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# Augmentation of NVP-BEZ235's anticancer activity against human lung cancer cells by blockage of autophagy

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Autophagy is a cellular lysosomal degradation pathway essential for regulation of cell survival and death to maintain homeostasis. This process is negatively regulated by mammalian target of rapamycin (mTOR) signaling and often counteracts efficacy of certain cancer therapeutic agents. NVP-BEZ235 (BEZ235) is a novel, orally bioavailable dual PI3K/mTOR inhibitor that has exhibited promising activity against non-small cell lung cancer (NSCLC) in preclinical models. The current study focuses on evaluating the role of BEZ235 in regulating autophagy. BEZ235 was effective in inhibiting the growth of NSCLC cells including induction of apoptosis. It also potently induced the expression of type-II LC3, indicating induction of autophagy. When BEZ235 was used in combination with the lysosomal or autophagic inhibitor chloroquine (CQ), enhanced inhibitory effects on monolayer growth and colony formation of NSCLC cells was observed. In addition, enhanced induction of apoptosis was also detected in cells exposed to the combination of BEZ235 and CQ. Moreover, the combination of BEZ235 and CQ was more effective than each single agent alone in inhibiting the growth of NSCLC xenografts in nude mice. Thus, induction of autophagy by BEZ235 appears to be a survival mechanism that may counteract its anticancer effects. Based on these, we suggest a strategy to enhance BEZ235's anticancer efficacy by blockade of autophagy.

## Introduction

Non-small cell lung cancer (NSCLC) has remained the leading cause of cancer-related deaths and has a poor 5-y survival rate (<16%) despite improvements in therapeutic options.[1] Thus novel agents or efficacious therapeutic regimens are urgently needed.

It is known that phosphoinositide-3-kinase (PI3K)/Akt signaling and its regulated mTOR axis promotes cancer cell proliferation and survival and is often activated in human cancers. Moreover, activation of this signaling pathway is associated with resistance to cytotoxic chemotherapy.[2,3] Therefore, targeting the PI3K/Akt/mTOR signaling pathway has become an attractive therapeutic strategy.[4,5] In NSCLC, the PI3K/Akt/mTOR signaling pathway is aberrantly activated, largely due to frequent mutations of its upstream regulators including K-Ras, LKB1 and epidermal growth factor receptor (EGFR).[6-8] Thus, the PI3K/Akt/mTOR signaling pathway is also a promising therapeutic target for NSCLC.

Consequently, small molecule drugs that target this signaling pathway have been actively developed and tested preclinically and clinically.[5,9,10] One such compound is NVP-BEZ235 (BEZ235), a novel and orally available dual PI3K and mTOR inhibitor. This compound potently and reversibly inhibits both class I PI3K and mTOR kinase catalytic activity by competing at their ATP-binding site.[11] BEZ235 is currently being studied in phase I/II clinical trials. A preclinical study has shown that BEZ235 causes marked tumor regression in mouse lung adenocracinomas initiated by expression of p110-α H1047R, but is ineffective in mutant K-Ras-initiated models unless combined with a MEK inhibitor.[12] In contrast, another recent study has demonstrated that BEZ235 effectively induces a striking growth-inhibitory effect against both oncogeneic K-Ras-induced lung adenocarcinomas in transgenic mice and human NSCLC xenografts harboring K-Ras mutation in nude mice.[13] In certain types of NSCLC (e.g., with EGFR mutation), BEZ235 alone does not induce apoptosis but does so when combined with a MEK inhibitor,[14] suggesting a necessity to combine with other agents to improve its anticancer efficacy.

Autophagy is a cellular lysosomal degradation pathway that is essential for regulation of cells survival and death to maintain cellular homeostasis.[15,16] One of the key regulators of autophagy is mTOR, which is the major inhibitory signal that shuts off autophagy in the presence of growth factors and abundant nutrients.[16] Accordingly, inhibition of mTOR signaling (e.g., by the mTOR inhibitor rapamycin) induces autophagy.[17] Autophagy can be either a pro-survival or death mechanism depending on

