were harvested 8 h after 2 consecutive treatments with each compound, and the levels of protein in tumor lysates were determined by western blotting. (D) Tumors were harvested 24 h after 4 consecutive treatments with each compound. Protein expression levels in the tumor lysates were determined by western blotting.
Figure 1

A

PC-9  HCC827  PC-3  HCC2279

4.2 kb

1.3 kb

BIMEL

BIML

BIMS

β-actin

B

6

0

Caspase-3/7 activity (fold)

PC-9  HCC827  PC-3  HCC2279

Control

Gefitinib

C

PC-9

Scramble  BIM #1  BIM #2  siRNA

BIM EL

β-actin

HCC827

Scramble  BIM #1  BIM #2  siRNA

BIM EL

β-actin

Caspase-3/7 activity (fold)
Figure 2

A

Vorinostat 0 0.3 1 3 10 (μM)

BIM

Acetylated Histone H3

Histone H3

B

Vorinostat

Gefitinib

p-EGFR

p-Akt

p-Erk

Gefitinib

-- ++

- +- +

-- ++

- +- +

PC-3

HCC827

PC-3

C

BIM exon 2A (total BIM)

BIM exon 3-containing isoform

BIM exon 4-containing isoform

D

E3:E4 ratio

Control Gefitinib Vorinostat Combination

Caspase-3 activity (fold)

Control Gefitinib Vorinostat Combination

HCC827

PC-3
Figure 3

A  HCC827

- Control
- Gefitinib 25mg/kg

Tumor volume (mm³)

Days after treatment

B  PC-3

- Control
- Gefitinib 25mg/kg
- Vorinostat 40mg/kg
- Combination

Tumor volume (mm³)

Days after treatment
Figure 4

A

HCC827

Control

Gefitinib

PC-3

Control

Gefitinib

Vorinostat

Combination

B

HCC827

PC-3

Apoptotic cells per field

Control

Gefitinib

Control

Gefitinib

Vorinostat

Combination

C

HCC827

PC-3

Vorinostat

Gefitinib

BIM

β-actin

D

HCC827

PC-3

Vorinostat

Gefitinib

Cleaved Caspase-3

β-actin

Research.
EGFR-TKI resistance due to BIM polymorphism can be circumvented by in combination with HDAC inhibition

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