The Phosphoinositide 3-Kinase α Selective Inhibitor BYL719 Enhances the Effect of the Protein Kinase C Inhibitor AEB071 in GNAQ/GNA11-Mutant Uveal Melanoma Cells

Elgilda Musi, Grazia Ambrosini, Elisa de Stanchina, and Gary K. Schwartz

Abstract

G-protein mutations are one of the most common mutations occurring in uveal melanoma activating the protein kinase C (PKC)/mitogen-activated protein kinase and phosphoinositide 3-kinase (PI3K)/AKT pathways. In this study, we described the effect of dual pathway inhibition in uveal melanoma harboring GNAQ and GNA11 mutations via PKC inhibition with AEB071 (sotrastaurin) and PI3K/AKT inhibition with BYL719, a selective PI3Kα inhibitor. Growth inhibition was observed in GNAQ/GNA11-mutant cells with AEB071 versus no activity in wild-type cells. In the GNAQ-mutant cells, AEB071 decreased phosphorylation of myristoylated alanine-rich C-kinase substrate, a substrate of PKC, along with ERK1/2 and ribosomal S6, but persistent AKT activation was present. BYL719 had minimal antiproliferative activity in all uveal melanoma cell lines, and inhibited phosphorylation of AKT in most cell lines. In the GNA11-mutant cell line, similar effects were observed with ERK1/2 inhibition, mostly inhibited by BYL719. With the combination treatment, both GNAQ- and GNA11-mutant cell lines showed synergistic inhibition of cell proliferation and apoptotic cell death. In vivo studies correlated with in vitro findings showing reduced xenograft tumor growth with the combination therapy in a GNAQ-mutant model. These findings suggest a new therapy treatment option for G-protein–mutant uveal melanoma with a focus on specific targeting of multiple downstream pathways as part of combination therapy. Mol Cancer Ther; 13(5); 1044–53. ©2014 AACR.

Introduction

Uveal melanoma is one of the most common intraocular malignancies of the adult eye, affecting 6 individual per million per year. Most cases of uveal melanoma metastasize to the liver and have a poor survival rate after initial diagnosis. Few treatment options for this fatal disease exist (1). Chemotherapy is ineffective. There has been recent interest in targeting the mitogen-activated protein kinase (MAPK) pathway (2). MAPK activation is found to be present among 80% of uveal melanoma, although it rarely occurs through mutations such as BRAF (3, 4). Activation of MAPK in uveal melanoma can be attributed to mutations in GNA11 or GNAQ, an early event of malignant transformation (5, 6). Collectively these genetic alterations are present in 85% of diagnosed cases (5, 7). These genes encode members of the q class of G-protein α subunits involved in mediating signals between G-protein–coupled receptors (GPCR) and downstream effectors. Activation is through stimulation of phospholipase C-β (PLCβ), which cleaves phosphatidylinositol (4,5)-bisphosphate (PIP2) to inositol triphosphate (IP3) and diacylglycerol (DAG), which activates protein kinase C (PKC) further activating the MAPK pathway (2, 8).

To date, most experimental therapies used to treat GNAQ- and GNA11-mutant uveal melanoma have focused on the targeting of the MEK/ERK pathway. However, these mutations have been shown to activate other signaling pathways upstream of MEK, including PKC family members that are involved in cell proliferation and apoptosis. The PKC family is a group of serine/threonine kinases composed of different isoforms that are divided into (1) the classical (or conventional) PKC isoforms α, β, γ, I, II, and (3) the atypical PKC isoforms λ and ζ each having different roles and functions in cancer (9). Myristoylated alanine-rich C-kinase substrate (MARCKS) is a major PKC substrate expressed in many different cell types. MARCKS, when phosphorylated by PKC, has such roles as calcium/calmodulin binding and actin–membrane interactions (10–12).

