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Enhancing therapeutic efficacy of the MEK inhibitor, MEK162, by blocking autophagy or inhibiting PI3K/Akt signaling in human lung cancer cells

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Abstract

Human non-small cell lung cancer (NSCLC) displays activated MEK/ERK signaling due to a high frequency of K-Ras mutation and is thus a potential candidate for MEK-targeted therapy. The current study focuses on demonstrating the activity of MEK162, a MEK inhibitor under clinical testing, against NSCLC and exploring possible mechanism-driven strategies to enhance its therapeutic efficacy. MEK162 inhibits the growth of human NSCLC cell lines with varied potencies through induction of G1 cell cycle arrest and apoptosis. Moreover, it induces autophagy and accordingly the combination of MEK162 with the autophagy inhibitor, chloroquine, synergistically inhibits the growth of NSCLC cells and enhances apoptosis. MEK162 activates Akt signaling while effectively inhibiting MEK/ERK signaling. Accordingly, the combination of MEK162 and BKM120, a pan PI3K inhibitor, abrogates induced Akt activation and significantly augments therapeutic efficacy against the growth of NSCLC cells both *in vitro* and *in vivo*. Hence our findings warrant further evaluation of these rational combinations in the clinic.

Keywords

MEK162; BKM120; autophagy; lung cancer

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Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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1. Introduction

Ras, particularly K-Ras, is frequently mutated in human non-small cell lung cancer (NSCLC), especially in tumors with adenocarcinoma histology. Ras mutations often result in activation of the RAF/MEK/ERK pathway [1]. Thus, agents that target this signaling pathway may have potential for treatment of this type of cancer and hence have actively been investigated pre-clinically and clinically. A number of MEK inhibitors such as selumetinib (AZD6244) and trametinib (GSK1120212) have progressed into clinical trials [1]. A randomized phase II trial of docetaxel with and without selumetinib revealed that the combination had numerically superior overall survival and a statistically significant improvement in progression-free survival and objective response rate, albeit with some concern regarding increased adverse effects [2]. Despite a strong scientific rationale, the activity of MEK inhibitors appears to be similar in NSCLC patients with and without K-Ras mutations [1].

MEK162 (binimetinib; ARRY-162 or ARRY-438162) is an orally available non-ATP-competitive allosteric inhibitor of MEK1/2. MEK162 has shown effectiveness in inhibiting the growth of N-Ras mutant melanomas from patients in culture [3] and pancreatic cancer cells [4]. Moreover MEK162 combined with other targeted agents has displayed promising anticancer activity in different preclinical models. For example, the combination of MEK162 with a PKC inhibitor results in sustained inhibition of MEK/ERK signaling and enhances therapeutic efficacy against the growth of uveal melanoma cells both *in vitro* and *in vivo* [5]. The combination of MEK162 and imatinib (Gleevec), a tyrosine kinase inhibitor, synergistically suppresses the growth of gastrointestinal stromal tumor *in vitro* and *in vivo* [6]. The combination of MEK162 and perifosine, an Akt inhibitor, synergistically inhibits the growth of lung cancer cells *in vitro* and *in vivo* [7]. In the clinic, MEK162 has shown some activity in patients with N-Ras-mutated melanoma [8].

The preclinical activity of MEK162 against the growth of human NSCLC cells, the modulatory effects of MEK162 on the MEK/ERK and other signaling pathways such as phosphoinositide 3-kinase (PI3K)/Akt and mammalian target of rapamycin (mTOR) pathways, and the potential impact of genetic alterations on cell responses to MEK162 have not been studied and thus were the focus of this study. Moreover, we were interested in developing mechanism-driven combinations to enhance the therapeutic efficacy of MEK162 based on our understanding of the biology of MEK162 in NSCLC cells. Hence we also studied the efficacy of MEK162 combined with autophagy or PI3K inhibition on the growth of NSCLC cells *in vitro* and *in vivo*.