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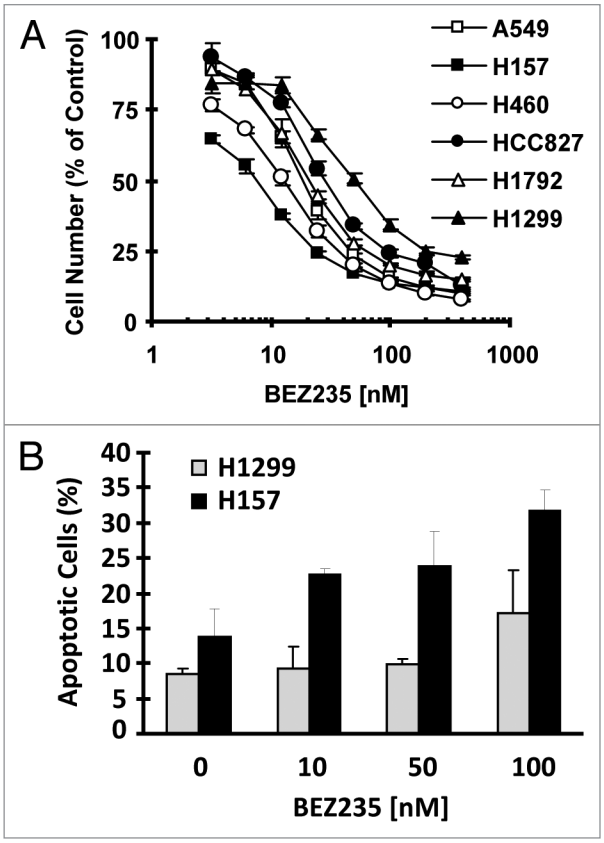


**Figure 1.** BEZ-235 inhibits cell growth (A) and induces apoptosis (B) in human lung cancer cells. (A) The indicated lung cancer cell lines were seeded in 96-well plates and then treated with different concentrations of BEZ235 as indicated on the second day. After 3 d, the cell numbers were estimated using the SRB assay. Points, means of four replicate determinations; bars, ± SD. (B) The indicated cell lines were plated in 6-well plates and then treated next day with different concentrations of BEZ235 as indicated. After 3 d, the cells were subjected to measurement of apoptosis using Annexin V staining. Columns, means of duplicate measurements; bars ± SD.

the circumstances,[15,16] thus generating variable impact on the outcome of cancer therapy.

In this study, we focused on addressing the role of BEZ235 on the induction of autophagy in human NSCLC cells and determining the impact of autophagy induction on its anticancer activity against NSCLC. We found that BEZ235 induced autophagy while inhibiting the growth of NSCLC cells. When combined with a lysosomal or authophagic inhibitor, the effects of BEZ235 on induction of apoptosis, inhibition of colony formation and suppression of xenografts in nude mice were substantially enhanced.

## Results

**BEZ235 inhibits cell growth and induces apoptosis in human NSCLC cells.** To determine the effects of BEZ235 on the growth of human NSCLC cells, we treated a panel of NSCLC cell lines with various concentrations of BEZ235 (0.5–200 nM) for 3 d and then estimated cell numbers with the SRB assay. All six tested cell lines responded well to BEZ235 treatment. The $IC_{50}$s of BEZ235 for these cell lines were within the range of 10–50 nM (**Fig. 1A**). Moreover, we determined whether BEZ235 induces apoptosis in NSCLC cells. As presented in **Figure 1B**, BEZ235 induced apoptosis in both H157 and H1299 cells. However, H1299 cells were less sensitive than H157 cell to BEZ235-induced apoptosis. Nearly 10-fold higher concentrations of BEZ235 were required to induce apoptosis in H1299 cells compared with H157 (100 vs. 10 nM). Thus, it appears that NSCLC cell lines have variable sensitivities to undergo BEZ235-induced apoptosis.

**BEZ235 inhibits Akt and mTOR signaling in human NSCLC cells.** We next determined whether BEZ235 inhibits PI3K/Akt/mTOR signaling pathway in human NSCLC cells. We treated three NSCLC cell lines, H157, H1299 and A549, with increasing concentrations of BEZ235 for 12 h and then harvested the cells for detection of phosphorylation of certain key proteins in the signaling pathway by protein gel blot analysis. As shown in **Figure 2**, BEZ235 reduced the levels of p-Akt, p-4EBP1 and p-S6 in a concentration-dependent manner, indicating that BEZ235 inhibits the PI3K/Akt/mTOR signaling pathway in human NSCLC cells. Interestingly, we noted that BEZ235 at 10 nM increased p-Akt levels in H1299 cells although it inhibited Akt phosphorylation at high concentrations (e.g., 50–100 nM).