PI3K/AKT signaling pathways are also activated in the setting of G-protein mutations in uveal melanoma (2). There are also signaling interactions involved with GNAQ and some PI3K isoforms (13). The PI3K/AKT pathway has been implicated in uveal melanoma, as well as in...
many other cancers, to drive cell survival and cell migration (14, 15). PI3K mediates phosphorylation of PIP2 to phosphatidylinositol (3,4,5)-tris-phosphate (PIP3), which activates AKT to promote tumor growth and proliferation. The PI3Ks are classified as class IA and IB. Class IA consist of heterodimers with an inhibitory adaptor unit (p85) as well as a catalytic subunit (p110). There are 3 known isoforms of the class IA p110 subunits, which include p110α, p110β, and p110δ. Class IB consists of p110γ and a regulatory subunit, p101 (16). p110α (PI3Kα) has been linked to activation of AKT in cell lines. It has been shown that siRNA knockdown of p110α in C2C12 myoblasts reduced phosphorylation of AKT stimulated by insulin-like growth factor-I whereas silencing of p110β did not affect AKT (17). Although AKT phosphorylation has been shown to be increased in uveal melanoma (18, 19), it remains to be determined whether a pan-PI3K or AKT inhibitor can be clinically developed in this or other diseases because of concerns for systemic toxicity. Rather, an inhibitor of PI3Kα, by providing isoform specificity for the pathway, may avoid toxicity and off target effects thus far associated with this class of drugs (20). This will be particularly relevant in uveal melanoma where activation of multiple downstream pathways, including PI3K/AKT, MEK, and PKC, drive tumor growth. Combination therapies will ultimately be absolutely essential to maximize effective growth inhibition.

Toward this end, we elected to evaluate dual target inhibition of PI3Kα and PKC in GNAQ- and GNA11-mutant uveal melanoma cells. It has been previously reported that PKC inhibitors have antiproliferative activity in vitro against uveal melanoma cells carrying GNAQ mutations (21, 22). AEB071 (sotrastaurin) is a potent inhibitor of many forms of PKC shown to have antiproliferative activity in other cell types as well (23). It is currently being evaluated in a phase I clinical trial for patients with metastatic uveal melanoma (NCT01430416). BYL719 is a next generation PI3Kα-specific inhibitor, which has been tested in a broad cancer cell line panel (24) and currently under clinical evaluation to assess efficacy in PI3Kα-driven tumors (25). We now show that the combination of the PKC inhibitor AEB071 with BYL719, a selective PI3Kα inhibitor, results in an enhanced antitumor effect because of inhibition of both PKC and PI3K/AKT signaling in G-protein–mutant uveal melanoma cells in vitro as well as in vivo. These findings would support the clinical development of this drug combination such that both of these pathways are inhibited in patients with uveal melanoma.

Materials and Methods

Cell culture

All cell lines were maintained in RPMI 1640 supplemented with heat inactivated 10% FBS supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin and maintained at 37°C and 5% CO2. 92.1 cells were provided by Dr. W. Harbour (Washington University, St. Louis, MO). Omm1.3 and Mel270 have been provided by Dr. B. Ksander (Harvard Medical School, Boston, MA). Omm1 was kindly provided by Dr. B. Bastian (University California of San Francisco, San Francisco, CA). Mel290 was from David Folberg (University of Illinois, Chicago, IL). C918 were obtained from Dr. David H. Abramson (Memorial Sloan Kettering Cancer Center, New York, NY) originally from David Folberg. As previously reported, uveal melanoma cell lines have been sequenced for the presence of activating mutations in codons 209 (exon 5) and 183 (exon 4) of GNAQ and GNA11. Two cell lines had Q209L mutation (22, 23), whereas Omm1.3 and Mel270 had Q209P mutation (23). Omm1 (GNA11) cell line had a Q209L mutation. A karyotype test was also performed for each cell line in 2012, confirming the authenticity of the cell lines as reported previously (27).