2. Materials and Methods

2.1 Reagents

MEK162 and BKM120 were supplied by Novartis Pharmaceuticals Corporation (East Hanover, NJ). AZD6244 was purchased from Selleckchem (Houston, TX). Chloroquine and rabbit polyclonal anti-actin antibody were purchased from Sigma Chemical Co. (St. Louis, MO). Antibodies against ERK, p-ERK1/2 (T202/Y204), Akt, p-Akt (S473), p-S6 (S235/S236), p-4EBP1 (T37/46), 4EBP1, p-70S6K (T389), 70S6K, PRAS40, p-PRAS40 (T246),

p-GSK3 α / β (S21/9), p-90RSK2 (S380), caspase-8 and poly(ADP-ribose)polymerase (PARP), respectively, were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Mouse monoclonal S6 and p90RSK2 antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse monoclonal cyclin D1 antibody was purchased from Dako (Carpinteria, CA). Rabbit polyclonal microtubule-associated protein light chain 3 (LC3) antibody was purchased from Novus Biologicals, Inc. (Littleton, CO).

2.2. Cell lines and cell culture

The human NSCLC cell lines used in this study were described previously [9]. A549/LC3-YFP cells were described previously [10]. 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% CO₂ and 95% air.

2.3. Growth inhibition 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 [11]. Combination index (CI) for drug interaction (e.g., synergy) was calculated using the CompuSyn software (ComboSyn, Inc.; Paramus, NJ).

2.4. Colony formation assay

The effects of the given drugs on colony formation on plates were measured as previously described [12].

2.5. Cell cycle analysis

Cells were harvested after a given treatment and stained with propidium iodide for cell cycle analysis as described previously [13, 14].

2.6. Detection of apoptosis

Apoptosis was evaluated with a Cell Death Detection ELISA^{Plus} kit (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's instructions. Caspase and PARP cleavage were also detected by Western blot analysis as described below as additional indicators of apoptosis.

2.7. Detection of autophagy

Autophagosomal membrane-bound type II LC3 (LC3-II) was detected by Western blotting and fluorescence imaging as an indication of autophagy.

2.8. Western blot analysis

Preparation of whole cell protein lysates and Western blot analysis were described previously [15, 16].

2.8. Lung cancer xenograft and treatments

Animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Emory University. Five to 6 week old female athymic (nu/nu) mice were

ordered from Charles River Labs (Wilmington, MA) and housed under pathogen-free conditions in microisolator cages with laboratory chow and water *ad libitum*. A549 cells at 3×10^6 in serum-free medium were injected subcutaneously into the flank region of nude mice. When tumors reached a size of approximately 100 mm³, the mice were randomized into four groups (n = 6/group) according to tumor volumes and body weights for the following treatments: vehicle control, MEK162 (5 mg/kg/day, og), BKM120 (7.5 mg/kg/day; og), and their combination. Tumor volumes were measured using caliper measurements once every two days and calculated with the formula $V = \pi(\text{length} \times \text{width}^2)/6$. At the end of the treatments, mice were sacrificed with CO₂. The tumors were then removed, weighed, and frozen in liquid nitrogen. Certain portions of tumor tissues were homogenized in protein lysis buffer for preparation of whole-cell protein lysates for Western blotting to detect the given proteins. The statistical significance of differences in tumor sizes between two groups was analyzed with two-sided unpaired Student's *t* tests (for equal variances) or with Welch's corrected *t* test (unequal variances) by use of Graphpad InStat 3 software. Results were considered to be statistically significant at $P < 0.05$.

3. Results

3.1. MEK162 inhibits the growth of human NSCLC cell lines with varied potencies

To determine whether MEK162 effectively inhibits the growth of human NSCLC cells, we treated a panel of 14 NSCLC cell lines harboring different genetic mutations (Table 1) with varying concentrations (0.04–10 μM) of MEK162 in comparison with another MEK inhibitor AZD6244 for 3 days and then measured changes in cell number. Both MEK162 and AZD6244 reduced cell numbers in a concentration-dependent manner with IC₅₀s ranging from 0.015 μM to >10 μM. Clearly MEK162 and AZD6244 have varying potencies against the growth of different cell lines. We arbitrarily divided these cell lines into resistant and sensitive groups using an IC₅₀ of 5 μM as a cutoff. We compared cell sensitivities with genetic mutations in these cell lines and found no apparent relationship between cell sensitivity and mutation of p53, PTEN, PIK3CA, EGFR, LKB1 or CDKN2A. However we found that 61% (7/11) of cell lines sensitive to both MEK162 and AZD6244 possessed mutant K-Ras or N-Ras (H1299) in comparison with 0% (0/3) of the resistant cell lines ($P < 0.05$ with Fisher's exact test). This suggests that Ras mutant NSCLC cells may respond better to MEK162 or AZD6244.

