Protein Kinase C Inhibitor AEB071 Targets Ocular Melanoma Harboring GNAQ Mutations via Effects on the PKC/Erk1/2 and PKC/NF-κB Pathways

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Abstract

Somatic GNAQ mutations at codon 209 have been identified in approximately 50% of uveal melanomas and have been reported to be oncogenic through activating PLCβ/PKC/Erk1/2 pathways. We hypothesized that protein kinase C (PKC) may provide new opportunities for therapeutic targeting of uveal melanoma carrying GNAQ mutations. To test this hypothesis, uveal melanoma cells harboring wild-type or mutant GNAQ were treated with the PKC inhibitor AEB071 (sotrastaurin) or infected with lentivirus-expressing short hairpin RNAs (shRNA) targeting PKC isoforms. Notably, AEB071 at low micromolar concentrations significantly inhibited the growth of uveal melanoma cells harboring GNAQ mutations through induction of G1 arrest and apoptosis. However, AEB071 had little effect on uveal melanoma cells carrying wild-type GNAQ. AEB071-mediated cell inhibition in the GNAQ-mutated uveal melanoma was accompanied by inhibition of extracellular signal–regulated kinase (Erk)1/2 phosphorylation, NF-κB, decreased expression of cyclin D1, survivin, Bcl-xL, and XIAP, and increased expression of cyclin-dependent kinase inhibitor p27 Kip1. AEB071 suppressed the expression of PKCα, β, δ, ε, and θ in GNAQ-mutated uveal melanoma cells. Our findings from shRNA-mediated knockdown studies revealed that these PKC isoforms are functionally important for uveal melanoma cells harboring GNAQ mutations. Furthermore, inhibitors of Erk1/2 and NF-κB pathways reduced viability of uveal melanoma cells. Together, our findings show that AEB071 exerts antitumor action on uveal melanoma cells carrying GNAQ mutations via targeting PKC/Erk1/2 and PKC/NF-κB pathways. Targeted PKC inhibition with drugs such as AEB071 offers novel therapeutic potential for uveal melanoma harboring GNAQ mutations.

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Introduction

Uveal melanoma is the most common primary intraocular malignant tumor in adults, with an incidence of 7 cases per million annually (1). Approximately half of patients with uveal melanoma develop metastases to the liver within 15 years of initial diagnosis. With no effective treatment modality available the median survival time of patients with uveal melanoma with metastasis is less than 6 months (2).

The etiology of uveal melanoma has not been fully understood. Mutations in the GNAQ gene have been identified in approximately 50% of uveal melanoma and 83% blue nevi (3–5). The GNAQ gene encodes for the α subunit of q class of heterotrimeric GTP-binding proteins (G proteins) that are composed of 3 subunits (Gα, Gβ, and Gγ) and transduce signals from 7-transmembrane G-protein–coupled receptors (GPCR) to intracellular cascades (6). Activation of GPCRs results in exchange of GDP for GTP on the Gα subunit, resulting in the dissociation of the GTP bound form of Gα from Gβγ. Both Gα and Gβγ can then activate downstream cellular signaling pathways. The signal is terminated when GTP is hydrolyzed to GDP by the intrinsic GTPase activity of the Gα subunit.

The majority of GNAQ mutations occur at codon 209 within the GTPase catalytic domain, resulting in loss of the intrinsic GTPase activity and constitutively activation of GNAQ. Expression of mutated GNAQ results in melanocyte transformation and increased extracellular signal–regulated kinase (Erk)1/2 phosphorylation, indicating that mutant GNAQ behaves as a dominant acting oncogene (4, 5). Currently, there are no available therapies targeting GNAQ.