**BEZ235 induces autophagy in human NSCLC cells.** Given the critical role of mTOR in negatively regulating autophagy,[16,17] we then studied whether BEZ235 induces authophagy in human NSCLC cells. By protein gel blotting, we detected increased levels of type II LC3 (LC3-II) expression, a lysosome-bound form of LC3, in cells exposed to BEZ235 (**Fig. 3A**). Moreover, we detected punctate staining of YFP-LC3 in cells infected with lentiviral YFP-LC3 when exposed to BEZ235 ranging from 10–100 nM (**Fig. 3B**), indicating the formation of autophagosomes. Collectively, these data demonstrate that BEZ235 induces autophagy in human NSCLC cells.

**Inhibition of autophagy enhances the effects of BEZ235 on suppressing cell growth and induction of apoptosis.** Autophagy can be a cell survival or death mechanism.[18] To determine whether induction of autophagy by BEZ235 is a survival or death mechanism, we analyzed the effects of BEZ235 on cell growth and apoptosis in the presence of the lysosomal protease inhibitor chloroquine (CQ) in several human NSCLC cells. As shown in **Figure 4A**, the combination of BEZ235 and CQ was more potent than either agent alone in inhibiting the growth of the NSCLC cells lines ($p < 0.01$ or 0.001). Furthermore, we examined apoptosis in cells exposed to BEZ235 alone, CQ alone and their combination. As presented in **Figure 4B**, both BEZ235 and CQ had a weak effect on the induction of apoptosis in both A549 and H1299 cell lines; however, the combination of BEZ235 and CQ was much more potent than either agent alone in inducing apoptosis. The highest levels of cleaved PARP was observed in cells exposed to the combination than in cells exposed to either agent alone (**Fig. 4C**). Thus, it is clear that inhibition of autophagy enhances the ability of BEZ235 to induce apoptosis.

To further demonstrate the impact of autophagy blockade on BEZ235's effect on inhibition of NSCLC cell growth, we conducted a colony formation assay which allows for long-term

repeating treatments. As shown in **Figure 5**, BEZ235 alone at the tested conditions partially inhibited the growth of colony formation of NSCLC cells. In the presence of CQ, this effect was substantially enhanced in both A549 ($p < 0.001$) and H1299 ($p < 0.05$ or 0.001). In A549 cells, the combination of BEZ235 and CQ eliminated cell colonies in comparison with either agent alone that only partially inhibited the formation and growth of colonies. These results further support the notion that inhibition of autophagy enhances the effects of BEZ235 on inhibiting cell growth and inducing apoptosis.

**BEZ235 combined with autophagy blockade exhibits enhanced antitumor activity against NSCLC xenografts in nude mice.** We further tested whether inhibition of autophagy enhances the anticancer activity of BEZ235 in vivo. In A549 xenograft model, treatment with the BEZ235 alone or CQ alone did not significantly inhibit the growth of xenograft tumors; however the combination of BEZ235 and CQ significantly ($p < 0.001$) suppressed tumor growth compared with vehicle control treatment (**Fig. 6A**). The combination did not significantly reduce mouse body weight (**Fig. 6B**), indicating the lack of significant toxicity. These data provide in vivo evidence for enhancement of efficacy of BEZ235 by preventing autophagy.

## Discussion

In the current study, we examined the effects of BEZ235 on the growth of a panel of NSCLC cell lines and found that BEZ235 effectively inhibited the growth of these cell lines with $IC_{50}$s of lower than 100 nM. Moreover, BEZ235 induced apoptosis in NSCLC cells as demonstrated in other types of cancer cells.[19,20] Thus, it is clear that BEZ235 is effective in inhibiting the growth of NSCLC cells. It was previously reported that BEZ235 treatment is ineffective in mutant K-Ras-initiated mouse lung adenocarcinoma model although generating marked tumor regression in mouse lung adenocracinomas initiated by mutant p110α.[12] In contrast to this study, another recent study has demonstrated that BEZ235 effectively induces a striking growth-inhibitory effect against both oncogeneic K-Ras-induced lung adenocarcinimas in transgenic mice and human NSCLC xenografts harboring K-Ras mutation in nude mice.[13] In our study, most of the tested cell lines (i.e., A549, H522, H460, H1792 and H157) have mutated K-Ras[21] and are equally sensitive as other cell lines without K-Ras mutation (e.g., HCC827) to BEZ235. Thus our findings support the efficacy of BEZ235 in NSCLC cells with K-Ras mutation.