We also detected the basal levels of certain signaling pathway proteins (particularly of the MEK/ERK, mTOR and Akt signaling pathways) in the tested NSCLC cells (Figure 1B) to explore whether their levels may impact cell sensitivity to MEK162. Unexpectedly, we found that 2 of 3 (67%) resistant cell lines (i.e., H596 and EKVX) had very high levels of p-ERK1/2, whereas only 3 of 11 (27%) sensitive cell lines (i.e., H1792, H23 and H157) expressed comparably high levels of p-ERK1/2. Interestingly, some of the sensitive cell lines displayed very high levels of p-Akt (e.g., H1299, A549 and H157) in comparison to some resistant cell lines (e.g., H596 and EKVX) which had very low levels of p-Akt. There was not a clear association between p-S6 levels and the sensitivity to MEK162.

3.2. MEK162 induces G1 arrest, apoptosis and autophagy

To explore the mechanism by which MEK162 suppresses the growth of NSCLC cells, we then examined the effect of MEK162 on cell cycle and apoptosis. MEK162 at relatively low concentration ranges 1 μ M (e.g., 0.5 and 1 μ M) induced G1 arrest in three sensitive NSCLC cell lines (Figure 2A). At 1 μ M and particularly 3 μ M, MEK162 also induced increased DNA fragmentation (Figure 2B) and cleavage of caspase-8, caspase-3 and PARP (Figure 2C) in H157 and A549 cells, but only minimally in H522 cells, indicating that MEK162 also induces apoptosis in some sensitive NSCLC cell lines. Hence MEK162 inhibits the growth of some NSCLC cells through induction of both cell cycle arrest and apoptosis. We noted that MEK162 at 3 μ M caused minimal increase in both DNA fragmentation and caspase cleavage in the sensitive H522 cells although they underwent G1 arrest, suggesting that MEK inhibits the growth of this cell line primarily through induction of cell cycle arrest.

Autophagy is another important mechanism that regulates cell survival or death [17]. We next determined whether MEK162 induces autophagy in these cell lines by detecting increase in type II LC3 (LC3-II), an autophagosome membrane-bound form of LC3. We found that LC3-II levels were increased in every tested cell line, especially in H522 cells, in Western blot analysis (Figure 2C). In agreement, we detected increased punctuate staining of LC3 in A549 cells stably expressing LC3-YFP after treatment with MEK162 (Figure 2D). Collectively it is clear that MEK162 induces autophagy in NSCLC cells.

3.3. MEK162 suppresses MEK/ERK and mTOR signaling while activating Akt signaling

Given that MEK162 is a MEK inhibitor, we determined whether it indeed inhibits the MEK/ERK signaling pathway in NSCLC cells. We found that, even at 0.5 μ M, MEK162 sufficiently decreased the levels of both p-ERK1/2 and p-RSK2 (a well-known substrate of ERK1/2) in both sensitive (H522 and A549) and resistant (EKVX) NSCLC cell lines (Figure 3A), indicating that MEK162 effectively inhibits MEK/ERK signaling regardless of cell sensitivity.

It has been recently suggested that suppression of mTOR complex 1 (mTORC1) signaling is associated with cell response to MEK inhibition in B-Raf-mutant melanoma cells [18]. Therefore, we evaluated whether MEK162 suppressed mTORC1 signaling in human NSCLC cells. Similar to its effect on p-ERK1/2, MEK162 at 0.5 μ M effectively decreased p-70S6K levels in all 3 tested cell lines. Interestingly, it reduced the levels of p-S6, a well-known substrate of p-70S6, only in the two sensitive cell lines tested, A549 and H522. MEK162 weakly decreased p-4EBP1 levels in A549 cells, but had no effect on p-4EBP1 levels in H522 and EKVX cell lines (Figure 3). Hence, it appears that suppression of S6, but not p-70S6K or 4EBP1, is associated with increased cell sensitivity to MEK162.

