

# BYL719, a selective inhibitor of phosphoinositide 3-Kinase $\alpha$ , enhances the effect of selumetinib (AZD6244, ARRY-142886) in KRAS-mutant non-small cell lung cancer

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**Summary** *Purpose* KRAS is frequently mutated in non-small cell lung cancers (NSCLC), resulting in activation of the MEK/ERK pathway. Because there are currently no drugs that target oncogenic KRAS, MEK inhibitors have been tested clinically as a possible treatment option for patients with NSCLC. However, KRAS-mutant cancers exhibit resistance to MEK inhibitors. Therefore, a combinational strategy is necessary for effective therapy. To address this, we investigated the therapeutic effects of combining selumetinib, a MEK1/2 inhibitor, with BYL719, a PI3K $\alpha$  inhibitor. *Methods* We evaluated the effects of selumetinib and BYL719 in vitro and in vivo in NSCLC cell lines. *Results* The combination of BYL719 and selumetinib resulted in synergistic cytotoxic activity compared with the single agents alone in KRAS-mutant NSCLC cells. At the molecular level, we found that AKT activation strongly influenced the sensitivity of KRAS-mutant NSCLC cells to selumetinib. Selumetinib upregulated phospho-AKT and phosphorylated BAD at ser136, which is responsible for intrinsic drug resistance in KRAS-mutant NSCLC cells. In contrast, inhibition of the PI3K/AKT pathway by BYL719 hindered selumetinib-induced BAD phosphorylation and increased the antitumor efficacy of selumetinib. Furthermore, selumetinib and BYL719 combination therapy showed synergy in the suppression of A549 xenograft tumor growth. On analysis of the pharmacodynamics, selumetinib and BYL719 together resulted in effective inhibition

of both p-ERK and p-AKT expression in tumor tissue. *Conclusion* Taken together, these data suggest that combination treatment with selumetinib and BYL719 is a promising therapeutic approach to overcoming resistance to MEK inhibitors.

**Keywords** MEK inhibitor · PI3K $\alpha$  inhibitor · KRAS · Non-small cell lung cancer · Combination therapy

## Introduction

KRAS is a member of the RAS family of oncogenes, a collection of small guanosine triphosphate (GTP)-binding proteins that activate intracellular signaling pathways to regulate cell proliferation, differentiation, and survival [1]. KRAS mutations predominantly arise as single amino acid substitutions at residues G12, G13, or Q61, and confer transforming capacity. Activating KRAS mutations are found in 15–30 % of patients with non-small cell lung cancer (NSCLC) and are associated with poor responses to conventional treatment regimens [2–4]. These mutations lead to impaired GTPase activity and constitutive activation of KRAS effectors, including RAF/MEK/ERK and the phosphoinositide 3-kinase (PI3K)/AKT signaling pathway [1, 4]. However, there are currently no effective therapies for mutant KRAS, since KRAS itself has proven difficult to directly target with small molecules [5, 6].

An alternative approach to targeting mutant KRAS involves using small molecule inhibitors that target KRAS effectors (e.g. RAF/MEK/ERK). As the MEK/ERK pathway is critical for cell growth, survival, and transformation, MEK inhibitors have been actively investigated as a treatment option for various cancers [7, 8]. Selumetinib is an orally active MEK inhibitor that inhibits both basal and induced ERK phosphorylation in numerous types of cancer cells [9]. Although selumetinib has shown activity in vitro and in several tumor xenografts models, a subset of tumors, particularly

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NSCLC, are resistant to this inhibition [7, 10–12]. Resistance to inhibition of the MEK/ERK pathway may result from mutations in MEK1 or activation of the PI3K pathway [13, 14]. Recently, it has been reported that a high level of AKT activation is associated with resistance to the MEK inhibitor selumetinib in cases of lung cancer, and that dual inhibition of the AKT and ERK pathways increased antitumor activity by selumetinib [15–17]. In addition, several studies have also demonstrated that simultaneous inhibition of MEK and PI3K leads to tumor regression in *KRAS*-mutant cancer cell models [18–20].

PI3K/AKT signaling pathways are also activated in a variety of cancers including NSCLC, and PI3K is the second best-characterized RAS effector [21, 22]. PI3K converts PIP<sub>2</sub> to PIP<sub>3</sub>, which activates AKT to promote cell growth and proliferation. The PI3Ks are organized into three classes (I–III). Class I PI3Ks are further divided into three class IA isoforms, PI3K $\alpha$ , PI3K $\beta$ , and PI3K $\delta$ , whereas PI3K $\gamma$  constitutes class IB. As PI3K $\alpha$  is ubiquitously expressed and has been linked to AKT activation, PI3K $\alpha$  appears to be an ideal target for drug development [22]. In this regard, targeting PI3K represents a promising combination treatment strategy in *KRAS*-mutant NSCLC.