The novel finding in this study is that BEZ235 potently induces autopagy in NSCLC cells while

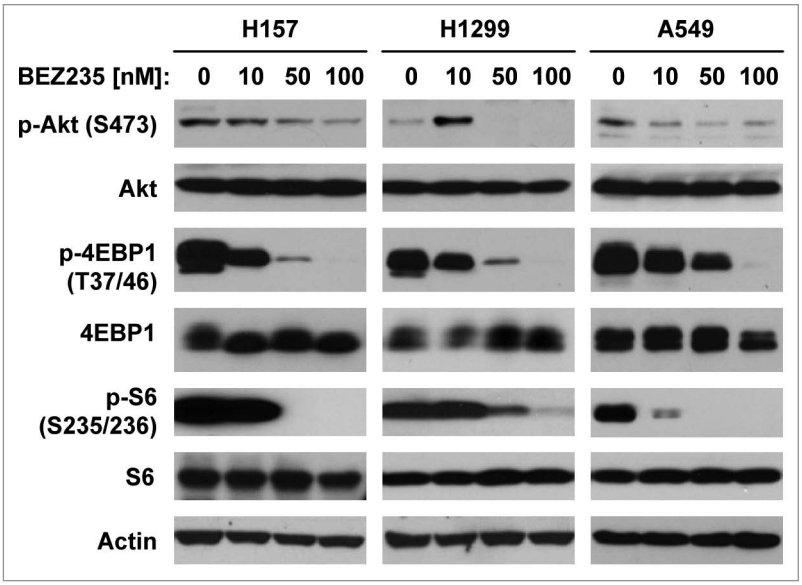


**Figure 2.** BEZ-235 inhibits Akt and mTOR signaling in human lung cancer cells. The indicated cell lines were plated in 10 cm-diameter cell culture dishes and treated next day with the given concentrations of BEZ-235 for 12 h. The cells were then harvested for preparation of whole-cell protein lysates and subsequent protein gel blot analysis.

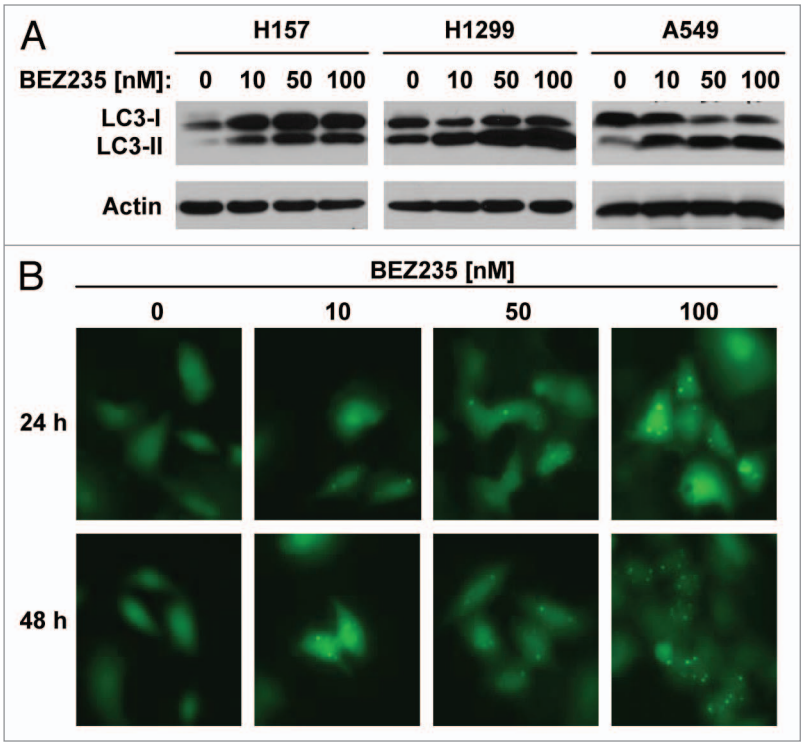


**Figure 3.** BEZ-235 induces type II LC3 expression. (A) The same whole-cell protein lysates as described in **Figure 2** were used for detection of LC3 with protein gel blot analysis. (B) A549/LC3-YFP cells were plated in 6-well plates and then treated with indicated concentrations of BEZ-235. After 24 and 48 h, fluorescence images recorded taken with a fluorescence microscopy.