MEK inhibition has been suggested to activate Akt signaling [19, 20]. Hence we also examined the effects of MEK162 on Akt signaling in NSCLC cells. We found that MEK162, particularly at lower concentration ranges (e.g., 0, 5 and 1 μ M), increased phosphorylation levels of Akt and its downstream substrates PRAS40 and GSK3 in the 3 tested cell lines irrespective of their sensitivity to MEK162 (Figure 3), indicating that

MEK162 at low concentration ranges activates Akt signaling in NSCLC cells. We noted that MEK162 at 3 μ M had reduced or no effect on increasing p-Akt levels. Whether this phenomenon suggests that high concentration of MEK162 may activate or inhibit additional pathway(s) that prevent Akt phosphorylation needs further investigation.

Cyclin D1 is a key protein that promotes cell cycle transition from G1 to S phase through positively regulating the activity of cyclin-dependent kinase [21]. We found that MEK162 at the tested concentration ranges (0.5 to 3 μ M) decreased cyclin D1 only in the two sensitive cell lines, A549 and H522 (Figure 3). This finding suggests that suppression of cyclin D1 is an important mechanism contributing to MEK162-induced G1 arrest.

3.4. Blockade of autophagy augments the inhibition of cell growth and induction of apoptosis by MEK162

Autophagy can be a cell survival or death mechanism [17, 22]. We observed that H522 cells, which did not undergo apoptosis upon MEK162 treatment, had relatively higher basal and induced (by MEK162) levels of LC3-II than two other sensitive cell lines, A549 and H157, which underwent apoptosis post MEK162 treatment (Figure 2). Therefore we speculated that autophagy induced by MEK162 is probably a protective or survival mechanism and that blockage of autophagy may enhance the growth-inhibitory effects of MEK162. To test this hypothesis, we compared the effects of MEK162 on cell growth and apoptosis in the absence and presence of the lysosomal protease inhibitor, chloroquine, a widely used autophagy inhibitor, in H522 and A549 cell lines. The combination of MEK162 and chloroquine was more potent than either of the agents alone in inhibiting cell growth (Figure 4A). The CIs for the combinations of chloroquine (using 20 μ M as an example) with different concentrations of MEK162 were < 1, indicating synergistic effects. As presented above, MEK162 exerted minimal apoptosis-inducing effect in the H522 cell line. However the combination of MEK162 and chloroquine substantially increased cleavage of caspase-8, caspase-3 and PARP (Figure 4B) and DNA fragmentation (Figure 4C) in comparison with the effects caused by MEK162 or chloroquine alone. Moreover, we detected higher levels of LC3-II in cells exposed to the combination of MEK162 and chloroquine than those treated with MEK162 alone. This result indicates that MEK162 induces autophagic flux, further supporting the notion that MEK162 induces autophagy. Thus, inhibition of autophagy clearly enhances the potency of MEK162 in inhibiting cell growth and inducing apoptosis.

3.5. Inhibition of PI3K/Akt signaling synergizes with MEK162 to inhibit the growth of NSCLC cells in vitro and in vivo

To evaluate the impact of Akt signaling activation on the growth-inhibitory efficacy of MEK162, we treated A549 and H522 cell lines with MEK162 alone, BKM120 (a pan-PI3K inhibitor) alone, or their combination. As expected, we detected elevation of p-Akt levels in cells treated with MEK162 alone, but not in cells exposed to the combination of MEK162 and BKM120 (Figure 5A), indicating that the combination abrogates Akt activation induced by MEK162. Following this finding, we then determined the impact of the MEK162 and BKM120 combination on the growth of NSCLC cells. As shown in Figure 5B, the combination of MEK162 and BKM120 was more effective than each agent alone in inhibiting the growth of the tested cell lines. The CIs were < 1 for most combinations,

indicating synergistic inhibition of the growth of NSCLC cells. In a long-term colony formation assay, which allows us to repeat treatments, MEK162 and BKM120 at the tested concentration alone partially suppressed colony formation of NSCLC cells; however the combination almost eliminated colony formation and drastically reduced colony numbers (Figure 5C). Thus, it is clear that the combination is much more effective than either single agent in inhibiting the colony formation and growth of NSCLC cells. In agreement, the combination of MEK162 and BKM120 was also more potent than each single agent in inducing G1 arrest (Figure 5D). Thus, we believe that enhanced G1 arrest contributes to augmented growth-inhibitory effects induced by the combination of MEK162 and BKM120.