The protein kinase C (PKC) family is a widely expressed group of serine/threonine kinases comprising multiple
isoforms that can be divided into 3 structurally and functionally distinct subgroups (7, 8). These are the conventional PKCs (PKCα, PKCβ, and PKCγ), which are activated by diacylglycerol (DAG) and phospholipids and are Ca2+ dependent; the novel PKCs (PKCδ, PKCe, PKCθ, and PKCζ), which are also activated by DAG and phospholipids, but are not Ca2+ dependent; and the atypical PKCs, which do not require DAG or Ca2+ for activation. PKCs regulate key biologic processes including cell proliferation, apoptosis, differentiation, angiogenesis, tumor development, and chemoresistance (7, 9–15). PKCs are involved in GNAQ-mediated activation of the MAPK/Erk1/2 pathways (6, 16). It has been known that GNAQ transduces signals from GPCRs to phospholipase Cβ (PLCβ; ref. 6). PLCβ enzymes catalyze the hydrolysis of phosphatidylinositol biphosphate to release inositol trisphosphate and diacylglycerol that function as second messengers propagating downstream of PKCs. Active PKCs further activate Erk1/2 through the RAF/MAPK/Erk1/2 pathway (16). Using short hairpin RNA (shRNA)-mediated downregulation of PKC isoforms β, ε, and θ we have recently shown that these isoforms are functionally important for GNAQ-mutated uveal melanoma cells (17). The oncogenic properties of mutant GNAQ and the important PKC roles in GNAQ-mediated Erk1/2 activation and GNAQ-mutated uveal melanoma cells (4, 16) suggested that PKC may provide new opportunities for therapeutic intervention of uveal melanoma carrying GNAQ mutations. To test this hypothesis, uveal melanoma cells carrying wild-type GNAQ or GNAQ mutated at codon 209 were treated with the PKC inhibitor AEB071 (sotrasertin), a PKC inhibitor that has potent activity against classical and novel PKC isotypes (18). AEB071 selectively inhibited the growth of uveal melanoma cells harboring GNAQ mutations by targeting PKC/Erk1/2 and PKC/NF-κB pathways.

Materials and Methods

Cell lines

The sources and GNAQ mutational status of uveal melanoma cell lines C918, Ocm1, Ocm3, Mel285, Mel202, 92.1, and Omm1.3 have been described previously (19). Uveal melanoma cells were cultured in RPMI-1640 containing 10% FBS, 50 μg/mL penicillin, and 100 μg/mL streptomycin at 37° C and 5% CO2. These cell lines were recently authenticated by short tandem repeat PCR analysis at Biosynthesis Inc. Human epidermal melanocytes were purchased from Lifeline Cell Technology and grown in the medium provided by the company.

Viability assay

Cells were seeded in 96-well plates at 2 × 104 cells per well and incubated overnight followed by treatment with AEB071 (provided by Novartis) for 3 days. Cell viability was measured as previously described (17).

Cell-cycle analysis

Cells were collected by trypsinization and fixed in cold ethanol. After incubation with RNase A and PI, cell-cycle distribution was determined by flow cytometric analysis (fluorescence-activated cell sorting, FACS).

Analysis of apoptosis

Apoptotic cells were detected by Annexin V-fluorescein isothiocyanate staining and FACS as described previously (17). Compensation for AEB071 autofluorescence was conducted.

Knockdown of PKC isotypes by shRNA

The constructs (pLKO.1-puro) containing shRNA target sequences for PKCα, PKCδ, or GFP were provided by Dana-Farber Cancer Institute shRNA Core Facility. Lentivirus expressing PKC shRNA was produced as described previously (17). Cells were infected with virus for 3 days and cell viability was determined using MTS assay.

Immunoblotting

Preparation of whole-cell lysates and immunoblotting have been described previously (19). Antibodies against PKC isoforms were: PKCα (Cell Signaling #2056), PKCδ (Cell Signaling #2058), PKCθ (BD Biosciences #610090); PKC0 Thr538 (Cell Signaling #9377), PKCθ/δ Ser643/676 (Cell Signaling #9376), and PKCe (BD Biosciences #610085). Antibodies against Akt, phospho-Akt, Erk1/2, phospho-Erk1/2, cyclin D1, Bcl-XL, XIAP, survivin, HADC1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Cell Signaling Technology. Antibodies against PKCβII, p27kip1, and RelA (p65) were purchased from Santa Cruz Biotechnology. Actin antibody was purchased from Sigma-Aldrich. Protein signal intensity was measured using NIH ImageJ software and normalized to that of actin.

