Combined effects of EGFR tyrosine kinase inhibitors and vATPase inhibitors in NSCLC cells

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A B S T R A C T

Despite excellent initial clinical responses of non-small cell lung cancer (NSCLC) patients to epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs), many patients eventually develop resistance. According to a recent report, vacuolar H + ATPase (vATPase) is overexpressed and is associated with chemotherapy drug resistance in NSCLC. We investigated the combined effects of EGFR TKIs and vATPase inhibitors and their underlying mechanisms in the regulation of NSCLC cell death. We found that combined treatment with EGFR TKIs (erlotinib, gefitinib, or lapatinib) and vATPase inhibitors (bafilomycin A1 or concanamycin A) enhanced synergistic cell death compared to treatments with each drug alone. Treatment with bafilomycin A1 or concanamycin A led to the induction of Bnip3 expression in an Hif-1α dependent manner. Knock-down of Hif-1α or Bnip3 by siRNA further enhanced cell death induced by bafilomycin A1, suggesting that Hif-1α/Bnip3 induction promoted resistance to cell death induced by the vATPase inhibitors. EGFR TKIs suppressed Hif-1α and Bnip3 expression induced by the vATPase inhibitors, suggesting that they enhanced the sensitivity of the cells to these inhibitors by decreasing Hif-1α/Bnip3 expression. Taken together, we conclude that EGFR TKIs enhance the sensitivity of NSCLC cells to vATPase inhibitors by decreasing Hif-1α/Bnip3 expression. We suggest that combined treatment with EGFR TKIs and vATPase inhibitors is potentially effective for the treatment of NSCLC.

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Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide. Non-small cell lung cancer (NSCLC), mainly adenocarcinoma, squamous cell and large cell carcinoma, accounts for 85% of all lung cancers. The epidermal growth factor receptor (EGFR) is highly overexpressed in 40–80% of NSCLC patients (Herbst et al., 2008). Activated EGFR triggers the phosphatidylinositol 3-kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK) pathways, which play major roles in cell proliferation, survival, and transformation and therapeutic resistance (Brognard et al., 2001; Brognard and Dennis, 2002).

EGFR tyrosine kinase inhibitors (TKIs), including erlotinib, gefitinib, and lapatinib, act directly on the cytoplasmic domain of EGFR, inhibiting the activity of the EGFR pathway (Baselga, 2000). EGFR TKIs have shown dramatic therapeutic efficacy in patients with NSCLC with EGFR activating mutations and have been recommended as the standard first-line therapy in these patients (Nguyen and Neal, 2012; Soria et al., 2012). However, despite their excellent initial clinical responses, nearly all responding patients eventually develop drug resistance after a median period of approximately 10 months (Pao et al., 2005).

The drug resistance of cancer cells is likely associated with changes in the pH gradient between the extracellular environment and the cytoplasm. Vacuolar H + ATPase (vATPase), a multi-subunit enzyme, plays a major role in the regulation of cellular pH (Sasazawa et al., 2009; Nishisho et al., 2011). vATPase translocates protons into extracellular environment or intracellular compartments to avoid accumulation of protons within the cell cytosol (Nishi and Forgac, 2002). vATPase is mainly involved in cancer metastasis and invasion (Gu and Gruenberg, 2000; Gocheva and Joyce, 2007). In particular, it is overexpressed in NSCLC (Lu et al., 2013). Its expression is related to the pathological type and grade of cancer and is likely associated with chemotherapy drug resistance in NSCLC (Lu et al., 2013). Therefore, vATPase inhibitors may be promising cancer treatments. Bafilomycin A1 and concanamycin A, selective vATPase inhibitors, have been suggested to be potential anti-cancer agents (Bowman et al., 2004; Hong et al., 2006).

In the present study, we showed that the combined treatment of NSCLC cells with EGFR TKIs and vATPase inhibitors enhanced synergistic...
cell death. Treatment with vATPase inhibitors induced Bnip3 expression in a Hif-1α dependent manner. Knock-down of Hif-1α or Bnip3 by siRNA further enhanced cell death induced by bafilomycin A1, suggesting that Hif-1α/Bnip3 induction confers resistance to cell death induced by the vATPase inhibitor. Treatment with EGFR TKIs suppressed Hif-1α and Bnip3 expression induced by bafilomycin A1. These data suggest that EGFRs TKIs enhance cell sensitivity to vATPase inhibitors via down-regulating Hif-1α/Bnip3. Our findings suggest that combined treatment with EGFR TKIs and vATPase inhibitors is potentially effective for the treatment of NSCLC.

