

# Inhibition of esophageal cancer growth through the suppression of PI3K/AKT/mTOR signaling pathway

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**Background:** The phosphatidylinositol-3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) pathway is implicated in several cancers. AKT allosteric inhibitor MK2206 and dual PI3K and mTOR inhibitor BEZ235 are promising drug candidates with potential anti-tumor effects.

**Purpose:** In this study, we aimed to detect the activation of PI3K/AKT/mTOR pathway and assess the efficacy of MK2206 and BEZ235 in inhibiting esophageal cancer growth.

**Materials and methods:** We used three different systems including carcinogen-induced animal model, human esophageal squamous cell carcinoma (SCC) cell lines, and xenograft mouse model.

**Results:** Our data indicated that components of the PI3K/AKT/mTOR pathway were over-expressed and activated in esophageal SCC. MK2206 and BEZ235 inhibited cell proliferation, enhanced apoptosis, and induced cell-cycle arrest through downstream effectors SKP2, MCL-1, and cyclin D1 in esophageal SCC cells. MK2206 and BEZ235 also inhibited tumor growth in xenograft mice through the inhibition of AKT phosphorylation. MK2206/BEZ235 combination showed greater anti-tumor effect than MK2206 or BEZ235 alone. The enhanced efficacy of the combination was associated with the inhibition of phosphorylation AKT on both Thr308 and Ser473.

**Conclusion:** The combination of MK2206 and BEZ235 exhibits potent antitumor effects and may have important clinical applications for esophageal SCC treatment.

**Keywords:** PI3K/AKT/mTOR, MK2206, BEZ235, Esophageal squamous cell carcinoma

## Introduction

Esophageal squamous cell carcinoma (SCC) is a major public health concern with low 5-year survival rates worldwide.<sup>1</sup> Most patients present with advanced metastatic disease at the time of diagnosis, yet effective therapy for esophageal SCC remains limited due to resistance to traditional cytotoxic chemotherapeutic agents.<sup>2</sup> Therefore, there is an urgent need for improved therapies in patients with esophageal SCC.

Esophageal carcinogenesis is a multistage process associated with multiple critical genetic alterations.<sup>2-5</sup> One of the signaling pathways that is implicated in such alterations and frequently activated in esophageal SCC is the phosphatidylinositol-3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) pathway.<sup>6</sup> In this pathway, activation of PI3K phosphorylates AKT, the downstream effects of which can contribute to oncogenic processes by regulating cellular proliferation, survival, motility, angiogenesis, and metabolism/glucose homeostasis.<sup>7</sup> Indeed, strategies targeting this signaling cascade have shown promise for cancer therapies,<sup>8-11</sup> and thus inhibiting this PI3K/AKT/mTOR pathway may be an effective treatment strategy in esophageal SCC.

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The highly selective AKT inhibitor MK2206 has shown antitumor effects by promoting dephosphorylation of AKT in breast, gastric, neuroendocrine, endometrial, and other cancers.<sup>12–15</sup> Additionally, a novel imidazoquinoline derivative with high selectivity for class I PI3K and mTOR known as BEZ235 has shown antitumor effects against breast, prostate, pancreatic, neuroendocrine, and other cancers.<sup>16–19</sup> Both MK2206 and BEZ235 have proven to be competitive candidates for cancer therapy in clinical trials.<sup>8–11</sup> MK2206, however, showed limited clinical benefits in patients when used as a single agent in clinical trials.<sup>8</sup> It has been shown that single-inhibitor treatment can induce drug resistance by compensatory activation of up- and down-stream molecules,<sup>20</sup> and therefore, inhibitors targeting more than one molecule in the same signaling pathway may be useful to overcome this compensatory activation. As a dual PI3K and mTORC1/2 inhibitor, BEZ235 may show greater antitumor activity because blocking PI3K can initiate mTOR inhibition and compensatory activation of AKT.<sup>21</sup> Additionally, the combination of drugs targeting different mechanisms has shown synergistic efficacy in preclinical models.<sup>22,23</sup> The combination of MK2206 with trastuzumab, mitogen-activated protein kinase (MEK) inhibitor AZD6244, farnesyltransferase FTI-2153 all showed high efficacy both in vitro and in vivo.<sup>24–27</sup> The combination of BEZ235 with chemotherapy drugs, such as taxotere, sorafenib, enzastaurin, and perifosine, has similarly shown positive effects.<sup>11,28–30</sup> MK2206 or BEZ235 in combination with an mTOR inhibitor, such as rapamycin and everolimus, exerts synergistic antitumor activity in preclinical cancer models.<sup>26,31,32</sup> Overall, these results suggest that the combination of MK2206 and BEZ235 might have synergistic antitumor effects.

In this study, we hypothesized that targeting the PI3K/AKT/mTOR pathway may be an effective strategy for esophageal SCC treatment. We therefore examined the expression and activation of the PI3K/AKT/mTOR pathway in esophageal SCC cell lines and in a N-nitrosomethylbenzylamine (NMBA)-induced esophageal SCC rat model. We also investigated the antitumor effects of MK2206 and BEZ235 alone and in combination in esophageal SCC cell lines and xenograft animal model.

## Materials and methods

### Cell lines

Esophageal SCC cell lines KYSE 70, KYSE 150, KYSE 270 and KYSE 410 were obtained from DSMZ - German Collection of Microorganisms and Cell Cultures. A normal

esophageal epithelial cell line HET-1A was obtained from the American Type Culture Collection. KYSE 70, KYSE 150, KYSE 270, and KYSE 410 were cultured in RPMI-1640 or Ham's F12 supplemented with 5% FBS and HET-1A cells were cultured in serum-free LHC-9 medium.