Nuclear extract preparation and electrophoresis mobility shift assay

Nuclear extraction and NF-κB electrophoresis mobility shift assay (EMSA) kits purchased from Signosis were used to isolate nuclear extracts from uveal melanoma cells and conduct EMSA. The instructions provided by the manufacturer were followed.

Measurement of interleukin-6 in cell culture medium

Cells were treated with dimethyl sulfoxide (DMSO) or 5 μmol/L AEB071 for 72 hours in 6-well plates. Medium was collected and centrifuged to remove cells. Interleukin (IL)-6 in the supernatant was determined using the Human IL-6 Quantikine ELISA Kit purchased from R&D Systems.

Statistical analysis

Data are presented as mean ± SD. Differences between treated and control groups was analyzed using Student t test and considered significant with P ≤ 0.05.
Results

The PKC inhibitor AEB071 selectively inhibits growth of uveal melanoma cells harboring GNAQ mutations

We first evaluated the effect of AEB071 (Fig. 1A) on viability of a panel of 7 uveal melanoma cell lines (Table 1). Sequencing analysis confirmed that GNAQ is wild-type in cell lines C918, Ocm1, Ocm3, and Mel285, whereas codon 209 of GNAQ is mutated from CAA (glutamine) to CTA (leucine) in cell line Mel202 and 92.1 and to CCA (proline) in cell line Omm1.3. Cell lines Ocm1 and Ocm3 have BRAF V600E mutation (19, 20).

AEB071 at low micromolar concentrations significantly decreased viability of all 3 GNAQ-mutated cell lines (Fig. 1B). The IC50 value of AEB071 was approximately 0.8, 3, and 4 μmol/L for 92.1, Omm1.3, and Mel202 cells respectively. AEB071 had little effect on viability of 3 GNAQ wild-type cells up to 10 μmol/L (Fig. 1B and Supplementary Fig. S1). In addition, the viability of human epidermal melanocytes was not reduced by AEB071 (Fig. 1B) and may have been modestly increased by the inhibitor. Along with decreased viability, microscopic examination found morphologic alterations in AEB071-treated cells harboring GNAQ mutations but not wild-type GNAQ or normal melanocytes (Fig. 1C). Treated Mel202, 92.1, and Omm1.3 cells lost spindle shape and became flattened with increased size or round floating dead cells with condensed cytoplasm.

AEB071 induces G1 arrest in GNAQ-mutated uveal melanoma cells

To better understand growth inhibitory effect of AEB071 on GNAQ-mutated cells, we investigated whether cell-cycle progression was altered by drug exposure. AEB071 markedly increased the G1 phase population whereas decreasing the S-phase population in uveal melanoma cells harboring GNAQ mutations (Fig. 2A and B). There was no significant change in the cell-cycle pattern for cell lines carrying wild-type GNAQ (C918, Mel285, and Ocm3). A decrease in the S-phase population with a concomitant increase in the G2–M phase population was observed in Ocm1 cells. In agreement with this G1 arrest, AEB071 also significantly increased the accumulation of p27Kip1, while decreasing the expression of cyclin D1 in all 3 GNAQ-mutated cell lines tested (Fig. 2C). In comparison, the expression of p27Kip1 and cyclin D1 was not significantly altered by AEB071 in GNAQ wild-type cells. These findings suggest that AEB071 selectively induced G1 arrest in GNAQ-mutated cells through altering the expression of regulators critical for the G1 to S transition.