Because of the promising growth-inhibitory effects of the MEK162 and BKM120 combination against NSCLC cells *in vitro*, we lastly validated the efficacy of the combination against the growth of NSCLC tumors in mice. Whereas MEK162 and BKM120 alone at the tested doses only weakly inhibited the growth of A549 xenografts as measured by both tumor sizes and weights, the combination of MEK162 and BKM120 significantly inhibited the growth of A549 xenografts ($P < 0.01$ compared with vehicle control, MEK162 alone or BKM120 alone group) (Figure 6A). The combination did not significantly affect the body weight of mice, suggesting that the combination does not accordingly enhance toxicity. These data indicate that the combination indeed displays augmented anti-cancer activity without compromising safety *in vivo*.

By analyzing tumor tissues, we detected reduced levels of p-ERK1/2 in tumors exposed to MEK162 or MEK162 combined with BKM120 (Figure 6D), indicating that MEK162 treatment indeed inhibits its targeted MEK/ERK signaling *in vivo*. We also detected increased levels of p-Akt in xenograft tumors treated with MEK162, but decreased levels of p-Akt in tumors exposed to either BKM120 or the combination of MEK162 and BKM120 (Figure 6D), indicating that BKM120 activates Akt signaling *in vivo*, which can be abrogated by the presence of BKM120. p-S6 levels were effectively reduced in tumors treated with MEK162, BKM120 or their combination (Figure 6D).

4. Discussion

This study examined the growth inhibitory effects of MEK162 against a panel of 14 human NSCLC cell lines with different genetic mutations including Ras (K- and N-Ras), p53, LKB1, PTEN, PIK3CA, EGFR and CDKA2N (Table 1). These cell lines displayed varying degrees of sensitivity to MEK162, and another MEK inhibitor, AZD6244; i.e., some cell lines (e.g., H157 and H522) lines were intrinsically sensitive to MEK162 treatment, while others (e.g., H596 and EK VX) were intrinsically resistant (Figure 1). Whereas there was no clear relationship between cell sensitivity and the presence of other mutations including p53, LKB1, PTEN, PIK3CA, EGFR and CDKA2N, we demonstrated that all Ras-mutant cell lines were within the group of sensitive cell lines (61%; 7/11). Whether this suggests that Ras-mutant NSCLCs are likely respond better to MEK-targeted therapy needs further investigation. Our finding is, however, consistent with that of another study showing better sensitivity of K-Ras-mutant NSCLC cells to AZD6244 [23]. In other types of cancers, K-Ras or N-Ras mutations have been suggested to be associated with better response to MEK inhibitors [3, 24, 25].

A previous study showed no clear association between MEK and PI3K pathway activation and sensitivity to MEK inhibition by AZD6244 in NSCLC cells [23]. Another study using colon cancer cell lines found that cell lines resistant to AZD6244 exhibited low or no ERK activation and/or strong PI3K signaling [26]. However, a lack of correlation between cell sensitivity and ERK activation in colon cancer cells was also reported [27]. In our study, we found no clear association between cell sensitivity and baseline activation of MEK/ERK, PI3K/Akt or mTOR signaling in NSCLC cell lines (Figure 1B). Interestingly, we noted that some resistant cell lines (e.g., H596 and EKVX) possessed very high basal levels of p-ERK1/2 and very low levels of p-Akt, implying activated MEK/ERK signaling with suppressed PI3K/Akt signaling. In contrast, some sensitive cell lines (e.g., H358, H460, H1299 and A549) had relatively low basal levels of p-ERK1/2 and high levels of p-Akt. Hence it appears that the baseline activation status of MEK/ERK signaling in NSCLC cells poorly predicts the sensitivity or resistance of NSCLC cells to MEK162 or MEK-targeted therapy.