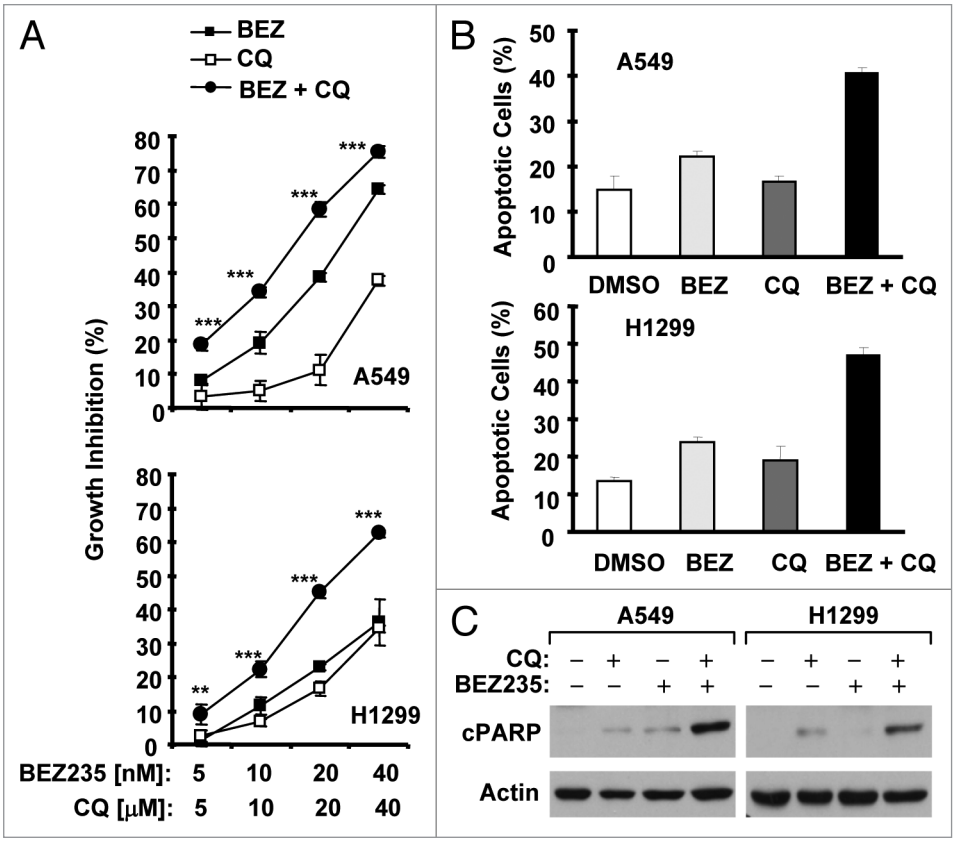


**Figure 4.** BEZ235 in combination with CQ exerts enhanced effects on inhibiting the growth (A) and on inducing apoptosis (B and C) of human lung cancer cells. (A) Given cell lines were seeded in 96-well plates and then treated next day with BEZ235, CQ and their combination as indicated for 3 d. The cell numbers were then estimated using the SRB assay. Points, mean of four replicate determinations; bars ± SD **$p < 0.01$ and ***$p < 0.001$ compared with BEZ235 alone or CQ alone. (B and C) The indicated cell lines were seeded in 6-well plates and then treated on the second day with 25 nM BEZ-235, 20 μM CQ and their combination. After 48 h, the cells were harvested for measurement of apoptosis using Annexin V staining (B) and for detection of PARP cleavage with protein gel blot analysis (C). Columns, means of duplicate measurements; bars ± SD.