In this study, we investigated the efficacy of a combination of selumetinib and BYL719, a novel specific PI3K $\alpha$  inhibitor with pharmacologic and biologic properties [23, 24]. Here, we present evidence that combining these two inhibitors in cases of *KRAS*-mutant NSCLC results in enhanced antitumor activity in vitro as well as in vivo. These findings support the potential clinical use of this drug combination in patients with *KRAS*-mutant NSCLC.

## Materials and methods

### Cell lines and cell cultures

Human NSCLC cell lines A549 and NCI-H2009 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in RPMI-1640 containing 10 % FBS at 37 °C in a humidified atmosphere containing 5 % CO<sub>2</sub>.

### Chemical reagents and antibodies

Selumetinib (AZD6244, AstraZeneca) and BYL 719 (Novartis) were dissolved in dimethyl sulfoxide (DMSO) to a 10 mM concentration and stored in small aliquots at –20 °C until further use. Antibodies to p-Akt (Ser473), Akt, p-ERK1/2 (Thr202/Thy204), ERK1/2, p-BIM (Ser69), BIM, p-BAD (Ser136), BAD, PARP, and  $\beta$ -actin were purchased from Cell Signaling Technology. Anti-14-3-3 was purchased from Santa Cruz Biotechnology.

### Cell viability assay and combination index analysis

Cells were seeded on a 96-well plate, allowed to adhere overnight, and treated with a dilution series of test compounds for 72 h. Cell viability was determined using a Cell Counting Kit (Dojindo Molecular Technologies) according to the manufacturer's instructions. Combination index (CI) data were generated using CompuSyn (Combosyn). A CI of 1 indicated an additive drug interaction, whereas a CI of <1 was synergistic and a CI of >1 was antagonistic.

### Cell cycle and apoptosis assay

Cell cycle analysis was performed after 24 h of treatment. Cells were fixed with ice-cold 70 % ethanol, stained with propidium iodide, and analyzed by flow cytometry (BD Biosciences).

### Western blot analysis

Cells were lysed in PRO-PREP™ protein extraction solution (iNtRON Biotechnology) supplemented with a protease and phosphatase inhibitor cocktail (Sigma). Equal amounts of protein were then subjected to SDS-PAGE (NuPAGE 4–12 % Bis-Tris Gel; Invitrogen) and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were then incubated with the aforementioned antibodies and developed by ECL.

### Immunoprecipitation

Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer supplemented with a protease and phosphatase inhibitor cocktail (Sigma). Equal amounts of protein were incubated with BAD, 14-3-3, or immunoglobulin G control antibodies overnight at 4 °C to allow the formation of immune complexes. Immune complexes were subsequently precipitated with protein A/G agarose beads (Santa Cruz Biotechnology) and analyzed using Western blot analyses with the aforementioned antibodies.