AEB071 induces apoptosis in GNAQ-mutated uveal melanoma cells

We next examined whether AEB071 promoted apoptosis in uveal melanoma cells. Treatment with 2 and 5 μmol/L AEB071 for 72 hours significantly increased Annexin V-positive (apoptotic) populations in GNAQ-mutated 92.1 and Omm1.3 cells (Fig. 3A). Further
demonstration of apoptosis was observed with caspase-3 cleavage in treated cells (Fig. 3B). For Mel202 cells, only a minimal increase in the Annexin V–positive cell population was observed after AEB071 treatment (Fig. 3A), but significant caspase-3 cleavage was induced (Fig. 3B). In contrast, AEB071 did not increase Annexin V–positive cell populations or caspase-3 cleavage in uveal melanoma cells harboring wild-type GNAQ (Fig. 3A and B). AEB071 further inhibited the expression of the antiapoptotic proteins survivin, Bcl-xL and XIAP, in a dose-dependent manner in Mel202, 92.1, and C918 cells (Fig. 3C). Under the same conditions, survivin, Bcl-xL, and XIAP were not affected or were modestly increased in GNAQ wild-type uveal melanoma cells (Fig. 3C). These results indicate that AEB071 selectively induced apoptosis in uveal melanoma cells harboring GNAQ mutations.

AEB071 inhibits expression and phosphorylation of PKC isoforms in uveal melanoma cells

To better understand differential responses of uveal melanoma cells to AEB071 based on GNAQ mutational status, we examined inhibition of PKC isoforms by AEB071 in uveal melanoma cells. Immunoblotting showed that treatment with AEB071 for 6 hours in the absence of serum resulted in decreased PKCβ/θ Ser643/676 phosphorylation in Mel202, 92.1, and C918 cells and PKCβ Thr505 phosphorylation in Mel202 cells (Fig. 4A). It has been reported that expression of PKC isoforms can be downregulated by PKC inhibition (21). We thus examined the expression of PKC isoforms after prolonged AEB071 treatment (5 μmol/L for 24 hours) in the presence of 10% FBS. Uveal melanoma cell lines showed varying reduction in PKC expression after AEB071 treatment (Fig. 4B). PKCα was reduced in some wild-type (C918 and Ocm1) and in GNAQ-mutated (92.1 and Omm1.3) cells, whereas PKCβ was reduced only in mutated cells (Mel202 and 92.1). PKCθ was decreased in all GNAQ-mutated but only in one wild-type (Ocm3) cells. PKCe was downregulated in all 3 GNAQ wild-type cell lines and 2 mutated cell lines (Mel202 and 92.1). These findings suggest that AEB071 may have greater overall inhibitory effect on multiple PKC isoforms in uveal melanoma cells with mutated GNAQ: PKCα, β, δ, ε, and/or θ expression was suppressed by AEB071 in GNAQ-mutated cells whereas PKCα and PKCβ expression was affected in GNAQ wild-type cells.

PKC isoforms are functionally important for uveal melanoma cells harboring GNAQ mutations

As the expression of PKCα and δ was suppressed by AEB071 in both GNAQ wild-type and mutated uveal melanoma cells, we next investigated whether these isoforms were of functional importance for uveal melanoma cells harboring GNAQ mutations.