Chemicals

AEB071 (sotrastaurin) and BYL719 were supplied by Novartis and dissolved in dimethyl sulfoxide (DMSO) at 10 mmol/L concentration and stored aliquoted at −20°C.

Cell viability assays and combination index analysis

Cells were plated in a 96-well plate and treated with AEB071, BYL719, or DMSO at indicated concentrations for a period of 5 days. Viability was assessed using Cell Counting Kit from Dojindo Molecular Technologies as per manufacturer’s instructions. The combination index (CI) values were calculated using the CompuSyn software (Combosyn) as developed by Chou and colleagues (28). Briefly explained, the plots generated by the CompuSyn software demonstrate the Y-axis CI values, where CI <1, =1, and >1 indicate synergism, additive effect, and antagonism, respectively. The X-axis represents the fractional activity, which reflects the fraction of cells inhibited by the treatments relative to vehicle control. For combination index studies, the concentrations tested included AEB071 (0, 125, 250, 500, and 1,000 nmol/L) and BYL719 (0, 250, 500, 1,000, and 2,000 nmol/L).

Gene silencing

Experiments with siRNAs were performed as previously described (29). Briefly, the cells were transfected using Lipofectamine RNAiMax (Invitrogen) according to manufacturer’s instructions. Next day, cells were treated with drug for an additional 24 hours. Cells were then harvested and lysates were collected for Western Blot analysis. siRNA for human PI3Kα was purchased from Thermo Scientific, On Target plus SMARTpool (L-003018-00), and control siRNA (sc-37007) was from Santa Cruz Biotechnology.

Western blots

Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer supplemented with protease inhibitor cocktail tablet (Roche Diagnostics) and 1 mmol/L Na3 VO4. Equal amounts of protein were loaded and separated on a 4% to 12% PAGE gel (Invitrogen). Proteins

PI3Kα and PKC Inhibition Are Synergistic in Uveal Melanoma

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were transferred to polyvinylidene difluoride membranes, which were blocked in 5% nonfat dried milk. Membranes were then incubated with primary and secondary antibody and developed by ECL. Antibodies used to probe were p-AKT (Ser473), Pan AKT, p-S6 (S240/244), S6 ribosomal protein, p-MARCKS (S152/156), MARCKS, α-tubulin, ERK1/2, cleaved PARP (Cell Signaling), and p-ERK1/2 (Y204) (Santa Cruz Biotechnology). For time course evaluation, media from plated cells was aspirated and freshly prepared drug in media was added to the plates. Cells were then harvested and lysed in RIPA buffer at 2, 6, and 24 hours.

**Flow cytometry**

Cell-cycle analysis was performed as previously described (30, 31). Briefly, cell-cycle analysis was performed after 72 hours of treatment. Cells were ethanol fixed, stained with propidium iodide and MPM-2 antibody, and analyzed by flow cytometry.

**Xenograft studies**

Six- to eight-week SCID female mice bearing subcutaneously injected 92.1 tumors (7 mice/group) of 100 mm³ diameter were treated with vehicle, AEB071 (80 mg/kg/d) 3 times a day and/or BYL719 orally (50 mg/kg/d) every day as single agents and in combination, 5 days/week for 2 weeks. After 2 weeks, 2 animals from each group were sacrificed and tumors were collected to analyze for hematoxylin and eosin, and terminal deoxynucleotidyl transferase nick end labeling (TUNEL) staining as previously described (31). Tumors were measured every 2 to 3 days with calipers, and tumor volumes were calculated by the formula \( V = \frac{4}{3}\pi r^3 \), where \( r \) is the larger diameter + smaller diameter)/4. Toxicity was monitored by weight loss. Experiments were carried out under institutional guidelines addressing the proper and humane use of animals. The Memorial Sloan-Kettering Cancer Center Animal Care and Use Committee and Research Animal Resource center approved this study. The study is also in accordance of the Principles of Laboratory Animal Care (NIH Publication No. 85-23, released 1985).