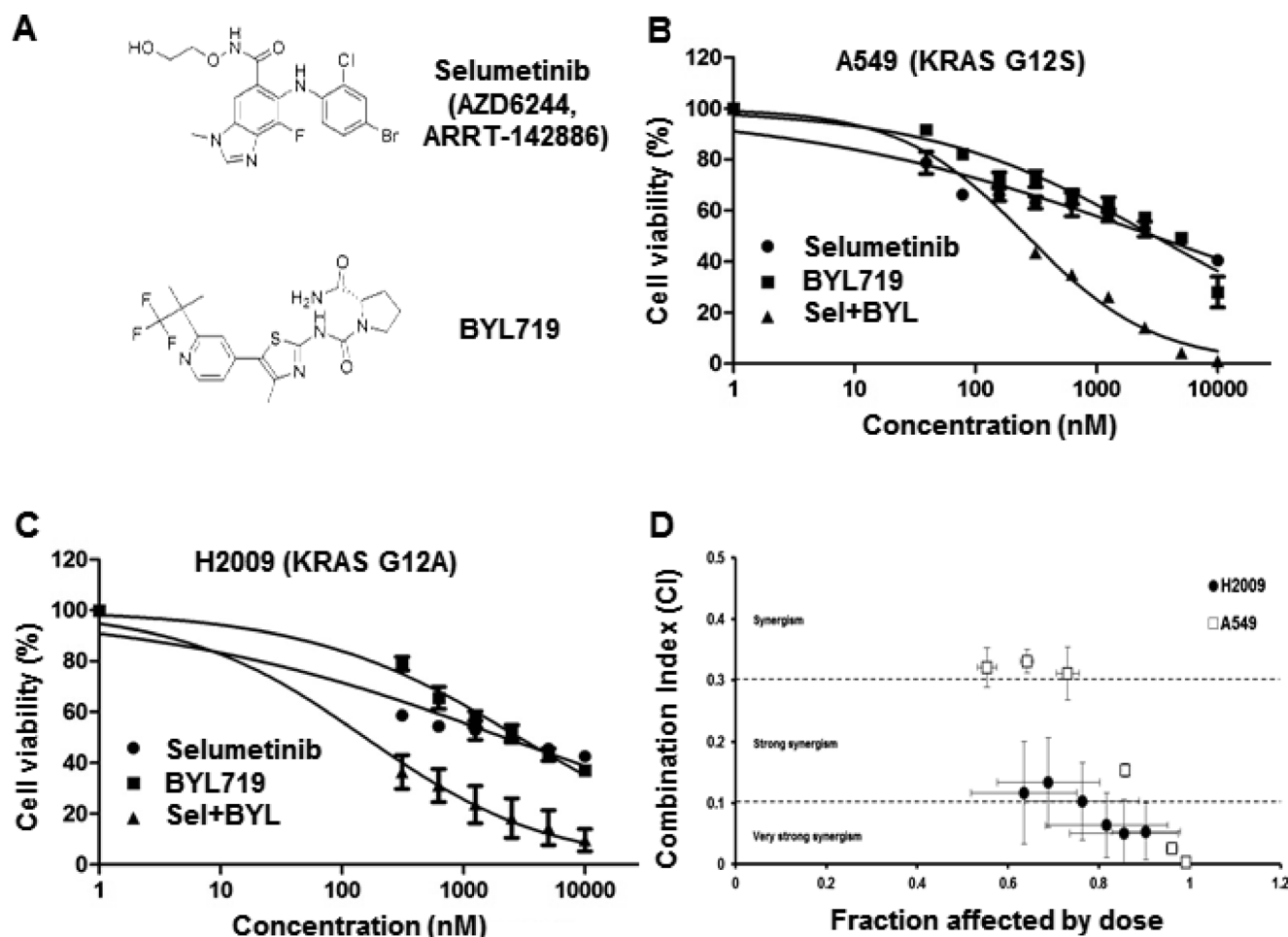
### Xenograft studies

All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the Samsung Biomedical Research Institute (SBRI). SBRI is an Association of Assessment and Accreditation of Laboratory Animal Care International (AAALAC International) accredited facility and abides by the Institute of Laboratory Animal Resources (ILAR) guidelines. Six-week-old BALB/c female nude mice were injected subcutaneously with A549 cells ( $5 \times 10^6$ ). When tumor sizes reached approximately 100 mm<sup>3</sup>, mice were randomized into four groups of 10–12 mice each. Each group of mice was dosed via daily oral

gavage with vehicle, selumetinib (25 mg/kg/d, twice per day), BYL719 (25 mg/kg/d), or a combination of both. The selumetinib vehicle contained 0.5 % hydroxypropyl methylcellulose plus 0.1 % Tween-80, and the BYL719 vehicle contained 0.5 % hydroxypropyl methylcellulose. All mice received both vehicles. Tumor volumes were determined using calipers and were calculated using the following formula:  $V = (L \times W^2)/2$  (L, Length; W, width). Toxicity was monitored according to weight loss. Three days after treatment was initiated, the tumors were removed for Western blot analysis 3 h after the last dose of medication or vehicle was administered.

#### Statistical analysis

All numerical data are presented as the mean  $\pm$  SEM. Statistical analyses were carried out using GraphPad Prism (GraphPad software). *P*-values  $<0.05$  were considered to be statistically significant.