### NMBA-induced esophageal SCC in rats

The current study is part of a large-scale investigation on carcinogenesis of esophageal SCC performed in the Ohio State University (OSU).<sup>5</sup> This animal study protocol was reviewed and approved by the OSU Institutional Animal Care and Use Committee (IACUC; Protocol No.: 2009A0054-R2). We strictly followed the guidelines set by the National Institutes of Health (NIH) on the Animal Health and Care Protocols and university IACUC. The euthanasia procedure was executed in compliance with the American Veterinary Medical Association (AVMA) guidelines for the Euthanasia of Animals. Male F344 rats were treated with NMBA (0.30 mg/kg b.w.) or a solution of 20% dimethyl sulfoxide (DMSO) in water (the solvent for NMBA) 3 times per week for 5 weeks. At 29 weeks, all rats were euthanized by CO<sub>2</sub> asphyxiation and subjected to gross necropsy. The esophagus of each rat was excised and opened longitudinally. Tumors larger than 0.5 mm in a single dimension were counted, mapped, and the esophageal tissues were collected as previously described.<sup>3,4</sup>

### Western blot

Proteins were extracted from cultured cells or frozen esophageal epithelium. Protein concentration was measured using a DC Protein Assay Kit (Bio-Rad, Hercules, CA) according to the manufacturer's recommendations. Protein samples with NuPAGE LDS Sample Buffer and NuPAGE sample reducing agent (Invitrogen, Carlsbad, CA) were heated at 100°C for 10 mins. After cooling at room temperature for 5 mins, proteins were fractionated by 4–12% NuPAGE Novex Tris-acetate gel electrophoresis (Invitrogen, Carlsbad, CA). Proteins were then transferred to an Invitrolon polyvinylidene difluoride (PVDF) membrane. The PVDF membrane was blocked with 10% (w/v) dry milk in TBS for 1hr and then incubated with first antibodies overnight at 4°C. Specific first antibodies: phospho-mTOR<sup>Ser2481</sup>, phospho-FOXO3a<sup>Ser253</sup>, phospho-S6 ribosomal protein<sup>Ser235/236</sup>, phospho-p70S6 ribosomal protein<sup>Thr389</sup>, phospho-ERK<sup>Thr202/204</sup>, S6 ribosomal protein, phospho-AKT<sup>Ser473</sup>, phospho-AKT<sup>Thr308</sup>, AKT, p110α, p110β, PI3K(p85), mTOR, ERK, FOXO3a, 4EBP1, S6, p70S6, CDK2, SKP2, cyclin D1, cleaved-caspase3, full length-caspase3, MCL-1, GAPDH and secondary antibodies:

horseradish peroxidase (HRP)-linked anti-mouse/rabbit antibody was bought from Cell Signaling Technology (Danvers, MA, USA). The immunoreactive bands were detected with an Immun-star<sup>TM</sup> WesternC<sup>TM</sup> Kit (Bio-Rad Laboratories) using Molecular Imager ChemiDoc XRS (Bio-Rad Laboratories) and analyzed by Image Lab Software (version 2.0, Bio-Rad Laboratories). The experiments were repeated three times.

## Cell proliferation assay

The proliferation of cells was assessed using the WST-1 kit (Cayman, Ann Arbor, Michigan). KYSE cells were seeded in a 96-well plate at a density of 2000 cells/well in 100  $\mu$ L medium. The next day, cells were treated with MK2206 or BEZ235. Forty-eight hours after the treatment, 10  $\mu$ L WST-1 reagent were added directly to the cell culture medium and incubator for 2 hrs. Cell viability was then detected by plate reading at 550 nm using Omega Microplate Reader (BMC Labtech., Offenburg, Germany). The experiments were repeated three times.

## Cell flow cytometry

Apoptosis was quantified by Annexin VI-APC staining (BD) as previously described, and the cell cycle was assessed using DNA staining with PI (BD). Detailed cycle analysis of esophageal cells was performed by quantifying G0-G1, S, and G2-M phases by propidium iodide staining using CycleTEST PLUS kit (Becton Dickinson) according to the manufacturer's recommendations. For the quantification of G0 and M phases, 10<sup>6</sup> cells were permeabilized with 1 mL of ice-cold ethanol (2 hrs, -20°C). Following two washes with PBS, 1% fetal bovine serum, and 0.25% Triton X-100 (PFT), the cells were stained in 200  $\mu$ L PFT for 30 mins at room temperature in the dark, either with 1  $\mu$ g of propidium iodide (BD) and 5  $\mu$ L of FITC-conjugated anti-human Ki67 mAb (BD), respectively. The experiments were repeated three times.

## Xenograft animal model

Animal care and experiments were approved by OSU IACUC (Protocol No.: 2013A00000143-R1). In total, 1 $\times$ 10<sup>6</sup> cells in 100  $\mu$ L PBS mixed with equal volume of Matrigel (BD Bioscience, San Jose, CA) were injected into male NCr nu/nu nude mice (Taconic Farm, NY). The treatment was initiated 1 week after the inoculation. MK2206 was dissolved in 30% captisol and BEZ235 was dissolved in 1:9 (v/v) NMP:PGE300. Animals were divided into 4 groups randomly and treated with different drugs 3 times a week (Monday, Wednesday and Friday) by oral gavage for 2 weeks. Group 1 was treated with a

vehicle control; Group 2 was treated with 90 mg/kg MK2206; Group 3 was treated with 15 mg/kg BEZ235; Group 4 was treated with 30 mg/kg MK2206 combined with 5 mg/kg BEZ235. Tumor volumes were evaluated twice every week after initial detection. The tumor sizes were measured by a digital caliper. The tumor volume in mm<sup>3</sup> was calculated by the formula: Volume = (width)<sup>2</sup> x length x  $\pi$ /6.

## Statistical analysis

Data were expressed as mean values and SD. Statistical significance of differences observed between experimental groups was determined using one-way analysis of variance and Tukey's multiple comparison test. Results were described using significance values \*, \*\*/###, and \*\*\*/#### indicating  $P < 0.05$ ,  $P < 0.01$ , and  $P < 0.001$ , respectively.