**Statistical analysis**

All *in vitro* experiments were carried out at least 2 to 3 times. SE was calculated as the SD divided by the square root of number of samples. Statistical analysis for *in vivo* studies was determined by the 2-sided *t* test. We chose *P* = 0.05 as statistically significant in individual comparisons.

**Results**

**AEBO71 inhibits cell proliferation in GNAQ/GNA11-mutant uveal melanoma cell lines with inhibition of the PKC/ERK1/2 pathway**

We evaluated the cell growth effect of the PKC inhibitor, AEB071 (structure Fig. 1A), utilizing 6 uveal melanoma cell lines with different genotypes. The cell lines included GNAQ-mutant cell lines 92.1, Mel270, Omm1.3, and the GNA11-mutant cell line Omm1. We also included wild-type cell lines C918 and Mel290, without GNAQ/GNA11 mutations. We examined the single-agent antiproliferative effect on all cell lines utilizing increasing concentrations 0 to 2 μmol/L of AEB071. We observed a dose-dependent inhibition of proliferation with Glc values ranging from 250 to 500 nmol/L for the GNAQ- and GNA11-mutant cell lines (Fig. 1B), whereas the cell lines with no mutations (wild type) were not inhibited by the drug up to the highest concentration of 2 μmol/L. We next examined target inhibition of PKC signaling with increasing concentrations of the drug from 0 to 1,000 nmol/L (Fig. 1C). AEB071 inhibited p-MARCKS, a PKC substrate, and pS6 in all the cell lines, independently of the mutational status. We also found an inhibition of ERK phosphorylation only in the GNAQ-mutant cells. There was a slight inhibition of pERK at lower doses also in the GNA11-mutant cells, but not in the wild-type cells at any concentrations. This is consistent with previous reports indicating that AEB071 inhibits ERK1/2 phosphorylation in GNAQ-mutant cell lines (22). Phosphorylation of AKT at Ser473 was minimally affected in the GNAQ-mutant cells, whereas it increased in the GNA11-mutant and wild-type cells. In Mel290 (wild type), the activation of AKT in response to AEB071 was particularly evident, indicating a feedback mechanism, possibly dependent on EGFR, which has been reported to be overexpressed in this cell line (32).

**Silencing of PI3Kα enhances the antiproliferative effects of the PKC inhibitor in GNAQ-mutant uveal melanoma cell lines**

To explore whether selective PI3Kα inhibition contributes to the PKC inhibitory effects in uveal melanoma, we performed gene silencing of p110α with or without the presence of AEB071 (Fig. 2A). Depletion of p110α inhibited AKT phosphorylation in the GNAQ-mutant (92.1, Omm1.3) and wild-type (C918) cells. There was no AKT inhibition by p110α siRNA in Mel270 and this was still maintained at basal levels in the presence of AEB071, and in Mel290. However, treatment with AEB071 in the presence of p110α siRNA suppression induced PARP cleavage only in the mutant cells, under which condition p-MARCKS, p-ERK, p-AKT, and p-S6 were inhibited (Fig. 2A). This corresponded to a significant decrease in cell viability in the GNAQ-mutant cells (Fig. 2B). In contrast, the wild-type cell lines showed no PARP cleavage, and the C918 cells showed an increase in cell viability. This enhancement of cell growth in the wild-type cell line with
PI3Kα suppression and AEB071 may be attributed to the absence of ERK1/2 decrease as seen with the GNAQ-mutant cells (Fig. 2A).