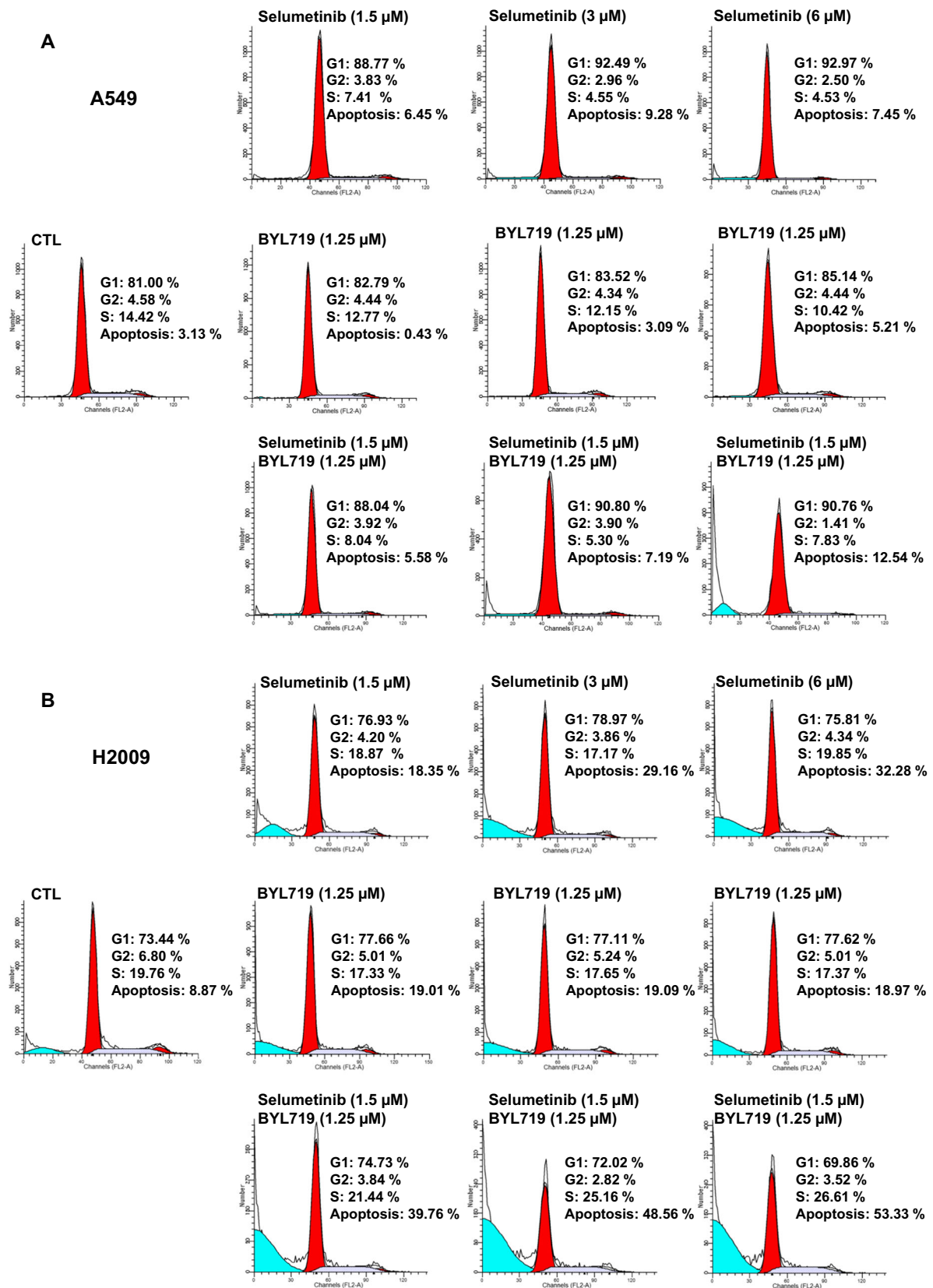
**Fig. 1** A combination of selumetinib and BYL719 was more efficacious than selumetinib alone in the treatment of *KRAS*-mutant lung cancers. **a** Structure of selumetinib and BYL719. **b** A549 and **c** NCI-H2009 cells were treated with various concentrations of selumetinib, BYL719, or selumetinib plus BYL719 for 72 h. Cell viability was measured using

## Results

### PI3K $\alpha$ and MEK inhibitors exhibit synergistic effects

To test whether a PI3K $\alpha$  inhibitor in combination with a MEK inhibitor have a synergistic effect on MEK inhibitor-resistant cells, we first evaluated the sensitivity to MEK inhibitors in *KRAS*-mutant NSCLC cell lines (A549 and NCI-H2009) by assessing cell viability. Cells were treated with the selumetinib (Fig. 1a) at concentrations ranging from 1 to 10,000 nM for 72 h. Both cell lines had a selumetinib IC<sub>50</sub> of  $\geq 1$   $\mu$ M (Fig. 1b and c). It was previously reported that 1  $\mu$ M was close to the average plasma concentration of selumetinib achieved in patients at the maximum tolerated dose for this agent [7]. Thus, we considered these cell lines to be resistant to selumetinib. We next examined the effects of a PI3K $\alpha$  inhibitor, BYL719, at increasing concentrations (Fig. 1a) from 1 to 10,000 nM. BYL719 reduced cell viability in a manner similar to selumetinib in both cell lines (Fig. 1b and c). The combination

CCK-8 assays. *Results* represent the mean of three independent experiments. *Error bars*, SEM. **d** Combination index plots of selumetinib and BYL719 in A549 and NCI-H2009 cells. Most of the data points were presented with CI  $<1$ , indicating that the selumetinib-BYL719 combination is synergistic



**Fig. 2** Treatment with selumetinib and BYL719 synergistically induced cell apoptosis in *KRAS*-mutant cells. **a** A549 and **b** H2009 cells were treated with selumetinib, BYL719, or a combination of the two at the indicated concentrations for 24 h. Flow cytometric histograms of cells

stained with propidium iodide were analyzed using Modifit software (BD Bioscience). Percentages of cells in apoptotic sub-G<sub>1</sub>, G<sub>1</sub>, S, and G<sub>2</sub>/M phases are presented



of selumetinib and BYL719 significantly reduced cell viability in A549 and NCI-H2009 cells (Fig. 1b and c). To determine whether the antitumor effects obtained with different doses of selumetinib and BYL719 combinations were synergistic, we evaluated the combination index (CI). After treatment with various concentrations (0.125–4  $\mu\text{M}$ ) of selumetinib, BYL719, and selumetinib plus BYL719, the CI was measured for each cell line. As shown in Fig. 1d, the combination treatment produced a strong synergistic effect in both cell lines (CI of A549: 0.003–0.321 and CI of H2009: 0.002–0.005).