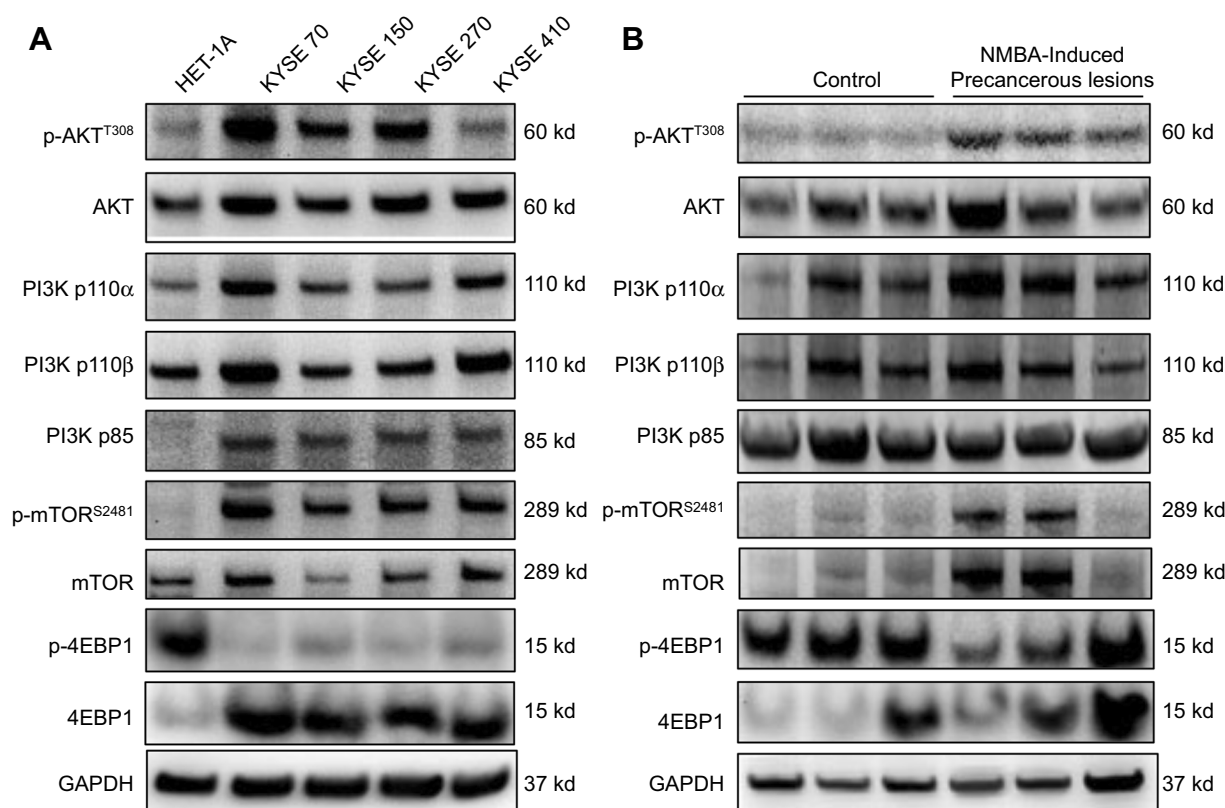
## Results

### High expression of PI3K, AKT, and mTOR is detected in both human esophageal SCC cell lines and rat esophageal tissues using an NMBA- induced esophageal SCC model

To verify the role of the PI3K/AKT/mTOR pathway in esophageal SCC, we compared the expression of PI3K, AKT, and mTOR in: 1) the normal esophageal epithelial cell line HEA-1A with esophageal SCC cell lines (Figure 1A) and 2) the normal rat esophageal epithelium with NMBA-induced rat precancerous esophageal tissue (Figure 1B). Results showed that compared to the HET-1A normal esophageal cell line, the esophageal SCC cell lines (KYSE 70, KYSE 150, KYWE 270, KYSE 410) exhibited elevated expression of p-AKT<sup>T308</sup>, p-mTOR<sup>S2448</sup>, p100 $\alpha$  PI3K, p110 $\beta$  PI3K, and p85 PI3K (Figure 1A). Furthermore, the expression of these molecules was elevated in the NMBA-induced precancerous esophageal tissue compared with normal esophageal epithelium (Figure 1B). These results underscore high expression of key components of the PI3K/AKT/mTOR pathway in esophageal SCC in both human cell lines and carcinogen-induced animal model of esophageal SCC.

### MK2206 and BEZ235 inhibit esophageal cancer cell proliferation

Next, we examined the inhibitory effects of MK2206 or BEZ235 on proliferation of esophageal SCC cell lines. Results highlighted that MK2206 and BEZ235 significantly



**Figure 1** Activation of AKT, PI3K, and mTOR in human esophageal cancer cell lines and NMBA-induced rat esophageal SCC. **(A)** Phosphorylation of AKT, PI3K, and mTOR in normal esophageal epithelial cell line HET-1A and esophageal SCC cell lines KYSE 70, KYSE 150, KYSE 270, and KYSE 410; **(B)** phosphorylation of AKT, PI3K, and mTOR in rats treated with NMBA.

inhibited cell proliferation in KYSE150 (Figure 2A and B). Specifically, both p-AKT<sup>T308</sup> and p-AKT<sup>S473</sup> were significantly decreased after MK2206 treatment, but p-AKT<sup>S473</sup> was less decreased than p-AKT<sup>T308</sup>. In accordance with these results, the p-S6 was decreased by MK2206. p-mTOR was only reduced at high doses of MK2206 treatment (Figure 2C). p-AKT<sup>T308</sup>, p-AKT<sup>S473</sup>, p-mTOR, and p-P70S6K were all significantly decreased after BEZ235 treatment (Figure 2D). Moreover, compared with MK2206, p-AKT<sup>S473</sup>, p-mTOR, and p-P70S6K decreased more after BEZ235 treatment. Similar results were found in KYSE 70, KYSE 270, and KYSE 410 cell lines (data not shown).