Materials and Methods

Cell culture and reagents. A549 and H460 lung cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI1640 medium (Welgene, Daegu, Republic of Korea) supplemented with 10% fetal bovine serum. Bafilomycin A1, concanamycin A, and 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO, USA), and erlotinib, gefitinib, and lapatinib were obtained from Selleck (Houston, TX, USA).

Measurement of cell viability. Cell viability was assessed by measuring the mitochondrial conversion of MTT to a colored product. After treatment, cell were incubated with the MTT reagent and solubilized in DMSO. The amount of converted MTT was calculated by measuring absorbance at 570 nm. The results are expressed as the percentage of MTT reduction, assuming that the absorbance of control cells was 100%. The MTT experiments were repeated three times.

Evaluation of cell death. Cell death was measured by annexin V-FITC and propidium iodide (PI) staining, according to manufacturer’s instructions (BD Biosciences, San Jose, CA, USA). Briefly, cells were collected, washed with cold PBS and suspended in annexin V binding buffer. After staining with annexin V-FITC and PI, cells were analyzed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA). Each experiment was repeated three times.

Measurement of caspase activation. Active caspases were detected using a CaspaTag™ Caspase 3/7 in situ Assay Kit (Chemicon, Temecula, CA, USA), according to the manufacturer’s instructions. The green fluorescence signal directly corresponds to the amount of active caspase.
present in the cell. Stained cells were analyzed with a FACSscan flow cytometer. Each experiment was repeated two times.

**RNA isolation and reverse transcription-PCR analysis.** Total RNA was isolated from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). cDNA primed with oligo dT was prepared from 2 μg of total RNA using M-MLV reverse transcripase (Invitrogen). The following specific primers were used for PCR: Bnip3 (5′-GTATACAAACAGGACGTCATG-3′ and 5′-CAAGAATATGCCCCCTTCTCA-3′; 253 bp product), Hif-1α (5′-CTCAAAGTGACCAGCCTC-3′ and 5′-CCCTGAGTAGGTTTCTGCTC-3′; 460 bp) (Leonard et al., 2003) and β-actin (5′-GGATTCCCTATGTGGGCGACAG-3′ and 5′-GGGCTTGAGGATCTTCATG-3′; 438 bp product) (Jin et al., 2009). PCR products were subjected to electrophoresis on 1.5% agarose gels and visualized with ethidium bromide. Each experiment was repeated three times.

**Real-time PCR.** Real-time PCR assays were conducted using QuantiTect SYBR Green PCR Kit (Qiagen, Valencia, CA, USA) on a Roche LightCycler 96 real-time PCR machine (Roche Diagnostics GmbH, Roche Applied Science, Penzberg, Germany). Primer sequences for real-time PCR were as follows: Bnip3 (5′-TTTACGAGGAACGCTTTGAGA-3′ and 5′-AGCTGCTGGAGAGGTTC-3′; 253 bp product), Hif-1α (5′-CTCAAAGTGACCAGCCTC-3′ and 5′-CCCTGAGTAGGTTTCTGCTC-3′; 460 bp) (Leonard et al., 2003) and β-actin (5′-GGATTCCCTATGTGGGCGACAG-3′ and 5′-GGGCTTGAGGATCTTCATG-3′; 438 bp product) (Jin et al., 2009). PCR products were subjected to electrophoresis on 1.5% agarose gels and visualized with ethidium bromide. Each experiment was repeated three times.

**Statistical analysis**

Data were presented as the mean ± standard deviation (SD) of three independent experiments. Statistical analyses were determined by Student’s t tests. The p-value of less than 0.05 was considered to be statistically significant.