We next examined the effect of single-agent BYL719 (structure in Fig. 2C) on the same cell lines, utilizing concentrations ranging from 0 to 2 μmol/L (Fig. 2D). We observed inhibition of phosphorylation of AKT (Ser473) up to 1 μmol/L in most cell lines (Fig. 2D), even though there was reactivation at higher doses in Omm1.3, Mel270, and Mel290. Some of the cell lines, such as Omm1.3, Omm1, and Mel290 showed inhibition of p-ERK1/2 by BYL719. There is recent evidence supporting the inhibition of ERK1/2 phosphorylation by the selective PI3Kα inhibitor BYL719 (33). There was also significant inhibition of S6 phosphorylation in the Omm1.3 and Mel290 cell lines. However, PI3Kα/pAKT inhibition in itself was clearly not sufficient to have an antiproliferative effect in any cell line (Fig. 2E). The exception to this was Omm1, the GNA11-mutant cell line, in which cell growth inhibition was observed at 1,000 nmol/L, a concentration at which there was inhibition of p-ERK.

Figure 1. AEB071 reduces cell viability in G-protein-mutant cell lines with minimal impact on the AKT pathway. A, structure of AEB071. B, AEB071 as single agent selectively inhibits cell proliferation of GNAQ/GNA11-mutant cells. Cell lines were treated with 0, 62.5, 250, 500, 1,000, and 2,000 nmol/L of AEB071 for 5 days. Results represent the mean of 3 independent experiments. C, AEB071 inhibits PKC and mTOR pathways but not AKT. Western blot analysis of MARCKS, ERK, ribosomal S6, and AKT phosphorylation following drug treatments for 24 hours. α-Tubulin was used as a loading control.

The combination AEB071/BYL719 induces a synergistic decrease in cell viability in GNAQ/GNA11-mutant uveal melanoma cell lines

To examine the effects of the AEB071/BYL719 combination on cell viability, we performed combination index analysis utilizing the Chou–Talay method to determine whether the effects are synergistic (28). The combination of AEB071 and BYL719 at different doses exhibited a combination index (CI) <1, indicative of synergy and a fractional activity of more than 50% ([Fa] > 0.5) in all GNAQ/GNA11-mutant cell lines (Supplementary Fig. S1). CI values for wild-type cell lines C918 and Mel290 ranged from >1 to 30 with a (Fa) < 0.2, showing that the combination was in fact antagonistic in cells that are wild type for G proteins.

We next investigated the occurrences of molecular events with the combination treatment (Fig. 3). We treated 2 GNAQ (92.1, Mel270) and 2 wild-type (C918, Mel290) cell lines with concentrations of 500 nmol/L of AEB071 and 1 μmol/L of BYL719 respectively and examined the effect on downstream signaling at 2, 6, and 24 hours. In
Figure 2. Selective inhibition of PI3K\(\alpha\) enhances AEB071 antiproliferative effect in GNAQ-mutant cells. A, PI3K\(\alpha\) siRNA inhibits AKT phosphorylation in uveal melanoma cell lines. siRNA knockdown of p110\(\alpha\) isoform was performed with or without AEB071 in 92.1, Mel270, Omm1.3 (GNAQ mutant), and Mel290, C918 (wild type) cell lines. A nonspecific siRNA was used as control. Western blots of PI3K\(\alpha\), AKT, MARCKS, ERK, S6, and cleaved PARP were then performed. The nuclear protein Ku70 was used as a loading control. B, PI3K\(\alpha\) and PKC inhibition reduces cell proliferation in GNAQ-mutant uveal melanoma. Cell viability was assessed after transfection, with or without AEB071 for 5 days. C, structure of BYL719. D, BYL719 inhibits AKT (Ser473) phosphorylation, and/or p-ERK in some cell lines. Cells were treated with indicated concentrations of BYL719 for 24 hours, and analyzed for pAKT, pERK, pS6, and the respective total proteins. \(\alpha\)-Tubulin was used as a loading control. E, BYL719 as single agent has minimal to no effect on cell viability in uveal melanoma after 5 days of exposure. Concentrations tested include 0, 10, 100, and 1,000 nmol/L. Results represent the mean of 3 independent experiments.
contrast to single-agent AEB071 or BYL719, the combination therapy resulted in inhibition of p-MARCKS and p-AKT over time and all downstream signaling pathways such as p-ERK and p-S6 in the GNAQ-mutant cell lines. Single-agent BYL719 caused a slight activation of p-ERK that was detectable as early as 2 hours after drug exposure, especially in the 92.1 cells, but this activation was suppressed with the combination treatment. This would in fact suggest that the reactivation of p-ERK in this setting may be mediated through PKC. The reactivation of p-ERK at 6 hours, especially in the Mel270 cell line, along with all untreated controls, may reflect a DMSO effect. In the Mel290 wild-type cell line, there was activation of AKT to both AEB071 and BYL719 along with reactivation of p-MARCKS at later time points, suggesting that this cell line may have a dependency on other survival pathways such as EGFR (32). For the wild-type C918 cell line, p-MARCKS, p-AKT, and p-S6 are inhibited over time but minimal p-AKT inhibition occurs. We also tested the GNA11-mutant cell line, Omm1, with the combination therapy (Supplementary Fig. S2A). In these cells, AEB071 inhibited p-MARCKS and p-S6 but induced activation of p-AKT over time, which was inhibited by BYL719 in the combination therapy. Interestingly, BYL719 also inhibited pERK in this cell line. Thus, these results indicate that inhibition of PKC and PI3Kα together achieves a synergistic effect in GNAQ and GNA11 cells with inhibition of downstream survival pathways that is not achievable with single-agent therapy alone.