### Table 1. Uveal melanoma cell lines used and their GNAQ mutational status

<table>
<thead>
<tr>
<th>GNAQ Codon 209</th>
<th>Cell lines</th>
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<tbody>
<tr>
<td>Wild-type</td>
<td>C918, Mel285, Ocm1, Ocm3</td>
</tr>
<tr>
<td>Mutation</td>
<td>Mel202, 92.1, Omm1.3</td>
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Figure 2. AEB071 induces G1 arrest in uveal melanoma cells harboring GNAQ mutations. A, uveal melanoma cells were treated with DMSO or 5 μmol/L AEB071 for 24 hours and subjected to cell-cycle analysis. B, bar graphs of percentages of G1, S, and G2–M populations. C, AEB071 selectively increased p27 and decreased cyclin D1 expression in GNAQ mutant uveal melanoma cells. Cells were treated with 0, 2, or 5 μmol/L AEB071 for 72 hours and analyzed by immunoblot. WT, GNAQ wild-type; MT, GNAQ mutation.
melanoma cells by shRNA-mediated knockdown to better understand the mechanisms for the differential AEB071 response in GNAQ wild-type and mutated cells. PKCα and PKCδ expression was significantly downregulated by their shRNA in C918 and Mel202 cells (Fig. 4C). Interestingly, knockdown of PKCα or PKCδ significantly decreased viability of Mel202 and Omm1.3 cells, but failed to significantly decrease the viability of C918 cells (Fig. 4D), indicating that PKCα and PKCδ are functionally important in uveal melanoma cells harboring GNAQ mutations. We have previously found that PKC isoforms β, ε, and θ are functionally critical for GNAQ-mutated uveal melanoma cells (17). These findings together suggest that AEB071 suppressed growth of GNAQ-mutated uveal melanoma cells via inhibition of multiple PKC isoforms.

**AEB071 selectively decreases Erk1/2 phosphorylation in uveal melanoma cells harboring GNAQ mutations**

To further define the molecular mechanisms underlying the antiproliferative action of AEB071, we next assessed downstream effectors of PKC-mediated pathways that were potentially affected by AEB071. It has been previously reported that PKCα can activate the phosphoinositide 3-kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK) pathways as well as GSK3β Ser9 phosphorylation. The PKC inhibitor enzastaurin inhibits Akt and GSK3β Ser9 phosphorylation in some types of cancer cells (21–26). Similarly, AEB071 decreased GSK3β Ser9 phosphorylation in all uveal melanoma cell lines studied here (Supplementary Fig. S2). However, AEB071 only significantly inhibited Erk1/2 phosphorylation in GNAQ mutant cells whereas it had minimal effect on Akt phosphorylation in both GNAQ wild-type and mutated cells (Fig. 5). Total Akt and Erk1/2 levels were not significantly altered by AEB071 in any of the cell lines examined. These findings show AEB071-induced selective inhibition of the PKC/MAPK pathway in uveal melanoma cells carrying GNAQ mutations. We have found that Erk1/2 inhibition decreased uveal melanoma cell viability (19). Therefore, AEB071 may exert its antiproliferative effects in part through suppression of Erk1/2 activation in GNAQ-mutated uveal melanoma cells.

**AEB071 selectively inhibits NF-κB activity in GNAQ-mutated uveal melanoma cells**

PKC isoforms are involved in GPCR-mediated NF-κB activation (27–30). In most cells, NF-κB is present as a latent, inactive, IκB-bound complex in the cytoplasm. On activation, IκB is phosphorylated and targeted to degradation. The released NF-κB translocates into the nucleus where it drives the expression of its target genes (29). It is well known that aberrant NF-κB activity promotes tumorigenesis and metastasis (31). We next examined whether AEB071 altered NF-κB activity in uveal melanoma cells. In the absence of AEB071, nuclear p65 (RelA) levels are more or less similar among wild-type and mutant cell lines (Fig. 6A). In contrast, in the presence of AEB071, nuclear p65 levels were significantly decreased in GNAQ-mutated cells whereas they were minimally altered in wild-type cells (Fig. 6A). Consistent with this, reduced DNA binding activity of NF-κB was detected only in the nuclear extracts of GNAQ-mutated cells exposed to AEB071 (Fig. 6B). In agreement with
AEB071 selectively inhibited NF-κB in C918 cells (Fig. 6D). These findings together indicate that GNAQ-mutated Omm1.3 cells but not GNAQ wild-type is also in agreement with the selective downregulation of melanoma cells harboring GNAQ mutations. This notion of NF-κB is functionally important for uveal melanoma cells, both GNAQ wild-type and mutant uveal melanoma cell lines were treated with AEB071 for 72 hours and analyzed for the expression of Erk1/2 and Akt and their phosphorylation by immunoblot analyses. WT, GNAQ wild-type; MT, GNAQ mutation.