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**Fig. 2.** Different sensitivities of H460 and A549 NSCLC cells to bafilomycin A1. (A-D) H460 and A549 cells were treated with the indicated concentrations of bafilomycin A1 for 48 h. (A) Cell viability was measured by MTT assay. Data are presented as mean percentage of control ± SD (n = 3). (B) Cell death was detected as the percentage of annexin V and/or PI positive cells. Data are presented as the mean ± SD (n = 3). (C) Caspase-3/7 activity was evaluated with a Caspase™ in situ Assay Kit, as described in Materials and Methods. Representative data of two independent experiments are shown. (D) The protein levels of cleaved PARP and β-actin were estimated by western blot analysis. The blot is representative of three independent experiments. BAF, bafilomycin A1 and Ctrl, control.
Results

Effects of the EGFR TKIs erlotinib, gefitinib, and lapatinib in NSCLC cells

First, we investigated the effects of EGFR TKIs on cell viability in NSCLC cells. H460 and A549 cells were treated with various doses of the EGFR TKIs, erlotinib, gefitinib or lapatinib for 48 h, and then cell viability was detected by the MTT assay. As shown in Fig. 1A, all of the tested EGFR TKIs caused a slight dose-dependent inhibition in cell viability. A less than 25% decrease in cell viability was observed in both of the cell lines treated with 20 μM of each of the EGFR TKIs, respectively. The assessment of apoptosis based on annexin V/PI positivity and PARP cleavage revealed no cell death in either the H460 or A549 cells treated with the EGFR TKIs (Fig. 1B and C). These data suggest that these cells are resistant to EGFR TKIs, as reported previously (Bill et al., 2012).

Differing sensitivities of H460 and A549 NSCLC cells to baflomycin A1

According to a recent report (Lu et al., 2013), vATPase is over-expressed in NSCLC and is associated with chemotherapy drug resistance in NSCLC. To examine the effects of baflomycin A1 in H460 and A549 cells, cell viability was determined using the MTT assay, and cell death was assessed by annexin V-FITC/PI staining, caspase-3/7 activity and PARP cleavage. Baflomycin A1 decreased the viability of the A549 cells in a dose-dependent manner, but it barely affected the viability of H460 cell (at concentrations of up to 200 nM) (Fig. 2A). More than 60% cell death was observed in the A549 cells treated with 10 nM baflomycin, but no more than 10% cell death was observed in the H460 cells treated with 200 nM baflomycin (Fig. 2B). Further, caspase-3/7 activity and PARP cleavage were also observed following treatment with 5 nM baflomycin in the A549 cells but not in the H460 cells treated with 200 nM baflomycin A1 (Fig. 2C and D).

Fig. 3. The combined effects of lapatinib and vATPase inhibitors in NSCLC cells. (A, B) H460 cells were treated with either 100 nM baflomycin A1 or 50 nM concanamycin A together with the indicated concentrations of lapatinib for 48 h. (A) Cell viability was measured by MTT assay. Data are presented as mean percentage of control ± SD (n = 3, ***p < 0.001). (B) Cell death was detected as the percentage of annexin V and/or PI positive cells. Data are presented as mean ± SD (n = 3, ***p < 0.001). (C, D) H460 cells were treated with either 100 nM baflomycin A1 or 50 nM concanamycin A combined with 10 μM lapatinib for 48 h. (C) Caspase-3/7 activity was evaluated with a CaspaTag™ in situ Assay Kit, as described in Materials and Methods. Representative data of two independent experiments are shown. (D) The protein levels of cleaved PARP and β-actin were estimated by western blot analysis. The blot is representative of three independent experiments. (E) A549 cells were treated with the indicated concentrations of baflomycin A1 and/or 10 μM lapatinib for 48 h. Cell death was detected as the percentage of annexin V and/or PI positive cells. Data are presented as mean ± SD (n = 3, **p < 0.01). BAF, baflomycin A1; CON, concanamycin A; CTL, control.
expression induced by bafilomycin A1 than A549 cells.

**Combined effects of lapatinib and vATPase inhibitors in NSCLC cells**

Next, we investigated whether vATPase inhibitors can enhance the inhibitory effects of EGFR TKIs on cell viability. As shown in Fig. 3A, treatment with 100 nM bafilomycin A1 or 50 nM concanamycin A enhanced the inhibitory effects of lapatinib on the viability of the H460 cells. Co-treatment with the vATPase inhibitors and lapatinib resulted in a significant increase in apoptosis compared with treatments with each drug alone (Fig. 3B and E). Specially, bafilomycin A1 sensitized the A549 cells to lapatinib-induced cell death at concentrations of < 2 nM (Fig. 3E). The synergistic induction of cell death by lapatinib and the vATPase inhibitors was demonstrated by assessments of caspase-3/7 activity, and PARP cleavage (Fig. 3C and D).