### MK2206 and BEZ235 increase cell apoptosis

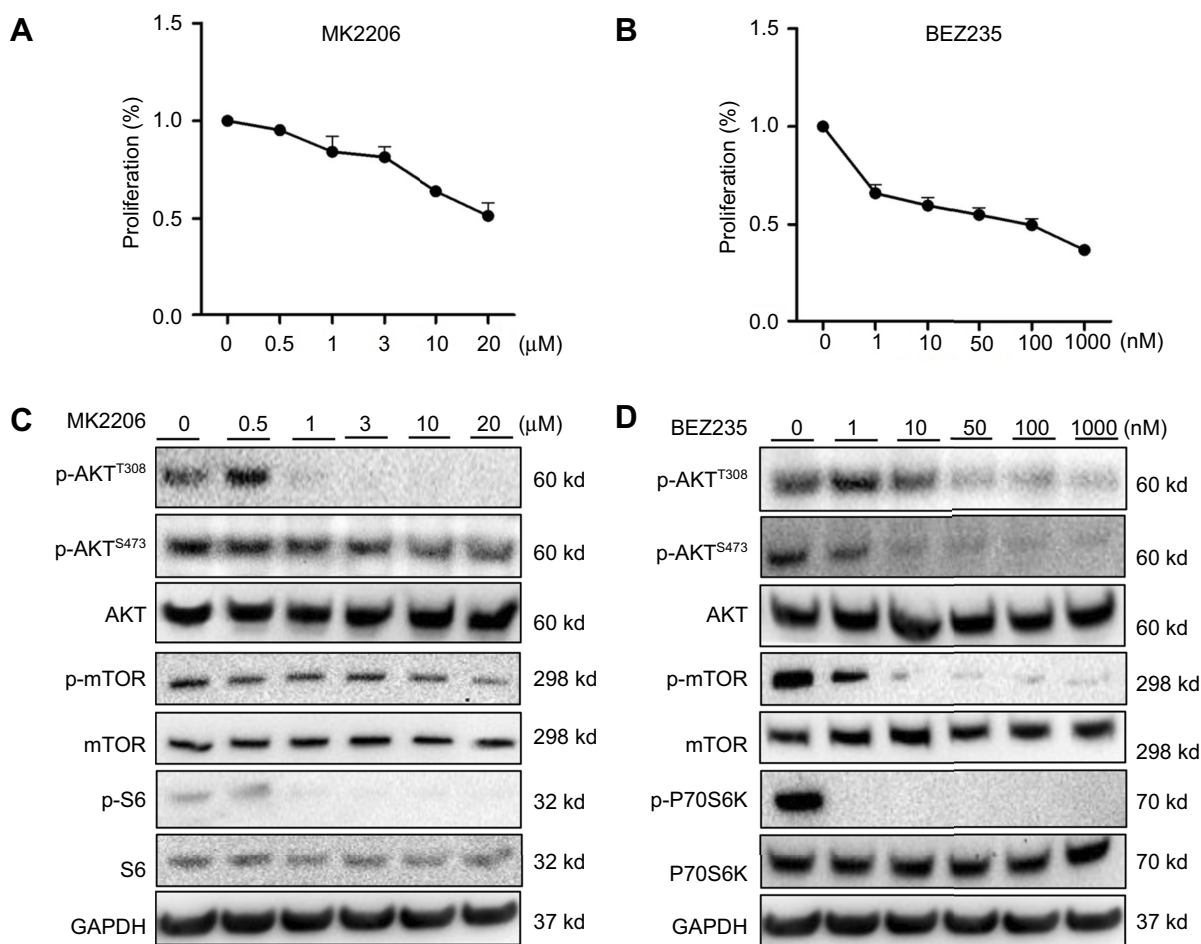
We next determined whether MK2206 or BEZ235 increased apoptosis in esophageal cancer cells. Cell apoptosis rates were measured after treatment with three different doses of MK2206 (3, 10, or 20 μM) and BEZ235 (10, 100, or 1000 nM) for 24 hrs. Results showed that both MK2206 and BEZ235 induced cell apoptosis dose-dependently. The 3 μM

dose of MK2206 and the 10 nM dose of BEZ235 both independently induced significant apoptosis in KYSE150 cells (Figure 3A and B). We then performed Western blot analyses to examine the changes in expression of apoptosis-related proteins MCL-1 and caspase-3 cleavage. The decreased MCL-1 and increased cleaved-caspase-3 were in line with increased apoptosis after MK2206 or BEZ235 treatment (Figure 3C and D). Similar results were found in KYSE 70, KYSE 270, and KYSE 410 cell lines (data not shown).

### MK2206 and BEZ235 induce cell cycle arrest

Cells were examined using the same doses in the apoptosis assay. As indicated in Figure 4A and B, MK2206 or BEZ235 treatment resulted in the accumulation of KYSE150 in the G2/M phase. Western blot analyses showed that cell cycle checkpoint proteins SKP2, CDK2 and cyclin D1 were decreased after MK2206 or BEZ235 treatment (Figure 4C and D). Similar results were found in KYSE 70, KYSE 270 and KYSE 410 cell lines (data not shown).





**Figure 2** MK2206 and BEZ235 inhibit cell proliferation and phosphorylation of AKT, PI3K, and mTOR in KYSE 150. **(A)** MK2206 inhibits cell proliferation in a dose-dependent manner; **(B)** BEZ235 inhibits cell proliferation in a dose-dependent manner; **(C)** MK2206 inhibits phosphorylation of AKT, PI3K, and mTOR; and **(D)** BEZ235 inhibits phosphorylation of AKT, PI3K, and mTOR. The values are expressed as mean; bars,  $\pm$ SE.