inhibiting cell growth and initiates apoptosis, evidenced by detection of increased levels of LC3-II and punctate staining of YFP-LC3 bound in autophagosome. Considering the important role of mTOR in negatively regulating autophagy, it is not surprising to detect autophagy in cells exposed to BEZ235, a dual PI3K/mTOR inhibitor. Since autophagy can be either a pro-survival or death mechanism depending on the circumstances,[15,16] we were particularly interested in the impact of autopahgy induction on the anticancer effects of BEZ235. It was reported that dual inhibition of PI3K and mTOR with the combination of rapamycin and LY294002 in glioma cells exhibits enhanced antitumor effect by synergistic induction of autophagy.[22] In our study, induction of autophagy by BEZ235 is clearly a survival mechanism that counteracts its antitumor efficacy based on the following findings: (1) the combination of BEZ235 with the lysosomal inhibitor CQ exerts enhanced effects on inhibiting the growth of human NSCLC cells in a monolayer culture assay (**Fig. 4A**); (2) the combination exhibits enhanced effect on inhibiting the formation and growth of NSCLC cell colonies in a long-term colony formation assay (**Fig. 5A and B**); (3) the presence of CQ substantially augments BEZ235-induced apoptosis (**Fig. 4B and C**); and (4) BEZ235 and CQ combination is more effective than BEZ235 or CQ alone in inhibiting the growth of NSCLC xenografts in nude mice (**Fig. 6A**). Our findings are in agreement with a recent report that the combination of BEZ235 and CQ exerts enhanced effects on induction of apoptosis and on inhibition of xenograft growth in glioma cells.[23]

We noted that the combination of BEZ235 and CQ exhibited much more impressive suppression on colony formation in A549 than in H1299 cells (**Fig. 5**), suggesting that different NSCLC cell lines have varied sensitivities to the combination treatment. We noted that BEZ235 at 10 nM, a concentration used in the colony formation assay, actually increased p-Akt levels in H1299 cells. Under the same condition, BEZ235 reduced p-Akt levels in A549 cells (**Fig. 2**). This observation is consistent with a previous report, in which BEZ235 increased Akt phosphorylation in some cancer cell lines at low doses (e.g., 10 nM).[20] Whether Akt activation induced by the low dose of BEZ235 attenuates the efficacy of the combination of BEZ235 and CQ on the growth of some NSCLC cells needs further investigation.

In our study, BEZ235 at 10 nM, a concentration within the $IC_{50}$ ranges in NSCLC cells (**Fig. 1A**), induced clear increase in LC3-II expression (**Fig. 3**), indicating that induction of autophagy in cells exposed to BEZ235 is a therapeutically relevant phenomenon. Thus, our finding on enhancement of efficacy of BEZ235 by blockade of autophagy may suggest a potential strategy to enhance therapeutic efficacy of dual targeting PI3K and mTOR signaling.

## Materials and Methods

**Reagent.** BEZ235 was supplied by Novartis Pharmaceuticals Corporation (East Hanover, NJ), dissolved in DMSO and stored at -80°C. CQ and rabbit polyclonal anti-actin antibody were purchased from Sigma Chemical Co. (St. Louis, MO). Rabbit polyclonal antibodies against Akt, p-Akt (S473), p-p70S6K (T389), p70S6K, p-4EBP1 (Thr37/46), 4EBP1 and poly(ADP-ribose)polymerase (PARP), respectively, were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Rabbit polyclonal microtubule-associated protein light chain 3 (LC3) antibody (NB100-2220) was purchased from Novus Biologicals, Inc. (Littleton, CO).