**Hif-1α expression is induced by bafilomycin A1 and mediates resistance to cell death**

Next, we investigated the differences in the sensitivities of these two cell lines to bafilomycin A1, the expression of apoptosis-related proteins was analyzed using a human apoptosis array kit (ARY009, R&D Systems). The induction of the Hif-1α protein was observed in cells treated with bafilomycin A1 (Fig. 4A). To further confirm whether bafilomycin A1 induced Hif-1α protein expression, we analyzed its expression by western blot. As shown in Fig. 4B and C, treatment with bafilomycin A1 resulted in an increase in Hif-1α protein expression in dose and time-dependent manners. However, the level of Hif-1α protein expression in the H460 cells treated with bafilomycin A1 was higher than that in the A549 cells. Several studies have reported that Hif-1α promotes tumor growth or suppresses apoptosis (Zhang et al., 2004; Gillespie et al., 2007; Kiliç et al., 2007). Bafilomycin A1 has also been found to restrict cell proliferation and tumor growth by inhibiting the degradation of the Hif-1α protein (Lim et al., 2006). Thus, to determine whether Hif-1α is involved in mediating resistance to apoptosis promoted by bafilomycin A1, we transfected Hif-1α siRNAs into H460 cells and then treated them with this vATPase inhibitor. Transfection of Hif-1α siRNAs significantly suppressed Hif-1α expression (Fig. 4D). Knockdown of Hif-1α led to enhanced cell sensitivity and PARP cleavage in response to bafilomycin A1 (Fig. 4D and E). Overexpression of HA-tagged Hif-1α significantly decreased the bafilomycin A1-induced death of the A549 cells (Fig. 4F and G). These data suggest that Hif-1α induction confers resistance to cell death by vATPase inhibitors. Interestingly, treatment with lapatinib resulted in a reduction in Hif-1α protein expression induced by bafilomycin A1 or concanamycin A (Fig. 4H), suggesting that this TKI enhanced cell sensitivity to the vATPase inhibitors by decreasing Hif-1α expression.

**Bnip3 expression is induced by bafilomycin A1 and mediates resistance to cell death**

Bnip3 is a known Hif-1 target gene that is involved in cell death and survival (Kothari et al., 2003; Daido et al., 2004; Kanzawa et al., 2005). We observed that Bnip3 expression increased in cells exposed to bafilomycin A1 and that treatment with Hif-1α siRNAs blocked Bnip3 induction by bafilomycin A1 (Fig. 5A and B), suggesting that this vATPase inhibitor induces Bnip3 expression in an Hif-1α-dependent manner. Treatment with lapatinib resulted in a decrease in Bnip3 expression induced by bafilomycin A1 or concanamycin A (Fig. 5C). Next, we investigated whether Bnip3 induction is involved in resistance to bafilomycin A1. Knock-down of Bnip3 led to enhanced cell death and PARP cleavage in response to bafilomycin A1 (Fig. 5D and E), suggesting that it mediates resistance to cell death induced by this vATPase inhibitor.

EGFR TKIs erlotinib and gefitinib decrease Hif-1α expression induced by bafilomycin A1 and enhance cell sensitivity to this vATPase inhibitor

We further investigated whether two other EGFR TKIs, erlotinib or gefitinib, sensitize cells to bafilomycin A1. As shown in Fig. 6A, erlotinib or gefitinib enhanced cell sensitivity to bafilomycin A1. Treatment of the cells with these EGFR TKIs led to a decrease in Hif-1α protein expression induced by this vATPase inhibitor (Fig. 6B). These results suggest that EGFR TKIs sensitize cells to bafilomycin A1. Moreover, this process is mediated via the downregulation of Hif-1α.