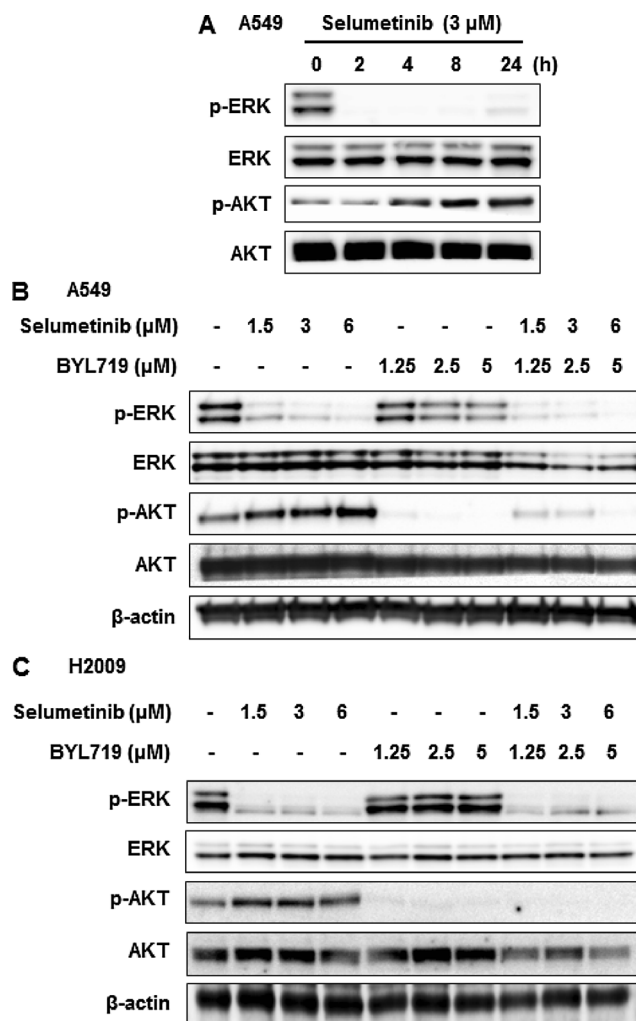
#### BYL719 enhances selumetinib-induced apoptosis

To test whether this reduction in cell viability was attributed to induction of apoptosis or growth arrest, we analyzed apoptosis and cell cycle profiles using A549 (Fig. 2a) and H2009 (Fig. 2b) cells. After 24 h of treatment, apoptosis was assessed by the detection sub- $G_1$  peak. We found that cell death was increased in a dose-dependent manner in combination-treated cells. The apoptosis was more significantly increased in H2009 cells. In the cell-cycle profile,  $G_1$  phase cells were increased by selumetinib and BYL719 in both cells. However the combination of selumetinib and BYL719 increased the  $G_1$  phase population in A549 cells. Thus, the combination treatment induces both cell death and cell-cycle arrest at the  $G_1$  phase.

#### BYL719 enhances selumetinib-induced apoptosis through BAD

A recent study reported that PI3K/AKT activation mediates resistance to MEK inhibitors [16, 25, 26]. Based on those results, we investigated the mechanism by which selumetinib and BYL719 cooperate to induce apoptosis in *KRAS*-mutant NSCLC cells. A549 cells were treated with 3  $\mu\text{M}$  selumetinib and levels of p-ERK and p-AKT were measured at 2, 4, 8, and 24 h. As expected, ERK phosphorylation was inhibited by selumetinib, but AKT phosphorylation was induced in a time-dependent (Fig. 3a) and dose-dependent (Fig. 3b) manner. BYL719 efficiently inhibited selumetinib-induced AKT phosphorylation in A549 (Fig. 3b) and H2009 (Fig. 3c). At high concentrations ( $\geq 2.5$   $\mu\text{M}$ ), BYL719 caused a slight reduction of p-ERK in A549 cells after 24 h of treatment. Similar to our results, it has been reported that lapatinib along with BYL719 inhibits ERK phosphorylation [27], and others have shown that PI3K inhibition actually enhances ERK activation in BT474 cells [28].

To determine the direct mediators of selumetinib/BYL719-induced apoptosis, we determined protein levels of pro-apoptotic Bcl-2 family members (BIM and BAD). Consistent with prior studies, suppression of p-ERK by selumetinib led to increased levels of BIM<sub>EL</sub>, and, to a lesser extent, of BIM<sub>L</sub> and