**AEB071/BYL719 combination induces apoptosis**

We next elected to determine whether this inhibition of cell growth was because of growth arrest or induction of apoptosis. We assessed cell-cycle effects of the drug combination with bi-parameter flow cytometry analysis for DNA content (detected with propidium iodide) and MPM-2, a mitotic marker, as previously described (31). Apoptosis was assessed by the detection of a sub-G1 peak and of PARP cleavage. The flow cytometry data indicated essentially minimal growth arrest (data not shown) with AEB071, but there was evidence of a sub-G1 peak with the combination therapy only in the GNAQ-mutant cell lines (Fig. 4A). Furthermore, the combination treatment induced PARP cleavage, an early apoptotic event.
marker, in GNAQ-mutant cells, whereas there was no evidence of PARP cleavage in the wild-type cell lines (Fig. 4B).

**AEB071/BYL719 combination inhibits tumor growth in vivo in a GNAQ-mutant xenograft model**

In view of the synergistic effects of the combination therapy observed in vitro, we elected to determine whether the combination therapy would be superior to single-agent therapy in a GNAQ-mutant xenograft mouse model. As shown in Fig. 5A, both single agents had a modest effect on inhibiting tumor growth at their respective single-agent MTDs. The combination therapy resulted in a significantly enhanced reduction in tumor volume when compared with either AEB071 or BYL719 alone (P = 0.049 vs. BYL719 and P = 0.022 vs. AEB071 at day 26). There was even a greater effect when compared with vehicle control (P = 0.016). Examination of the tumor lysates following drug therapy (day #26) confirmed the in vitro data indicating that the combination therapy resulted in concomitant inhibition of PKC and PI3K signaling with inhibition p-MARCKS, p-AKT, p-ERK, and p-S6 that was not observed with either single-agent alone. Toxicity was measured along with tumor volume by weight loss, which was less than 14.7% for all treatments (Fig. 5A, right). Examination of apoptosis by TUNEL indicates the presence of apoptotic cells only with the combination therapy (Supplementary Fig. S3).

The Omm1 (GNA11)-mutant xenograft mouse model was also performed with both single agents having an effect on inhibiting tumor growth at their respective single-agent MTDs, which seemed greater than that observed in the GNAQ xenograft. Furthermore, the combination therapy did not result in a significant reduction in tumor volume when compared with either AEB071 or BYL719 alone, although a trend favored the combination therapy (Supplementary Fig. S2B). For unclear reasons, the animals bearing the GNA11 xenografts experienced a 23.9% weight loss with combination therapy (Supplementary Fig. S2B, right).