## MK2206/BEZ235 combination enhances inhibition of cell proliferation

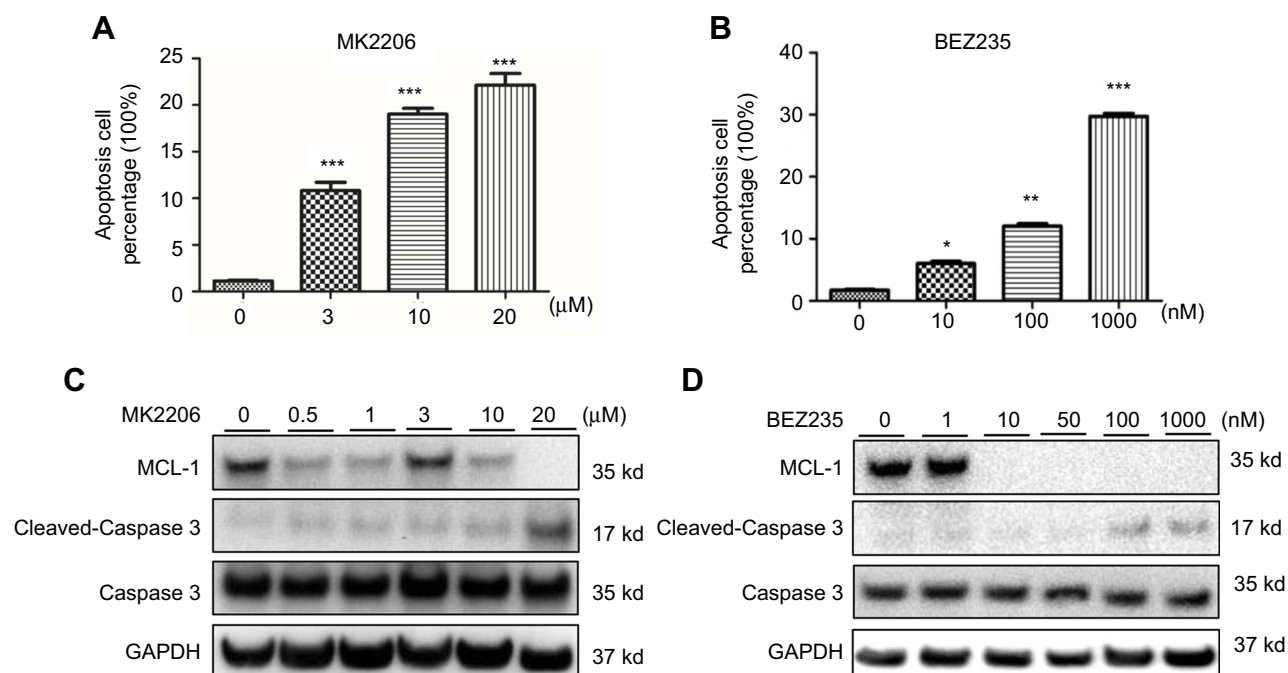
Given that the combination of inhibitors targeted more than one molecule in a signaling pathway, we examined the efficiency of the combination of MK2206/BEZ235 in esophageal cancer cells. We compared the cell proliferation among four treatment regimens: 1) 3  $\mu$ M MK2206+100 nM BEZ235; 2) 20  $\mu$ M MK2206; 3) 100 nM BEZ235; and 4) 1000 nM BEZ235. Our results showed that the combination of MK2206 and BEZ235 significantly inhibited cell proliferation compared with the high dose of MK2206 or BEZ235 alone in KYSE 150 (Figure 5A). Western blot analyses further showed that expression of p-AKT<sup>T308</sup>, p-AKT<sup>S473</sup>, and p-mTOR decreased more by MK2206 plus BEZ235 compared to MK2206 or BEZ235 alone (Figure 5B).

Similar results were found in KYSE 70, KYSE 270, and KYSE 410 cell lines (data not shown).

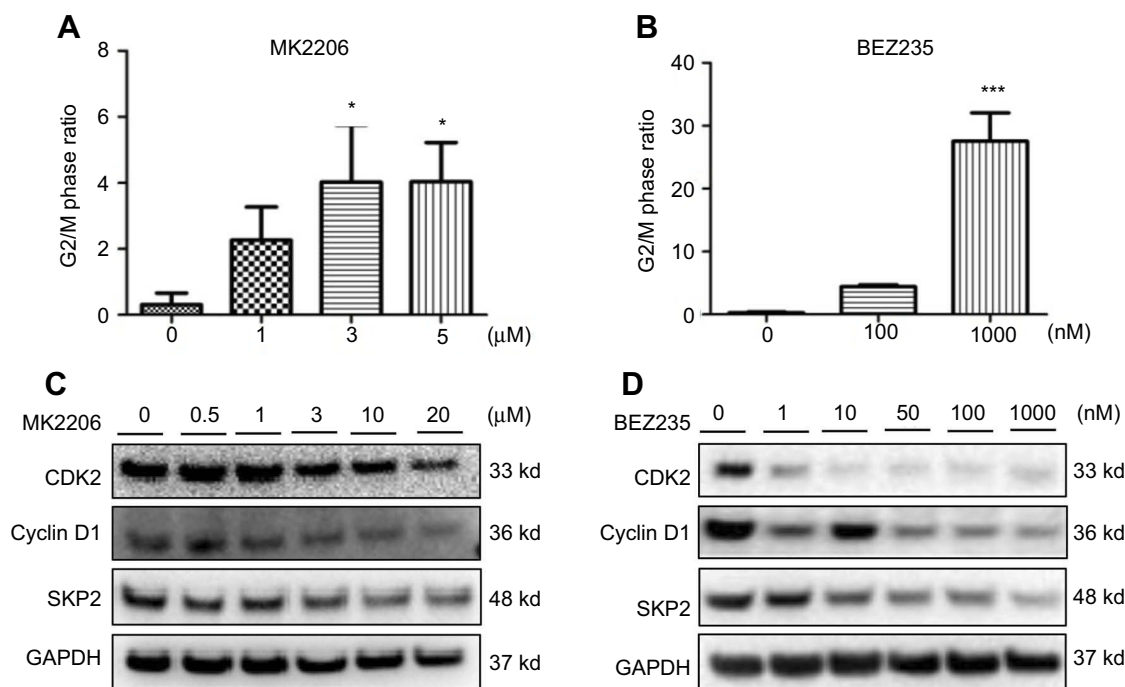
## MK2206/BEZ235 combination increases apoptosis and cell cycle arrest relative to individual agent