In this study, we also found that MEK162 effectively decreased the levels of both p-ERK1/2 and p-RSK2 with comparable potencies in both MEK162-resistant (EKVX) and sensitive (A549 and H522) cell lines. These data clearly indicate that MEK162 effectively inhibits MEK/ERK signaling; however the inhibition of this putative target may not account entirely for the mechanism by which MEK162 exerts its growth-inhibitory effect. There may be additional mechanism(s) beyond MEK inhibition. This important finding also suggests that inhibition of MEK/ERK signaling is unlikely to be a good pharmacodynamic marker to predict tumor response to MEK162.

A recent study has suggested that suppression of mTORC1 signaling is associated with cell response to MEK inhibition (e.g., by AZD6244) in B-Raf-mutant melanoma cells using p-S6 inhibition as a sole readout [18]. In our study, MEK162 did lead to decreased levels of p-70S6K, a well-known readout of mTORC1 suppression, in the tested cell lines although it had weak or no effect on decreasing p-4EBP1; but this effect was seen regardless of cell sensitivity to MEK162. Interestingly, the phosphorylation of S6, a well-known substrate of p-70S6K, was selectively inhibited by MEK162 in the sensitive cell lines, A549 and H522 (Figure 3). Hence, it is suppression of S6 phosphorylation, rather than inhibition of p-70S6K phosphorylation or mTORC1 signaling, that is associated with cell response to MEK162. In other words, our results do not support the role of mTORC1 inhibition in predicting response of NSCLC cells to MEK162. Moreover, our data also indicate that MEK162-induced suppression of S6 phosphorylation is unlikely secondary to inhibition of p70S6K, suggesting that there may be an additional kinase that phosphorylates S6 beyond p70S6K. p90RSK has been suggested to phosphorylate S6 at S235/236 [28]. In our study, MEK effectively suppressed phosphorylation of RSK2, but not S6, in EKVX cells (Figure 3), suggesting that suppression of ERK/RSK is also unlikely to be the mechanism underlying inhibition of S6 phosphorylation by MEK162.

We found that MEK162 induces both G1 cell cycle arrest and apoptosis in some sensitive cell lines (e.g., H157 and A549). Hence induction of both G1 arrest and apoptosis contributes to its growth-inhibitory effects against some NSCLC cells. We also noted an exceptional case in H522 cells, in which MEK effectively caused G1 arrest but only

minimally induced apoptosis, suggesting that MEK162 inhibits the growth of some NSCLC cells (e.g., H522) primarily via induction of G1 cell cycle arrest. The level of cyclin D1, a key protein promoting cell cycle transition from G1 to S phase [21], was selectively reduced in MEK162-sensitive cell lines (e.g., A549 and H522) (Figure 3). Thus suppression of cyclin D1 may be an important mechanism accounting for G1 arrest by MEK162.

Beyond G1 arrest and apoptosis, MEK162 also induces autophagy in NSCLC cells, as shown by the increased levels of LC3-II and LC3 puncta in treated cells (Figure 2). This phenotype occurred in all three tested sensitive cell lines. However, the basal and induced levels of LC3-II in H522 cells were higher than those in H157 and A549 cells that undergo apoptosis upon MEK162 treatment. Autophagy is a catabolic process involving the degradation and recycling of macromolecules and organelles and is often considered a survival mechanism in cancer cells that antagonizes or delays apoptosis in response to cancer therapeutic agents [17]. In our study, the combination of MEK162 and the autophagy inhibitor chloroquine synergistically inhibited the growth of NSCLC cells and clearly increased apoptosis even in H522 cells (Figure 4). Hence induction of autophagy by MEK162 in NSCLC cells is clearly a protective or survival mechanism that counteracts its antitumor efficacy. On the other hand, blockage of autophagy may be another effective strategy to enhance MEK162's anticancer efficacy.