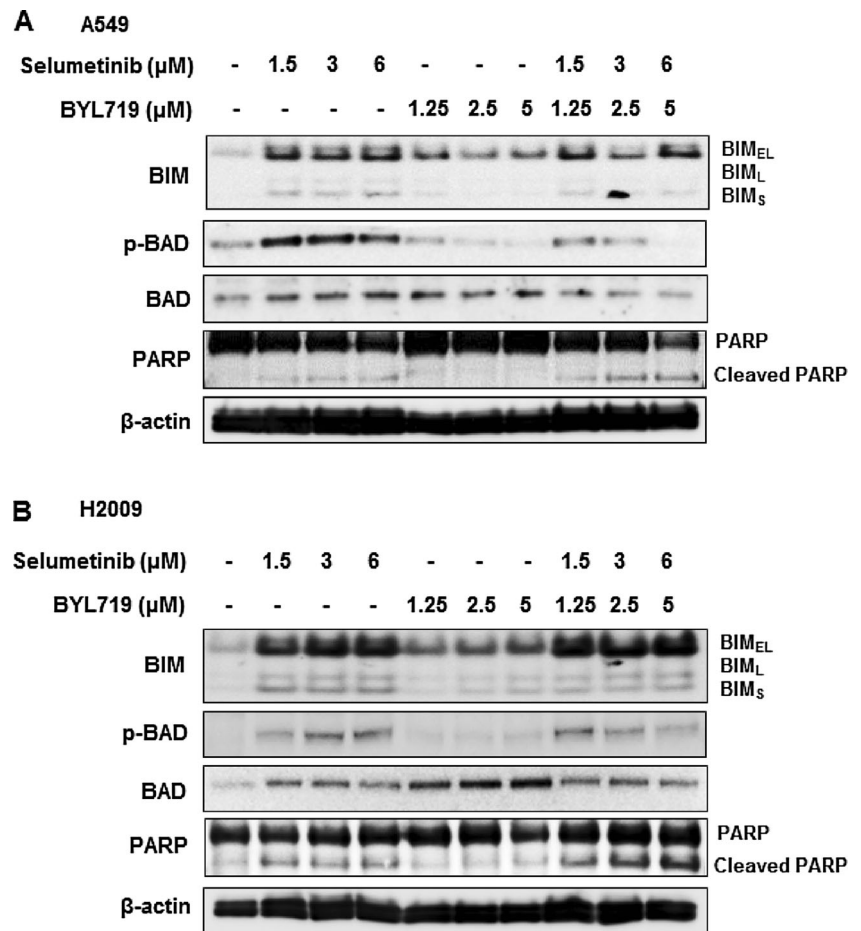


**Fig. 3** Pathway modulation in the *KRAS*-mutant cell line following MEK and PI3K inhibition. **a** A549 cells were treated with 3  $\mu\text{M}$  selumetinib for 0, 2, 4, 8, or 24 h. Cell lysates were immunoblotted for levels of p-ERK and p-Akt. **b** A549 and **c** H2009 Cells were treated with the indicated concentrations of selumetinib or BYL719 for 24 h. Western blot analysis was carried out to determine the levels of p-ERK and p-Akt using specific antibodies.  $\beta$ -actin was used as a loading control

BIM<sub>S</sub> which are well-known targets of MAPK signaling [29–33]. However, selumetinib /BYL719 did not induce obvious changes in BIM expression compared to selumetinib alone in A549 and H2009 cells (Fig. 4). Interestingly, p-BAD (ser136) was significantly induced by selumetinib alone, and we also observed a clear synergistic inhibition of p-BAD with the combination of selumetinib and BYL719 (Fig. 4). BAD can be phosphorylated at ser112 and ser136 by the MEK/ERK and the PI3K/AKT pathways, respectively [25], and this phosphorylation inactivates its pro-apoptotic function [34]. In accordance with reduced BAD phosphorylation, PARP cleavage was increased with combination therapy (Fig. 4).

Phosphorylation of BAD has been reported to promote BAD translocation from the mitochondria into the cytosol, interaction with the scaffold protein 14-3-3, and dissociation

**Fig. 4** Simultaneous suppression of PI3K sensitizes *KRAS*-mutant cells to MEK inhibitors. **a** A549 and **b** H2009 cells were treated with selumetinib, BYL719, or a combination of the two for 24 h. *Western blot* analysis was performed using the indicated antibodies to determine the biochemical response

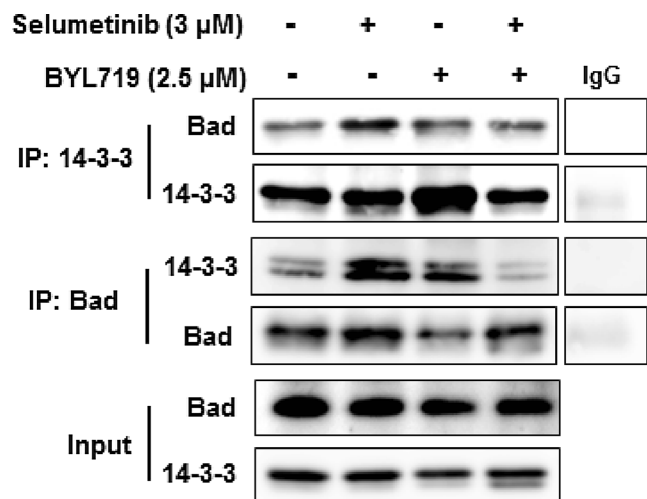


from Bcl-XL [35, 36, 34]. Immunoprecipitation of BAD revealed that when p-BAD levels were induced by selumetinib, a proportional increase in the amount of 14-3-3 associated with BAD was observed. However, BYL719 disrupted the association of BAD with 14-3-3 following p-BAD induction by selumetinib (Fig. 5). These results suggest that selumetinib-induced BAD phosphorylation may lead to the loss of pro-apoptotic activity and thereby contribute to the resistance of *KRAS*-mutant NSCLC cells to MEK inhibitors. Thus, BYL719 in combination with selumetinib effectively promotes apoptosis by blocking the ability of BAD to bind 14-3-3.