Figure 5. AEB071 selectively inhibited Erk1/2 phosphorylation in GNAQ-mutated uveal melanoma cells. Cells were treated with AEB071 for 72 hours and analyzed for the expression of Erk1/2 and Akt and their phosphorylation by immunoblot analyses. WT, GNAQ wild-type; MT, GNAQ mutation.

NF-κB target genes including survivin, Bcl-xL, XIAP, and cyclin D1, by AEB071 in GNAQ-mutated cells (Figs. 2C and 3C).

**NF-κB activity is functionally important for uveal melanoma cells**

To determine whether NF-κB activity is functionally important for uveal melanoma cells, both GNAQ wild-type and mutated uveal melanoma cells were treated with an inhibitor of IkB kinase (IKK)1/2 inhibitor that possesses potent NF-κB inhibition activity (32). This treatment resulted in the dramatic decrease in viability of both GNAQ wild-type and mutant uveal melanoma cell lines (Supplementary Fig. S3). These findings suggest that NF-κB activity is critically important for uveal melanoma cells and its suppression contributes to AEB071-induced growth inhibition of uveal melanoma cells harboring GNAQ mutations.

**Discussion**

There are currently no drugs available for targeting the oncogenic GNAQ mutations that occur frequently in
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Figure 6. AEB071 selectively inhibited NF-κB activation in uveal melanoma cells harboring GNAQ mutations. A, immunoblot analysis of nuclear RelA (p65) levels of uveal melanoma cells treated with 0 or 5 μmol/L AEB071 for 40 hours. HDAC1 (histone deacetylase 1) was used as loading control for nuclear extracts (56). B, EMSA analysis of NF-κB DNA-binding activity in the same nuclear extracts used in A. NF-κB/DNA complexes were determined as described in Materials and Methods. C, AEB071 increased IκBα protein levels in uveal melanoma cells with mutated GNAQ. Cells were treated with DMSO or 5 μmol/L AEB071 for 20 hours and subjected to immunoblot analysis of IκBα. D, AEB071 reduced IL-6 secretion from Omm1.3 cells. Cells were treated with DMSO or 5 μmol/L AEB071 for 72 hours. Medium was collected for ELISA analysis of IL-6. Data are presented as mean ± SD of percent secretion of 3 independent experiments. * P < 0.05.

primary and metastatic uveal melanoma. In the present study, we describe the first small-molecule inhibitor that selectively exhibits antiproliferative activity of uveal melanoma cells harboring GNAQ mutations: the novel PKC inhibitor AEB071 reduced viability of GNAQ-mutated uveal melanoma cell lines, but had little effect on those carrying wild-type GNAQ. AEB071-induced growth inhibition is associated with reduced expression of PKC isoforms α, β, δ, ε, and/or θ, accompanied by inhibition of Erk1/2 phosphorylation, and NF-κB activation. We have previously shown that PKCβ, PKCβ, and PKCδ are functionally important for GNAQ-mutated uveal melanoma cells and that inhibition of Erk1/2 by MAP–ERK kinase (MEK)1/2 inhibitors reduced uveal melanoma cell viability (17). Here, we show that PKCα, PKCδ, and NF-κB are also functionally important for GNAQ-mutated uveal melanoma cells. Together, our findings suggest that AEB071 may selectively exert antiproliferative activity on GNAQ-mutated uveal melanoma cells via targeting the PKC/Erk1/2 and PKC/NF-κB pathways.

AEB071-induced growth suppression of GNAQ mutant cells is associated with pronounced G1 arrest and induction of apoptosis. The molecular mechanisms for AEB071-induced G1 arrest involve altered expression of positive and negative regulators of transition through G1 phase of cell cycle, including cyclin D1 and p27Kip1 (33, 34). AEB071-induced apoptosis is associated with decreased expression of antiapoptotic proteins, yet the underlying molecular mechanisms remain to be revealed.