We next determined whether the combination of MK2206 and BEZ235 induces apoptosis and cell cycle arrest. Results showed that 20  $\mu$ M MK2206, 1000 nM BEZ235, and the combination of 3  $\mu$ M MK2206 plus 100 nM BEZ235 induced cell apoptosis by 22.5%, 30.8%, and 42.3% in KYSE 150, respectively (Figure 5C).

In cells treated with MK2206 or BEZ235 alone, we showed an increased number of cells in G2/M phase (Figure 5D). We compared the cells in G2/M phase after the combination treatment to the single drug



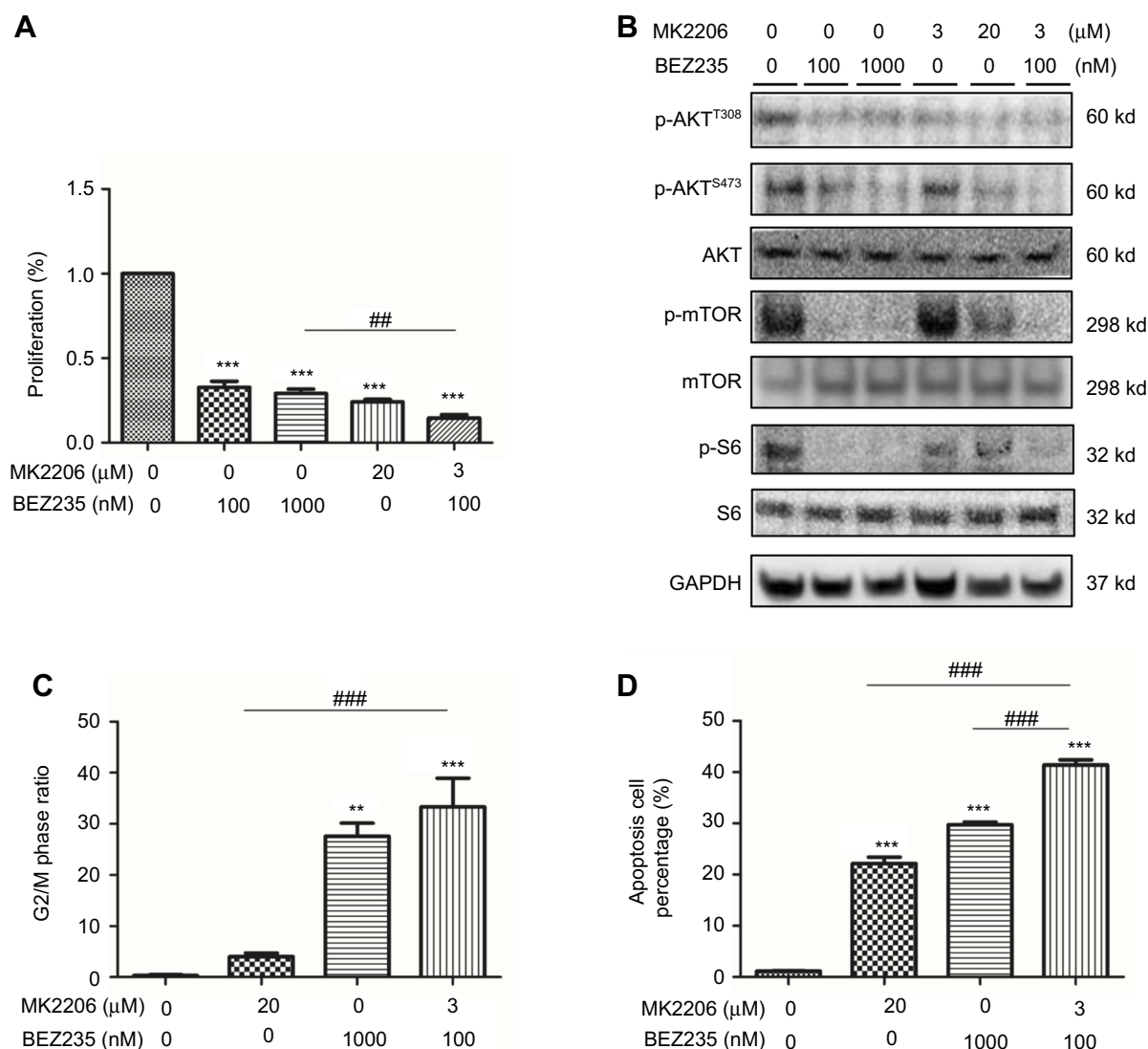
**Figure 3** MK2206 and BEZ235 induce apoptosis and apoptosis-related proteins in KYSE 150 in a dose-dependent manner. **(A)** MK2206 induces apoptosis; **(B)** BEZ235 induces apoptosis; **(C)** MK2206 reduces MCL-I expression and induces cleaved-caspase 3; and **(D)** BEZ235 reduces MCL-I expression and induces cleaved-caspase 3. The values are expressed as mean; bars,  $\pm$ SE. \* $P$ <0.05; \*\* $P$ <0.01; \*\*\* $P$ <0.001.