In our study, we observed that MEK162 increased the levels of p-Akt, p-PRAS40 and p-GSK3 (Figure 3), demonstrating that MEK162 activates Akt signaling while inhibiting MEK/ERK signaling. This finding is in agreement with other studies showing that MEK inhibition activates Akt signaling [19, 20]. When combined with the PI3K inhibitor BKM120, MEK162-induced increase in Akt phosphorylation in NSCLC cells was blocked (Figure 5A). Moreover, the combination of MEK162 and BKM120 synergistically inhibited the growth of NSCLC cells *in vitro*, including enhanced effects on arresting cancer cells in G1 phase and on suppressing colony formation and growth (Figure 5). This enhanced growth-inhibitory effect was further validated *in vivo* using a lung cancer xenograft model in nude mice. We found that the combination of MEK162 and BKM120 was well tolerated in mice, but significantly inhibited the growth of A549 xenografts in comparison with each agent alone, which only weakly inhibited tumor growth (Figure 6). As we observed *in vitro*, BKM120 increased p-Akt levels in A549 xenografts; this effect was abrogated by the combination of MEK162 and BKM120. Interestingly, BKM120 alone substantially increased p-ERK1/2 levels in A549 xenografts *in vivo* (Figure 6D) although it did not do so *in vitro* (Figure 5A), suggesting activation of MEK/ERK signaling. This effect was also abolished in the xenografts treated with the combination of MEK162 and BKM120. The *in vivo* activation of MEK/ERK signaling is likely due to sustained or repeated treatment with BKM120. Our findings in fact support the notion that the MEK/ERK and PI3K/Akt signaling pathways have a compensatory cross-talk [29]. Therefore our *in vitro* and *in vivo* findings as demonstrated in this study provide a scientific rationale for co-targeting both MEK/ERK and PI3K/Akt signaling as a strategy for NSCLC (e.g., with the combination of MEK162 and BKM120). This combination warrants further evaluation in the clinic.

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Highlights

1. MEK162, a MEK inhibitor under clinical testing, inhibits the growth of human non-small cell lung cancer (NSCLC) cells with varied potencies.
2. MEK162 induces G1 arrest, autophagy and/or apoptosis.
3. MEK162 activates Akt signaling while effectively suppressing the MEK/ERK signaling.
4. Blocking autophagy enhances MEK162's growth-inhibitory effects.
5. The combination of MEK162 and BKM120, a PI3K inhibitor, abrogates induced Akt activation and significantly augments therapeutic efficacy against the growth of NSCLC cells both *in vitro* and *in vivo*.

Figure 1. Human NSCLC cell lines display varied sensitivities to both MEK162 and AZD6244 (A) and varied basal activity of MEK/ERK and Akt signaling pathways (B)

A, The panel of human NSCLC cell lines as indicated were seeded in 96-well plates and then treated with different concentrations of MEK162 or AZD6244 ranging from 0.04 μM to 10 μM on the second day. After 3 days, cell numbers were estimated using the SRB assay. IC₅₀s, which were estimated from the growth inhibition curves, are presented as the means of 2–4 independent assays ± SDs. Five μM was used as a cut-off to divide cell lines into resistant and sensitive groups. #, K-Ras mutation; *, N-Ras mutation. Chemical structures of both MEK162 and AZD9244 are shown on the right panel. **B**, Whole-cell protein lysates were prepared from the indicated cell lines and used for Western blotting to detect the indicated proteins. LE, longer exposure.

Figure 2. MEK162 induces G1 arrest (A), apoptosis (B and C) and autophagy (C and D) in human NSCLC cells

A–C, The given cell lines were treated with indicated concentrations of MEK162 for 48h and then harvested for cell cycle analysis with flow cytometry (A), for assaying histone-associate DNA fragments with a cell death ELISA kit and for detection of proteins of interest as indicated by Western blotting. Columns, means of triplicate determinations; bars, $\pm$ SD. D, A549/LC3-YFP cells were seeded in a 24-well plate and treated with 3 μ M MEK162 for 48 h. Fluorescent images were then recorded with a fluorescence microscope.

Figure 3. MEK162 modulates MEK/ERK, PI3K/Akt and mTOR signaling pathways

The given cell lines were treated with indicated concentrations of MEK162 for 24 h. Whole-cell protein lysates were then prepared from these cell lines and used for Western blotting to detect proteins of interest.