**Discussion**

EGFR is highly expressed in NSCLC, and the activation of the EGFR signaling network results in tumor growth, the inhibition of apoptosis, cell migration and invasion, cellular differentiation, and transformation (Grandis and Sok, 2004; Merrick et al., 2006). Patients with NSCLC show excellent responses to treatment with EGFR TKIs, such as gefitinib and erlotinib (Nguyen and Neal, 2012; Soria et al., 2012). However, nearly all responding patients succumb to relapse because of drug resistance (Pao et al., 2005). In this study, we found that the EGFR wild-type A549 and NCI-H460 cell lines exhibited poor responses to EGFR TKIs (Fig. 1), as reported previously (Bill et al., 2012). Novel strategies are needed to delay or overcome resistance to EGFR TKIs.

Metastatic cancer cells display a more alkaline cytosolic pH and a more acidic extracellular pH relative to normal cells (Webb et al., 2011). The drug resistance of cancer cells is likely related to changes in the pH gradient between the extracellular environment and the cytoplasm. The primary role of vATPase is to pump protons from the cytosol into intracellular compartments or the extracellular space. This enzyme has been implicated in a number of critical physiological processes, including receptor-mediated endocytosis and intracellular trafficking, protein processing and degradation, the coupled transport of small molecules and ions, and the entry of various pathogens into cells (Forgac, 2007). Recently, overexpression of vATPase has been reported in NSCLC and has been associated with chemotherapy drug resistance in this type of cancer (Lu et al., 2013). In the present study, combined treatment with EGFR TKIs (erlotinib, gefitinib, or lapatinib) and vATPase inhibitors (bafilomycin A1 or concanamycin A) further inhibited cell viability in NSCLC cells compared with the use of a single vATPase inhibitor or EGFR TKI alone (Figs. 3 and 6). In addition, combined treatment with vATPase inhibitors and lapatinib significantly enhanced cell death compared with the use of a single vATPase inhibitor or lapatinib alone (Fig. 3). Interestingly, in the cells exposed to lapatinib, more than 50% cell death was observed for the H460 cells treated with 100 nM and for the A549 cells treated with 2 nM of bafilomycin A1 (Fig. 3). Bafilomycin A1 treatment resulted in a dose-dependent reduction in the viability of the A549 cells but did not result in a significant reduction in H460 cell viability (at concentrations of up to 200 nM) (Fig. 2). To elucidate the mechanism underlying these effects, the expression of apoptosis-related proteins was analyzed using a human apoptosis array kit (ARY009, R&D Systems). The induction of the Hif-1α protein was observed in the cells treated with bafilomycin A1 (Fig. 4A), consistent with previous studies (Lim et al., 2006, 2007). Lim et al. have reported that ATP6V0C, a component of vATPase, binds to the N-terminal end of Hif-1α, perturbing its structure so that it is unfavorable for pVHL binding, resulting in its stabilization (Lim et al., 2007). Bafilomycin stimulates ATP6V0C binding to Hif-1α (Lim et al., 2007). Specially, we found that Hif-1α expression in the H460 cells treated with vATPase inhibitors was greater than that in the A549 cells treated with these inhibitors (Fig. 4B and C). Because Hif-1α expression has been linked to the promotion of tumor growth or the suppression of apoptosis (Zhang et al., 2004; Gillespie et al., 2007; Kiliç et al., 2007), we examined whether Hif-1α expression affects cell sensitivity to bafilomycin. Knock-down of Hif-1α by siRNAs resulted in the increased sensitivity of the H460 cells to this vATPase inhibitor.
Fig. 5. Bafilomycin A1 induces Bnip3 expression in an Hif-1α-dependent manner, and Bnip3 mediates resistance to cell death induced by bafilomycin A1. (A) H460 or A549 cells were treated with the indicated concentrations of bafilomycin A1 for 48 h. The expression of Bnip3 mRNA was determined by real-time PCR (upper panel) and RT-PCR (low panel). Each experiment was repeated three times. (B) H460 cells were transfected with control or Hif-1α siRNA for 16 h, followed by treatment with 100 nM bafilomycin A1 for 48 h. (C) H460 cells were treated with 10 μM lapatinib and/or 100 nM bafilomycin A1 or 50 nM concanamycin A for 48 h. (B, C) The indicated mRNA levels were determined by RT-PCR. β-actin was used as an internal control. Three independent experiments were performed and representative results are shown. (D, E) H460 cells were transfected with control or Bnip3 siRNA for 16 h, followed by treatment with the indicated concentrations of bafilomycin A1 for 48 h. (D) Total protein isolated from cell lysates was subjected to western blot analysis for the measurement of cleaved PARP. β-actin used as an equal loading control for normalization. The blot is representative of three independent experiments. (E) Cell death was detected as the percentage of annexin v and/or PI positive cells. Data are presented as mean ± SD (n = 3, **p < 0.01). BAF; bafilomycin A1, CON; concanamycin A and CTL; control.