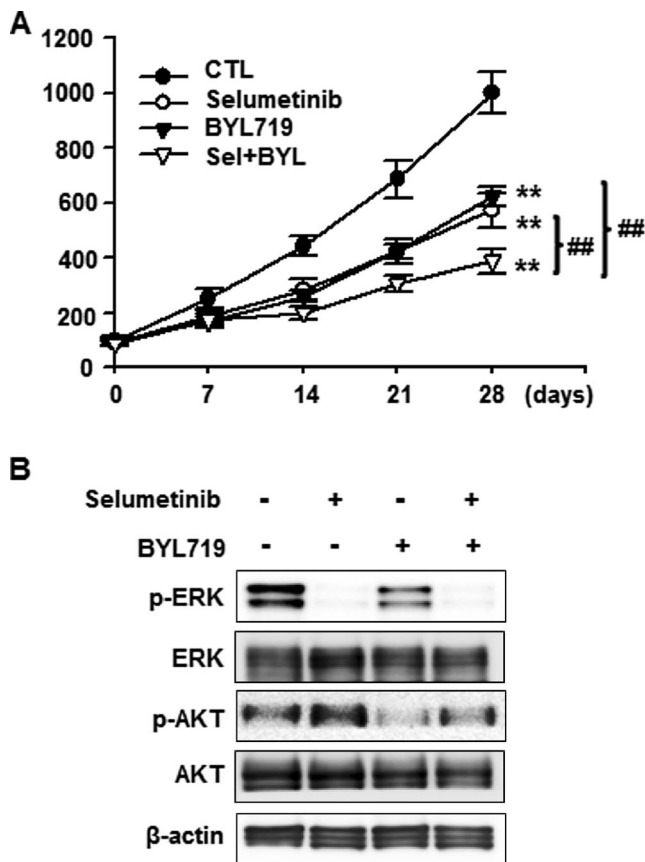
A combination of selumetinib and BYL719 leads to enhanced antitumor efficacy in the *KRAS*-mutant xenograft model

In light of the synergistic effects of the combination therapy observed *in vitro*, we investigated the efficacy of combining selumetinib and BYL719 *in vivo*. Mice bearing A549 xenografts were treated with vehicle, selumetinib, BYL719, or a combination of selumetinib and BYL719. As shown in Fig. 6a, selumetinib and BYL719 alone had a modest effect on the inhibition of tumor growth (55 and 65 % growth

compared with vehicle, respectively). The combination therapy resulted in a significantly enhanced reduction in tumor



**Fig. 5** BYL719 inhibits selumetinib-induced BAD association with 14-3-3. A549 cells were treated for 24 h with selumetinib, BYL719, or a combination of the two. Immunoprecipitation was performed with BAD, 14-3-3, or immunoglobulin G (IgG) control antibodies. BAD-associated 14-3-3, 14-3-3-associated BAD, total BAD, and total 14-3-3 were analyzed using Western blot. IgG was used as a *negative control*



**Fig. 6** The combination of selumetinib and BYL719 resulted in enhanced antitumor efficacy in a *KRAS*-mutant xenograft model. **a** A549 tumor cells were subcutaneously injected into the flank of Balb/c nude mice. Drug treatments began after the tumor reached a volume of  $\sim 100 \text{ mm}^3$  (day 0). All mice were treated with vehicle, selumetinib (25 mg/kg/d), BYL719 (25 mg/kg/d), or selumetinib plus BYL719 by oral gavage for 5 days each week for a total of 4 weeks. Error bars, SEM. ( $n=10\text{--}12$ ) \*\*,  $P<0.01$  for all groups vs. vehicle; ##,  $P<0.01$  for selumetinib+BYL719 vs. selumetinib or BYL719 alone. **b** Xenografts from A were harvested for Western blot analysis on treatment day 3, approximately 3 h after their last treatment. Tumors were subsequently lysed and Western blot analysis was performed for p-Akt, Akt, p-ERK, ERK, and  $\beta$ -actin

volume when compared with selumetinib alone ( $P=0.06$ ), BYL719 alone ( $P=0.0009$ ), or vehicle ( $P=0.00001$ ) at day 28. Furthermore, no significant differences in body weight were found between the four groups during the 28 days of treatment, and no obvious toxicities were observed (data not shown). To investigate the pharmacodynamic effects of combining selumetinib and BYL719 in vivo, A549 xenografts were collected 3 h after the final dose of selumetinib and/or BYL719 was administered on day 3. Tumor lysates were analyzed for p-ERK and p-AKT by immunoblotting. Consistent with the in vitro findings above, combination therapy inhibited selumetinib-induced AKT activation (Fig. 6b). Taken together, these results suggest that the combination of selumetinib and BYL719 has a synergistic therapeutic effect on *KRAS*-mutant NSCLC.