Figure 4. The combination of MEK162 and chloroquine synergistically inhibits the growth of NSCLC cells (A) and enhances induction of apoptosis (B and C)

A, The indicated cell lines were seeded in 96-well plates and treated the next day with different concentrations of MEK162 alone, chloroquine (CQ) alone and their respective combinations as indicated. After 48 h, cell numbers were estimated using the SRB assay. The data are means $\pm$ SDs of four replicate determinations. CIs for a representative 20 μ M CQ in combination with different concentrations of MEK162 were between 0.3 and 0.75 in H522 cells and around 0.4 in A549 cells. B and C, H522 cells were treated with indicated concentrations of MEK162 alone, chloroquine alone or their combinations for 48 h. The cells were then harvested for Western blotting to detect proteins of interest (B) and for assaying histone-associated DNA fragments with a cell death ELISA kit (C). Columns represent means $\pm$ SDs of triplicate determinations. SE, shorter exposure.

Figure 5. The combination of MEK162 and BKM120 effectively inhibits Akt activation induced by MEK162 (A) and enhances the inhibition of cell growth (B and C) and the induction of G1 arrest (D)

A, The given cell lines were treated with 100 nM MEK162, 500 nM BKM120 or their combination for 48 h and then harvest for Western blotting to detect the indicated proteins. B, The given cell lines were seeded in 96 well-plates and treated with different concentrations of MEK162 alone, BKM120 alone and their respective combinations. After 3 days, the cells were estimated using the SRB assay. The data are means $\pm$ SDs of four replicate determinations. The numbers in the graphs are CIs for different combinations. C, The indicated cell lines at a density of approximately 300 cells/well were seeded in 12-well plates. On the second day, cells were treated with 50 nM MEK162, 400 nM BKM120 or their combination. Treatments were repeated every 3 days. After 10 days, the plates were

stained for cell colonies with crystal violet dye. The colonies were then counted and representative pictures of colonies were taken with a digital camera. Columns represent means $\pm$ SDs of triplicate determinations. **, $P < 0.01$ (at least) and ***, $P < 0.001$ (at least) compared with all other three treatments. *D*, The indicated cell lines were treated with 100 nM MEK162, 500 nM BKM120 or their combination. After 48 h, the cells were harvested for cell cycle analysis with flow cytometry.

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Figure 6. The combination of MEK162 and BKM120 is significantly more effective than each single agent in suppressing the growth of NSCLC xenografts (A and B) without apparent toxicity in mice (C) and prevents Akt activation by MEK162 or ERK activation by BKM120 (D)

A, A549 xenografts were treated (once a day) with vehicle control, MEK162 (5 mg/kg, og), BKM120 (7.5 mg/kg, og) and their combination (MEK + BKM) starting on the same day after grouping. Tumor sizes (A) and mouse body weights (B) were measured as indicated. Each measurement is mean $\pm$ SEM (n = 6). After 21 days, the mice were sacrificed and the tumors were removed and weighed (C). Moreover, whole-protein cell lysates were also prepared randomly from 3 tumors in each group for Western blotting to detect the indicated proteins (D). * $P < 0.05$ (at least), ** $P < 0.01$ (at least) and *** $P < 0.001$ (at least) compared with all other three treatment groups.

Table 1

Genetic alterations in NSCLC cell lines used in this study

Cell line	Gene mutation						
	p53	PTEN	Ras	EGFR	LKB1	PIK3CA	CDKN2A
H596	Yes				Yes	Yes	
EKVX				Yes			Yes
H1975	Yes			Yes			Yes
HCC827	Yes		Yes (K-Ras)				
Calu-1	Yes		Yes (K-Ras)				
H1792	Yes		Yes (K-Ras)		Yes		Yes
H322M	Yes		Yes (K-Ras)				Yes
H358	Null		Yes (K-Ras)		Yes		Yes
H23	Yes	Yes	Yes (K-Ras)		Yes		
H460			Yes (K-Ras)		Yes	Yes	Yes
A549			Yes (K-Ras)		Yes		Yes
H1299	Null		Yes (N-Ras)				Yes
H522	Yes						
H157	Yes	Yes	Yes (K-Ras)		Yes		Yes

The information listed in this table was obtained from Sanger (<http://www.sanger.ac.uk/genetics/CGP/CellLines/>) or provided by Dr. J. D. Minna (University of Texas Southwestern Medical Center, Dallas, TX)