**Figure 4** MK2206 and BEZ235 induce G2/M accumulation and cell cycle-related proteins in KYSE 150 in a dose-dependent manner. **(A)** MK2206 induces G2/M accumulation; **(B)** BEZ235 induces G2/M accumulation; **(C)** MK2206 reduces SKP2, CDK2, and cyclin D1; and **(D)** BEZ235 reduces SKP2, CDK2, and cyclin D1. The values are expressed as mean; bars,  $\pm$ SE. \* $P$ <0.05; \*\*\* $P$ <0.001.

treatments. Our data showed that 20 μM MK2206, 1000 nM BEZ235, and 3 μM MK2206+100 nM BEZ235 led to 5.2%, 28.7%, and 33.5% of the cells accumulated in

the G2/M phase, respectively. Similar results were found in KYSE 70, KYSE 270, and KYSE 410 cell lines (data not shown).



**Figure 5** Combination of MK2206 and BEZ235 has better antitumor effects than MK2206 and BEZ235 alone. **(A)** The combination of MK2206 and BEZ235 at lower dose inhibits cell proliferation than MK2206 or BEZ235 alone at higher dose; **(B)** the combination of MK2206 and BEZ235 at lower dose inhibits AKT phosphorylation at Thr308 and Ser473 better than MK2206 or BEZ235 alone at higher dose; **(C)** the combination of MK2206 and BEZ235 at lower dose induces apoptosis better than MK2206 or BEZ235 alone at higher dose; and **(D)** the combination of MK2206 and BEZ235 at lower dose induces G2/M accumulation better than MK2206 or BEZ235 alone at higher dose. The values are expressed as mean; bars,  $\pm$ SE. \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; ### $P < 0.01$ ; #### $P < 0.001$ .

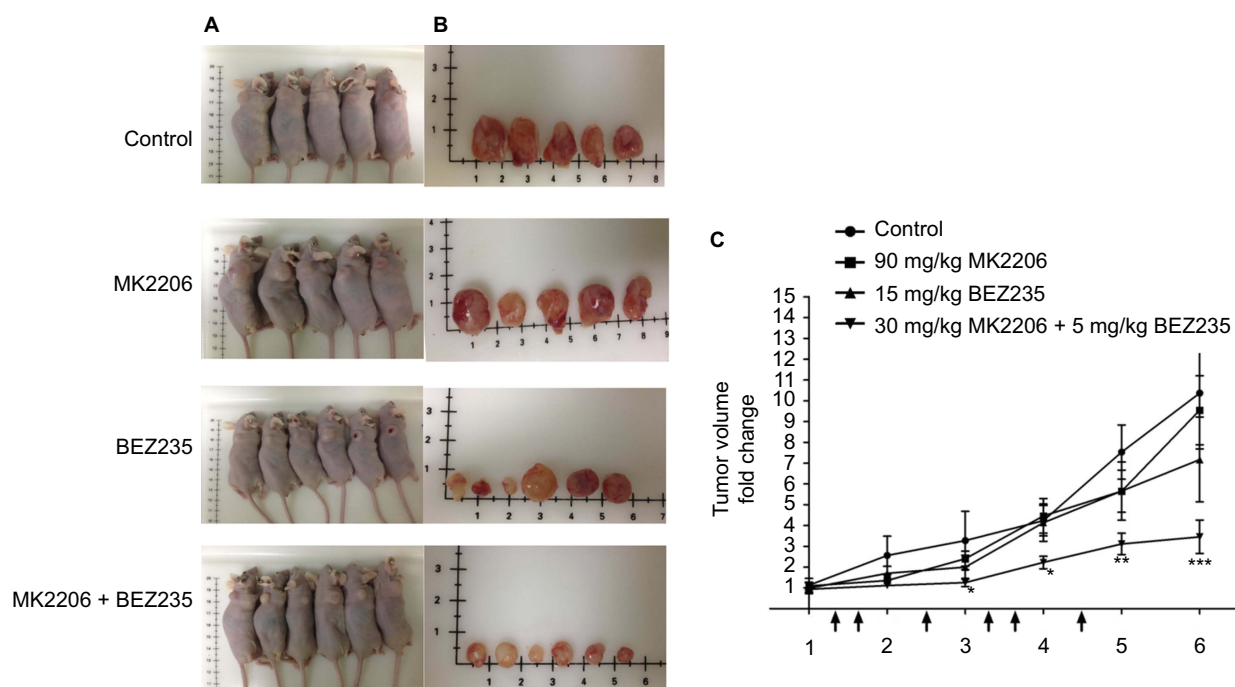
## MK2206/BEZ235 combination effectively suppresses in vivo xenograft growth relative to individual agent

We also examined whether the MK2206/BEZ235 combination had better antitumor effects than the single inhibitor in a KYSE 150 xenograft mouse model in vivo. As shown in Figure 6, MK2206 or BEZ235 alone only showed a slight inhibition of tumor growth, which was not significant compared to the control animals. The combination of MK2206 and BEZ235 at lower dose, however, significantly inhibited tumor growth compared to the control animals. Combined with the in vitro study, these results

verify the efficacy of the combination of MK2206 and BEZ235 on apoptosis and tumor inhibition in esophageal SCC.

## Discussion

This study evaluated the roles of two small molecular inhibitors, MK2206 and BEZ235, which target the PI3K/AKT/mTOR pathway, in esophageal SCC. Results highlight that there is high expression and activation of key components in the PI3K/AKT/mTOR pathway in both human esophageal SCC cell lines and NMBA-induced rat esophageal SCC model. Furthermore, results show



**Figure 6** Combination of MK2206 and BEZ235 has better antitumor effects than MK2206 or BEZ235 alone in xenograft animal model. (A and B) The images of the tumors in nude mice and (C) the tumor volume in control and treatment groups. The values are expressed as mean; bars,  $\pm$ SE. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

that MK2206 and BEZ235 inhibit proliferation, increase apoptosis, and induce cell cycle arrest in esophageal SCC. More importantly, the combination of MK2206 and BEZ235 exhibits enhanced antitumor effects as compared to MK2206 or BEZ235 alone. The results are consistent in both in vivo and in vitro analyses.