The PI3K/Akt and MAPK pathways are frequently activated in malignant tumors and are critical for cancer cell survival and proliferation (35–37). Erk1/2 activation is common in uveal melanoma and has been reported to play a crucial role in uveal melanoma development (38, 39). AEB071 inhibited Erk1/2 phosphorylation in GNAQ mutated but not GNAQ wild-type uveal melanoma cells, and had minimal impact on Akt phosphorylation. Similarly, we have recently found that PKC inhibitor enzastaurin inhibits phosphorylation of Erk1/2 but not Akt in GNAQ-mutated cell lines (17), although enzastaurin has been reported to inhibit Akt phosphorylation but not Erk1/2 phosphorylation in other types of cancer cells (24, 26, 40). The inhibition of Erk1/2 phosphorylation is therefore likely a common mechanism for the antiproliferative action of PKC inhibitors in GNAQ-mutated uveal melanoma cells. Further studies are needed to identify the PKC isoform(s) that are most crucial for Erk1/2 phosphorylation in GNAQ mutant uveal melanoma, and whose inhibition by AEB071 leads to decreased Erk1/2 phosphorylation. PKCβII and PKCβ are among the candidates, because activation of these isoforms triggers several signaling pathways including MAPK (9, 13, 17, 41), and siRNA downregulation of PKCβII decreased Erk1/2 phosphorylation in metastatic hepatocellular carcinoma cells (42).

In addition to the PKC/Erk1/2 pathway, activation of many GPCRs can trigger the PKC/IKK/NF-κB pathway through G proteins, including GNAQ (27–30, 43).
Overexpression of mutant GNAQ(Q209L) leads to constitutive activation of NF-κB, which is mediated by PKCδ and to a lesser extent PKCcα and PKCc in human umbilical vein endothelial cell (HUVEC; refs. 27, 28). It is not known whether the same PKC isoforms have comparable roles in uveal melanoma cells. Importantly, we show that AEB071 selectively repressed NF-κB activation in uveal melanoma cells harboring GNAQ mutations and this is accompanied by downregulation of multiple PKC isoforms, in particular PKCα, PKCδ, and PKCc. The downregulation of these PKC isoforms may contribute to AEB071 suppression of NF-κB activity. Comparison studies using an IKK inhibitor corroborate that the NF-κB pathway has critical functional roles in uveal melanoma cells. NF-κB inhibition might therefore be another mechanism for the antiproliferative action of AEB071 on uveal melanoma cells harboring GNAQ mutations. The association between PKC inhibition and decreased NF-κB activity in GNAQ-mutated uveal melanoma cells suggests that these cells might rely on GNAQ/PKC/NF-κB pathways for NF-κB activation. Other pathways presumably regulate NF-κB activation in GNAQ wild-type cells. For example, Ocm1 and Ocm3 cells have been shown to carry the common V600E BRAF mutation that constitutively activates the MAPK and NF-κB pathways (19, 20, 44–46). High c-Met expression has been found in C918 and Mel285 cells (47) and it has been reported that NF-κB can be activated by hepatocyte growth factor (HGF)/c-Met signaling (48, 49).

Our data indicate that multiple PKC isoforms including α, β, δ, ε, and θ are suppressed by AEB071 in GNAQ-mutated uveal melanoma cells whereas only PKCα and PKCδ were affected in GNAQ wild-type cells. The findings from shRNA knockdown studies confirmed the functional importance of PKCα and PKCδ in GNAQ-mutated uveal melanoma cells. Knockdown of these 2 PKC isoforms had no (PKCα) or little (PKCδ) affect on viability of GNAQ wild-type cells, suggesting that these 2 PKC isoforms are less important in GNAQ wild-type cells than in GNAQ-mutated cells. Similarly, our previous shRNA-mediated knockdown studies have found that PKCβ, PKCc, and PKCb are functionally more important for GNAQ-mutated than wild-type uveal melanoma cells (17). Together, these findings suggest that GNAQ-mutated uveal melanoma cells are more dependent on these PKC isoforms than GNAQ wild-type cells and that AEB071 exerts antiproliferative action on GNAQ-mutated cells via suppression of these PKC isoforms. These findings also provide a plausible explanation for the differential response/sensitivity of GNAQ wild-type and mutated uveal melanoma cells to AEB071.