**Discussion**

PKC, MAPK, and PI3K/AKT pathways are highly activated in uveal melanoma (3, 4, 18, 34). MEK/ERK signaling has been considered as a major target in uveal melanoma and clinical trials are underway targeting this pathway (35). Alternatively, dual-targeted therapy is now being considered as a treatment option as further studies elucidate the importance of targeting activated signal pathways. Previous studies have focused on combining inhibitors of MEK with mTOR and PI3K/AKT pathways (26, 36, 37). Targeted therapy directed against PI3K isoforms has usually been effective in cancers harboring mutations of the isoform but PI3K mutations have not been reported in uveal melanoma. Clinically, the benefit of targeting a specific isoform of PI3K has led to more complete target inhibition at lower doses resulting in less adverse effects (38). The blocking of AKT activation in other cell types through PI3Kε isoform has been widely studied (17, 39). The role of PI3Kε isoform in its active state allows for cell survival and migration, leading to tumor formation and metastases (40). We postulated that preventing this activation with a selective PI3Kε inhibitor would provide a means to inhibit AKT in these cells as part of a combinatorial approach. The G-protein oncogenic mutations have been shown to activate PKC. In fact, GNAQ mutations rely mostly on PKC and MAPK pathways for survival (26, 41). This present study demonstrates that inhibiting PKC and ERK1/2 signaling with...
AEB071 together with the PI3Kα inhibitor, BYL719, results in a synergistic antitumor effect in uveal melanoma harboring GNAQ and GNA11 mutations in vitro. Previous reports have shown that PKC inhibition has a negative regulatory effect on PI3K/AKT by stimulating Akt/PKB phosphorylation at Ser473 (42). This may explain the minimal single-agent effect of AEB071 on inhibiting pAKT in the GNAQ- and GNA11-mutant cell lines. Interestingly, our current results also show inhibition of ribosomal S6 phosphorylation with PKC inhibition and combination treatment. S6 overexpression has been reported as a prognostic marker for uveal melanoma and mTOR signaling may similarly provide a growth advantage to these cells (19). This could explain why inhibition of this pathway also enhances the antitumor effect we have observed in these cells. In the setting of GNAQ/GNA11 mutations, the inhibition of MARCKS, ERK, AKT, and S6 phosphorylation results in apoptosis by the combination treatment. Similar pathway targeting in pancreatic cells have shown an apoptotic outcome by sulforaphane treatment because of synergistic inhibition of PI3K/AKT and MEK/ERK (43).

The effects of this combination treatment demonstrate the importance of targeting parallel survival and growth pathways that are downstream of GNAQ and GNA11 in uveal melanoma. However, simply inhibiting several of these pathways may not be sufficient to inhibit tumor growth in vivo. For example, we previously reported that blockade of Torc1 and Torc2 with AZD8055 in combination with the MEK inhibitor selumetinib (AZD6244) in GNAQ-mutant cells is insufficient to suppress tumor growth in vivo (36). In contrast, we have shown that inhibition of p-AKT with MK2206, an allosteric inhibitor of AKT, in combination with selumetinib