## Discussion

Advances in the understanding of genetic alterations in NSCLC have given rise to therapies that target specific oncogenic pathways. At present, however, there are no effective therapies for *KRAS*-driven lung cancers, and mutations in *KRAS* are associated with poor prognosis and resistance to both adjuvant therapy and targeted EGFR TKIs [37, 38]. Since MEK/ERK and PI3K/AKT are two of the key pathways in the transformational activity of mutant *KRAS*, the combined inhibition of MEK and PI3K may constitute an anti-*KRAS* therapeutic strategy.

Selumetinib is a small-molecule inhibitor that is selective for MEK. It has been investigated in clinical trials for use in the treatment of advanced NSCLC, melanoma, and a variety of other cancers [7, 11, 39]. BIM, a member of the intrinsic apoptosis pathway, has been shown to mediate selumetinib-induced apoptosis in patients with lung cancer [29, 30, 33]. Although intrinsic and acquired resistance to this compound occurs, especially in NSCLC, the precise underlying mechanism remains controversial. Several specific genetic mutations have been suggested as predictors of sensitivity to MEK inhibitors, such as *BRAF*, *MEK1*, *PI3K $\alpha$* , and *PTEN* [40, 18, 41, 13]. However, more evidence indicates that feedback activation of compensatory pathways is responsible for resistance to MEK inhibitors, and identifying and targeting those pathways may help overcome resistance and induce synergistic antitumor effects [21, 42, 25, 12].

Previous studies have reported that resistance to selumetinib in lung cancer cells is mediated by AKT activation [17, 26, 14]. It has been shown that dual-agent combination therapy using selumetinib and the AKT inhibitor MK2206 resulted in synergistic effects on cell viability in vitro as well as tumor growth in *KRAS*-driven NSCLC tumors in vivo [17]. In another report, down-regulation of *PIK3CA* resensitized cells with co-occurring *KRAS* and *PIK3CA* mutations to MEK inhibition [14]. Collectively, these findings demonstrate that PI3K pathway activation is a major mechanism of resistance that impairs the efficacy of MEK inhibitors in *KRAS*-mutant cancers. Thus, we hypothesized that dual inhibition of MEK and PI3K could synergistically induce antitumor effects in *KRAS*-mutant NSCLC.

In this study we demonstrated that the combination of the MEK inhibitor selumetinib and the PI3K $\alpha$  inhibitor BYL719 can induce synergistic inhibition of tumor growth in vitro and in vivo. Previous studies reported that selumetinib-resistant cells expressed higher levels of p-Akt than sensitive cells, and that A549 cells showed persistent activation of AKT and were resistant to selumetinib [15, 16]. In the same manner, due to increased AKT phosphorylation, selumetinib caused only a partial suppression of cell viability in *KRAS*-mutant A549 cells in this study. In contrast, in the presence of both selumetinib and BYL719, AKT signaling was strongly



suppressed and a highly synergistic induction of apoptosis was observed. The PI3K/AKT and MEK/ERK pathways converge on the pro-apoptotic Bcl-2 homology 3 (BH3) family of proteins, which regulate apoptosis [30]. It has been recently reported that induction of p-BAD is required for selumetinib -resistance [25]. Therefore, we examined protein levels of this family and found that the combination of selumetinib and BYL719 led to a complete inhibition of phosphorylation of the serine 136 residue on the pro-apoptotic protein BAD. These findings provide evidence that selumetinib -induced S136 site phosphorylation occurs through the PK3K/AKT pathway. It has previously been shown that BAD is a downstream target that can integrate EGFR/ERK and PI3K/AKT signaling in PTEN-negative/EGFR amplified tumors, and that knocking down BAD significantly attenuates the effects of combined pathway inhibition in breast and colon cancer cells [43, 19]. In addition, phosphorylation of BAD sequesters it in 14-3-3 protein complexes at the plasma membrane, thereby inhibiting its pro-apoptotic action [35, 44, 45]. In this study, the combination of selumetinib and BYL719 induced synergistic inhibition of p-BAD along with increased PARP cleavage, which is consistent with previous reports.

In summary, we have shown that treatment with BYL719, a PI3K $\alpha$  specific inhibitor which is currently in early phase clinical trials, is able to overcome resistance to MEK inhibition in selumetinib-resistant cancer cell lines both in vitro and in vivo. These results suggest that the PI3K/AKT pathway could be activated in human cancer cells with intrinsic resistance to selective MEK inhibitors, and represent a rationale for the investigation of combined MEK and PI3K inhibitors for treatment of NSCLC.

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**Ethical standards** All experiments were performed in accordance with the regulation of the Institutional Animal Care and Use Committee (IACUC) at the Samsung Biomedical Research Institute (SBRI) and with the applicable laws and regulations of Korea.

**Conflict of interest** The authors declare that they have no conflict of interest.

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