GPCRs have a pivotal role in many physiologic functions and in multiple diseases, including tumorigenesis and metastasis of cancers (50). Frequent somatic mutations in GPCRs have been found in melanoma and mutations in GRM3, which is a glutamate receptor and a member of the metabolic GPCRs, activate the MAPK/ERK pathway, and promote growth and migration of melanoma cells (51). Furthermore, overexpression of diverse GPCRs were found in number of primary and metastatic cancers including melanoma, breast, prostate, non–small cell lung cancer, gastric tumors, head and neck squamous cell carcinoma, and diffused large B-cell lymphoma (52, 53). GPCRs can also influence cancer progression through cross-talk with growth factor receptors such as EGFR and insulin-like growth factor-1R, G-proteins (such as Gaq/11 and Ga12/13), chemokines, Hedgehog and WNT signaling pathways, regulation of the apoptotic response and viral factors. Various GPCRs can be involved in cancer through activating NF-κB (50). As more data linking GPCRs to cancer emerge, these receptors are attractive potential targets for tumor therapy. In particular, the GPCR/ERK and GPCR/NF-κB pathways could be valuable targets for innovative anticancer drug discovery.

We show that PKC inhibitor AEB071 (sotastaurin) at low micromolar concentrations exerts significant antiproliferative effect on GNAQ-mutated uveal melanoma cells through targeting the PKC/MAPK and PKC/NF-κB pathways. Our findings support PKCs as important targets for therapeutic intervention of uveal melanoma harboring GNAQ mutations. Clinical studies have shown that a blood concentration of 4 μmol/L was achieved within 5 hours after an oral dose of 500 mg AEB071 (18). AEB071 could thus be of therapeutic potential for uveal melanoma with GNAQ mutations. A clinical trial with AEB071 in patients with uveal melanoma is currently enrolling patients. AEB071 has primarily been studied as an anti-inflammatory agent for the treatment of diseases such as psoriasis and in preventing solid organ rejection following transplant (54, 55). How the immunosuppressive effects of this drug may influence the clinical efficacy will have to be determined, and monitoring of changes in antitumor immune responses as a function of treatment should be helpful to discern such changes. Given that preclinical models for uveal melanoma are difficult and mostly comprise xenograft studies, much of these questions will likely be answered by clinical investigation. There may be future opportunities to combine AEB071 or other PKC inhibitors with other rational small-molecule inhibitors (e.g., MEK or PI3K) or immune therapy such as ipilimumab to improve the efficacy and durability of any clinical activity. It would be also of great interest to investigate antitumor effects of other specific PKC inhibitors in melanoma and other cancers with GPCR mutations that may activate MAPK/ERK and/or NF-κB pathways.

Disclosure of Potential Conflicts of Interest
F.S. Hodi has served as a nonpaid consultant to Novartis and received clinical trial support from Novartis. No potential conflicts of interest were disclosed by the other authors.

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Conception and design: X. Wu, F.S. Hodi
Development of methodology: X. Wu, J.A. Fletcher, F.S. Hodi
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): X. Wu, J. Li, M. Zhu, J.A. Fletcher, F.S. Hodi
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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): X. Wu, M. Zhu, J.A. Fletcher, F.S. Hodi
Writing, review, and/or revision of the manuscript: X. Wu, M. Zhu, J.A. Fletcher, F.S. Hodi
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.A. Fletcher, F.S. Hodi
Study supervision: F.S. Hodi

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Molecular Cancer Therapeutics

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