Figure 5. AEB071/BYL719 combination inhibits in vivo tumor growth in a GNAQ-mutant xenograft model. A, AEB071/BYL719 inhibited tumor growth in a xenograft model with the GNAQ-mutant 92.1 cell line. Six- to eight-week SCID female mice were subcutaneously injected with 92.1 cells. Drug treatments began after tumors reached 100 mm³. Mice with tumors were treated 3 times daily with AEB071 (80 mg/kg/d) or once daily with BYL719 (50 mg/kg/d) or in combination for 5 days each week for a total of 2 weeks. Tumors were measured with calipers every 2 to 3 days. Tumor volume was compared between groups of mice at various time points. *P* value when compared with either AEB071 or BYL719 alone *P* = 0.049 versus BYL719 and *P* = 0.022 versus AEB071 at day 26. Toxicity was measured by weight loss (right). B, 2 animals from each cohort were sacrificed and tumors were collected on day 26. Tumors were lysed with RIPA buffer and Western blots were performed for pAKT, pERK, pS6, pMARCKS, and α-tubulin. QD, every day; TID, 3 times a day.
results in enhanced antitumor effects (26). This would suggest that the development of combination therapy for the treatment of GNAQ uveal melanoma will depend largely on the selective targeting of pathways that are critical for tumor growth. Based on our MK2206 and selumetinib data, inhibition of p-ERK in combination with inhibition of p-AKT seems to be the minimal requirement necessary to promote an antitumor effect with combination therapy. As AEB071 results in inhibition of PKC as well as p-ERK, this dual pathway inhibition, when combined with inhibition of P53Ko/AKT by BYL719, seems to provide a means to broaden pathway inhibition above and beyond MEK and AKT alone for cell growth inhibition of GNAQ/GNA11-mutant cells.

All together our findings indicate that the targeting of PKC/ERK, along with a selective P53Ko isoform inhibitor, cooperate in inhibiting growth in GNAQ-mutant uveal melanoma cells. In the GNA11 cell line, this same effect is ultimately achieved, although it is BYL719, the P53Ko inhibitor, rather than AEB071, the PKC inhibitor, that contributes to the inhibition of p-ERK. We also show a translatable GNAQ xenograft model of uveal melanoma demonstrating effective PKC/ERK and P53K/ AKT pathway inhibition, which results in enhanced in vivo tumor growth inhibition with the combination therapy. The combination effects in vivo on the GNA11 xenograft were less definitive despite synergy in vitro. There was unexpected weight loss with the combination therapy, which may have been because of the difference in mouse species (nude mice rather than SCID mice) needed to establish the GNA11 xenograft.

The combination of AEB071 and MEK162, the MEK inhibitor, has recently been reported in uveal melanoma xenografts (41). Our studies indicate that both AEB071 and BYL719 can inhibit p-ERK as single agents or as part of combination therapy. However, only BYL719 can inhibit p-AKT, which seems critical to the synergistic effect. In conclusion, our results suggest an effective new therapy for the treatment of uveal melanoma, and targeting these pathways with AEB071 and BYL719 could have therapeutic implications for the future treatment of patients with this disease. Based on these results, a phase Ib/II clinical trial of this drug combination in patients with metastatic uveal melanoma is now planned.

Disclosure of Potential Conflicts of Interest
G.K. Schwartz is a consultant/advisory board member for Novartis. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions
Conception and design: E. Musi, G. Ambrosini, G.K. Schwartz Development of methodology: E. Musi, E. de Stanchina, G.K. Schwartz Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): E. Musi, E. de Stanchina, G.K. Schwartz Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): E. Musi, G.K. Schwartz Writing, review, and/or revision of the manuscript: E. Musi, G. Ambrosini, G.K. Schwartz Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): E. Musi, G.K. Schwartz Study supervision: G. Ambrosini, E. de Stanchina, G.K. Schwartz

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Correction: The Phosphoinositide 3-Kinase \( \alpha \) Selective Inhibitor BYL719 Enhances the Effect of the Protein Kinase C Inhibitor AEB071 in GNAQ/GNA11-Mutant Uveal Melanoma Cells

In this article (Mol Cancer Ther 2014;13:1044–53), which was published in the May 2014 issue of Molecular Cancer Therapeutics (1), the authors regret that the \( y \)-axis of the small graph (right) in Fig. 5A is incorrectly labeled "tumor weight (gm)." It should instead read "weight (gm)." The corrected Fig. 5 is shown below.

In addition, in Supplementary Fig. S2B, the \( y \)-axis of the large graph (left) in Supplementary Fig. S2B was incorrectly labeled "tumor weight (gm)." It should instead read "tumor volume (mm\(^3\))." The online version of Supplementary Fig. S2B has been replaced with a corrected version.

Reference


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