Previous studies have identified that the PI3K/AKT/mTOR pathway is critical in carcinogenesis.<sup>7</sup> Phosphorylations at Thr308 and at Ser473 are both necessary for fully AKT activation.<sup>33,34</sup> Activated AKT can phosphorylate multiple substrates to regulate cellular processes, including proliferation, survival, motility, angiogenesis, and metabolism/glucose homeostasis.<sup>7</sup> Our results show that both MK2206 and BEZ235 inhibit esophageal SCC cell proliferation, increase apoptosis and that is associated with G2/M cell cycle accumulation. MK2206 and BEZ235 may inhibit AKT phosphorylations at Thr308 and Ser473. To explore the molecular mechanism associated with these findings, we detected several AKT downstream effectors related to cell cycle and apoptosis. Specifically, the expression levels of SKP2, MCL-1, and cyclin D1 are suppressed by MK2206 or BEZ235 treatment. SKP2 is overexpressed in oral cancer, breast cancer, gastric cancer, and prostate cancer. It displays M-phase-promoting function and regulates the degradation of p21 and p27, which is required for the activation of cyclin-

dependent kinase 1 (CDK1).<sup>35,36</sup> MCL-1 inhibits apoptotic cell death through ligation of the proapoptotic Bcl-2 family member Bak.<sup>37</sup> MCL can lead to constitutive transcription of cyclin D1, one of the cell cycle regulatory switches in actively proliferating cells.<sup>38</sup> Apoptosis and cell proliferation are linked by cell-cycle regulators such as cyclins and CDKs. Our data indicate that MK2206 and BEZ235 impact cell cycle and apoptosis, at least in part, through modulating cell-cycle regulators.

MK2206 binds to PH domain and it does not interact with the ATP-binding pocket to prevent the AKT membrane translocation and subsequent activation.<sup>9,39</sup> We found that the phosphorylation of AKT at Thr308 is significantly reduced by MK2206. S6 is a ribosomal protein, the downstream effector of AKT, which is inhibited by MK2206 in esophageal cancer cells. The phosphorylation of AKT at Ser473 was only slightly reduced by MK2206. As we mentioned earlier, phosphorylation at Thr308 and at Ser473 is both necessary for full AKT activation. A slight inhibition of phosphorylation at Ser473 may explain the less encouraging clinical benefits observed for single treatment of MK2206. In the present study, the approach targeting more than one molecule in the same pathway shows enhanced efficacy. As a dual PI3K-mTOR inhibitor, BEZ235 inhibits the activity of PI3K and mTORC1 as well as mTORC2. P70S6K, the downstream effector of



mTOR is significantly decreased by BEZ235, which indicates mTOR inhibition role of BEZ235 in esophageal SCC. Our results show that compared with MK2206, BEZ235 inhibits AKT phosphorylation at Thr308 and Ser473, and decreases the phosphorylation of mTOR significantly. mTOR is a key regulator of protein translation and cell proliferation, energy metabolism by phosphorylating its downstream markers p70S6k and 4E-binding protein (4EBP).<sup>40,41</sup> About 25% of the esophageal SCC patients have overexpression of p-mTOR,<sup>40,42</sup> which has been found to be an independent prognostic factor for poor survival in esophageal SCC.<sup>43</sup> PI3K/AKT and mTOR signaling networks are closely related and are subject to complex cross-talk and feedback interactions.<sup>44</sup> The activity of mTORC1 is tightly regulated by PI3K/AKT and MAPK signaling pathways. After being fully activated, AKT mediates the phosphorylation of TSC2 to inhibit the TSC1/2 complex then activates mTORC1 signaling.<sup>34</sup> The inhibition of mTOR could induce insulin receptor substrate-1 expression to activate AKT.<sup>21,45</sup> When p-mTOR is blocked in parallel with the inhibition of AKT phosphorylations at Thr308 and Ser473, BEZ235 at nmol level shows better inhibition on cell proliferation, apoptosis, and cell cycle arrest than MK2206 at mmol level. The xenograft mouse model analysis also shows reduced tumor size in the BEZ235 treatment group than MK2206 treatment group, although the difference did not reach statistical significance.

When combined with other chemotherapy or target therapy, MK2206 and BEZ235 have shown encouraging efficacy in several cancers both in vitro and in vivo.<sup>24–27</sup> In this study, we investigated the efficacy of MK2206/BEZ235 combination in esophageal SCC. Our study verifies increased antitumor efficacy in MK2206/BEZ235 combination compared with MK2206 or BEZ235 alone in vitro and in vivo. The combination of MK2206 and BEZ235 shows better inhibition effects on phosphorylation of AKT at Thr308 and Ser473.

In summary, the PI3K/AKT/mTOR pathway is implicated in esophageal SCC. Blocking the PI3K/AKT/mTOR pathway by MK2206 and BEZ235 could inhibit cell proliferation, induce cell apoptosis and cause cell cycle arrest through the inhibition of AKT phosphorylation, and downstream effectors SKP2, MCL-1, and cyclin D1. The combination of MK2206 and BEZ235 showed better antitumor effects than MK2206 or BEZ235 alone. The inhibition on phosphorylation ATK on Thr308 and Ser473 is critical for the antitumor effects in esophageal SCC. The better efficacy

of MK2206/BEZ235 combination is associated with the inhibition of phosphorylation ATK on Thr308 and Ser473. This study importantly has clinical application for MK2206/BEZ235 combination treatment in esophageal SCC.

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## Disclosure

The authors report no conflicts of interest in this work.

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