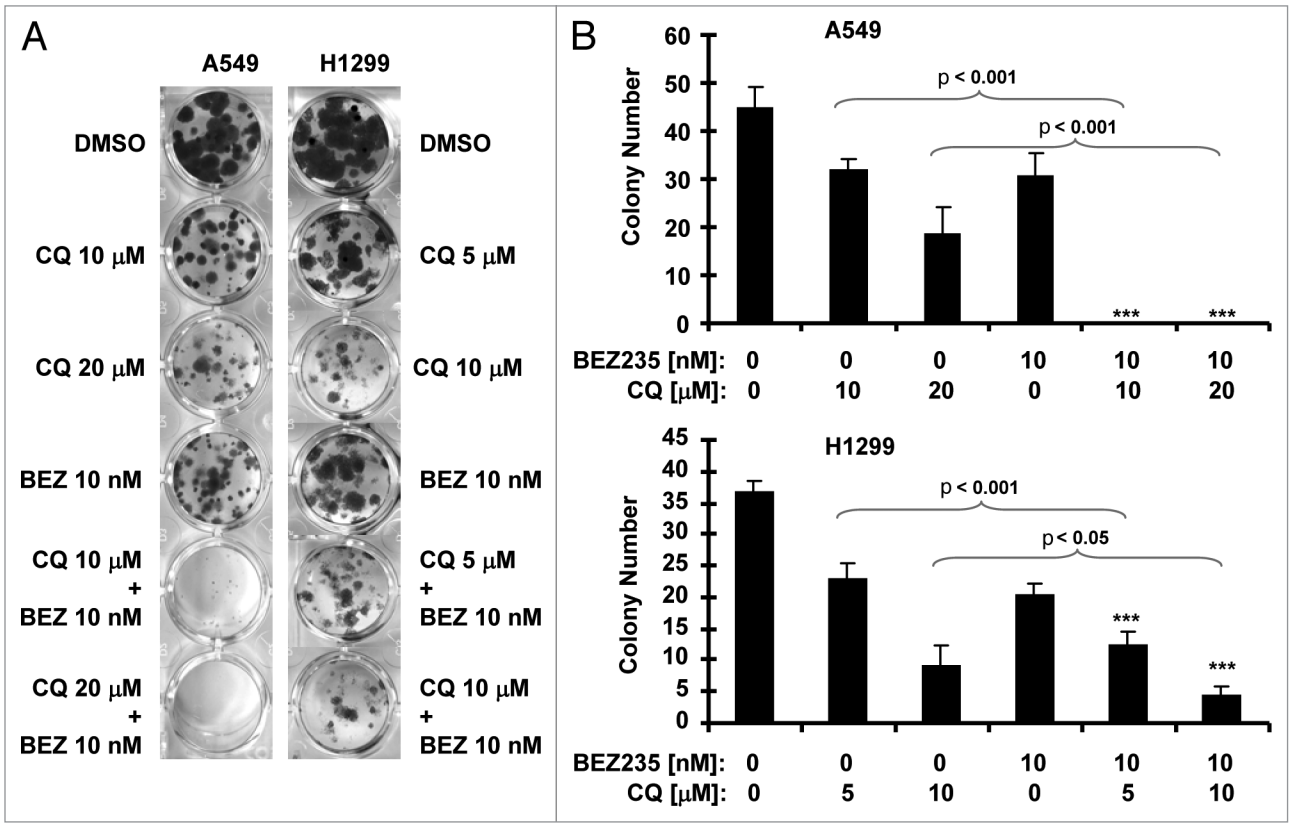


**Figure 5.** BEZ235 and CQ combination results in enhanced inhibitory effects on colony formation of lung cancer cells. The indicated cell lines at a density of approximately 200 cells/well were seeded in 24-well cell culture plates. On the second day, cells were treated with CQ, BEZ235 (BEZ), and their combinations as indicated. The same treatments were repeated every 3 d. After 12 d, the plates were stained for the formation of cell colonies with crystal violet dye. The representative pictures of the colonies were taken using a digital camera (A) and colonies were counted manually (B). Columns, means of four replicate measurements; bars ± SD ***p < 0.001 compared with BEZ235 (10 nM) alone.

**Cell lines and cell culture.** The human lung cancer cell lines used in this study were described previously in reference 24. A549/YFP-LC3 stable line was established by infecting A549 cells with lentiviruses carrying lentiviral YFP-LC3 expression construct as generated below. These cell lines were grown in monolayer culture in RPMI 1640 medium supplemented with 5% fetal bovine serum (FBS) at 37°C in a humidified atmosphere consisting of 5% $CO_2$ and 95% air.

**Generation of lentiviral YFP-LC3 expression construct.** LC3 cDNA, which was amplified with RT-PCR from total cellular RNA extracted from H157 cells with the following primers: LC3 BglII, 5'-ATA TAT AGA TCT CCG TCG GAG AAG ACC TTC-3', and LC3 EcoRI, 5'-GCG CGC GAA TTC TTA CAC TGA CAA TTT CAT-3', was cloned into pEYFP-C1 vector in the same reading frame with N-terminal YFP. The BamHI-YFP/LC3-EcoRI fragment was then obtained with PCR using the following primers and inserted into FUGW lentiviral vector[25] with BamHI and EcoRI link: BamHI YFP, 5'-ATA TAT GGA TCC ACC ATG GTG AGC AAG GGC-3' and LC3 EcoRI (same as above). The LC3 sequence was confirmed by sequencing.