Fig. 4. Hif-1α expression is induced by bafilomycin A1 and mediates resistance to cell death. (A) Cell lysates from H460 or A549 cells treated with 50 nM or 5 nM bafilomycin A1, respectively, for 24 h were analyzed using a human apoptosis array kit. (B) H460 or A549 cells were treated with the indicated concentrations of bafilomycin A1 for 48 h. Hif-1α protein and mRNA levels were determined by western blot and RT-PCR analysis, respectively. β-actin was used as an internal control. Three independent experiments were performed and representative results are shown. (C) H460 or A549 cells were treated with 100 nM (for H460) or 5 nM (for A549) bafilomycin A1, respectively, for the indicated durations. The protein levels of Hif-1α and β-actin were determined by western blot analysis. The blot is representative of three independent experiments. (D, E) H460 cells were transfected with control or Hif-1α siRNA for 16 h, followed by treatment with 100 nM bafilomycin A1 for additional 48 h. (D) The indicated protein and mRNA expression levels were determined by western blot and RT-PCR analysis, respectively. Three independent experiments were performed and representative results are shown. (E) Cell viability was measured by MTT assay. Data are presented as mean percentage of control ± SD (n = 3, **p < 0.01). (F, G) A549 cells were transiently transfected with control empty vector or HA-Hif-1α expression plasmid for 16 h, followed by treatment with 0, 7.5 or 10 nM of bafilomycin A1 for 48 h. (F) The protein levels of Hif-1α, cleaved PARP and β-actin were determined by western blot analysis. The blot is representative of three independent experiments. (G) Cell death was detected as the percentage of annexin v and/or PI positive cells. Data are presented as mean ± SD (n = 3, **p < 0.01). (H) H460 cells were treated with 10 μM lapatinib and/or 100 nM bafilomycin A1 or 50 nM concanamycin A for 48 h. Total protein isolated from cell lysates was subjected to western blot analysis for the measurement of cleaved PARP. β-actin used as an equal loading control for normalization. The blot is representative of three independent experiments. BAF; bafilomycin A1, CON; concanamycin A and CTL; control.

The vATPase inhibitors (Figs. 4H and 6B), suggesting that EGFR TKIs enhance cell sensitivity to vATPase inhibitors by decreasing Hif-1α expression. The mechanism underlying EGFR TKIs inhibit Hif-1α expression induced by vATPase inhibitors, remains to be investigated in the next study.
Several studies have implicated the Hif-induced putative BH3-only proapoptotic gene Bnip3 in hypoxia-mediated cell death (Chen et al., 1999; Guo et al., 2001). However, some studies have concluded that this gene can actually promote survival by increasing reparative autophagy (Tracy and Macleod, 2007; Zhu et al., 2013). Bellot et al. have reported that hypoxia-induced autophagy via Bnip3 is clearly a survival mechanism that promotes tumor progression (Bellot et al., 2006). We observed that bafilomycin A1 induced Bnip3 expression in an Hif-1α-dependent manner (Fig. 5). As shown in Fig. 5D and E, the silencing of Bnip3 resulted in an increase in cell sensitivity to bafilomycin A1, suggesting that its expression mediates cell resistance induced by bafilomycin A1.

These data suggest that EGFR TKIs enhance the sensitivity of NSCLC cells to vATPase inhibitors by decreasing Hif-1α/Bnip3 expression. We suggest that treatment with a combination of EGFR TKIs and vATPase inhibitors may represent a useful strategy for NSCLC therapy.

Transparency Document

The Transparency document associated with this article can be found, in online version.

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