**Cell survival assay.** Cells were cultured in 96-well cell culture plates and treated the next day with the agents indicated. Viable cell number was estimated using the sulforhodamine B (SRB) assay, as previously described in reference 24.

**Colony formation assay.** The effects of the given drugs on colony formation on plates were measured as previously described in reference 26.

**Detection of apoptosis.** Apoptosis was evaluated by Annexin V staining using Annexin V-PE apoptosis detection kit purchased from BD Biosciences (San Jose, CA) following the manufacturer's instructions. PARP cleavage was also detected by protein gel blotting as an additional indicator of apoptosis.

**Protein gel blot analysis.** Preparation of whole cell protein lysates and protein gel blot analysis were described previously in reference 27 and 28.

**Lung cancer xenografts and treatments.** Animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Emory University. Five- to six-week old female athymic (nu/nu) mice were ordered from Taconic (Hudson, NY) and housed under pathogen-free conditions in microisolator cages with laboratory chow and water ad libitum. A549 cells at 5 x $10^6$ in serum-free medium were injected s.c. into the flank region of nude mice. When tumors reached certain size ranges (~100 $mm^3$), the mice were randomized into four groups (n = 6/group) according to tumor volumes and body weights for the following treatments: vehicle control, BEZ235 (30 mg/kg/day, og), CQ (50 mg/kg/day; ip), and their combination. Tumor

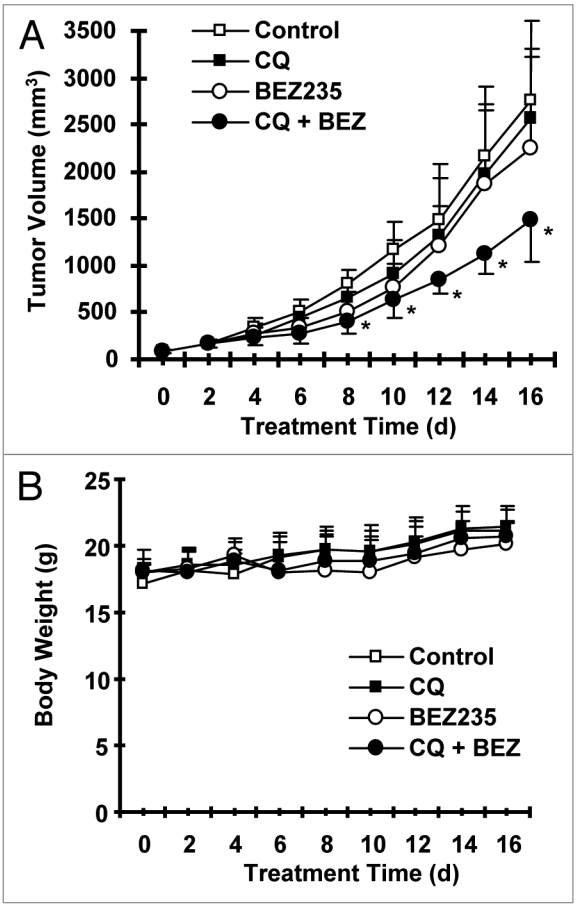


**Figure 6.** BEZ235 and CQ combination results in enhanced inhibitory effects on the growth of lung cancer xenograpts in mice. Mice with A549 xenograpts were treated with vehicle control, CQ (50 mg/kg) alone, BEZ-235 (30 mg/kg) alone and BEZ-235 plus CQ on the same day after grouping. After 16-d consecutive treatment (once daily), the mice were sacrificed. Tumor sizes (A) and body weight (B) were measured once every 2 d. Each measurement is a mean ± SD (n = 6). *p <0.001 compared with the vehicle control.

volumes were measured using caliper measurements once every 2 d and calculated with the formula V = π(length x width$^2$)/6.

**Statistical analysis.** The statistical significances among treatment groups in cell cultures were analyzed with one-way analysis of variance (ANOVA). The statistical significance of differences in tumor sizes between two groups was analyzed with two-sided unpaired Student's t-tests when the variances were equal or with Welch's corrected t-test when the variances were not equal. All of these analysese were done by use of Graphpad InStat 3 software (GraphPad Software; La Jolla, CA). Results were considered to be statistically significant at $p < 0.05$.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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