Oncogene (2014) **33,** 4724–4734

www.nature.com/onc

**ORIGINAL ARTICLE**

# Combined PKC and MEK inhibition in uveal melanoma with GNAQ and GNA11 mutations

X Chen[1,2], Q Wu[2], L Tan[3], D Porter[3], MJ Jager[4], C Emery[3] and BC Bastian[1,2]

Uveal melanoma (UM) is a genetically and biologically distinct type of melanoma, and once metastatic there is no effective treatment currently available. Eighty percent of UMs harbor mutations in the $G\alpha_q$ family members GNAQ and GNA11. Understanding the effector pathways downstream of these oncoproteins is important to identify opportunities for targeted therapy. We report consistent activation of the protein kinase C (PKC) and MAPK pathways as a consequence of GNAQ or GNA11 mutation. PKC inhibition with AEB071 or AHT956 suppressed PKC and MAPK signalling and induced G1 arrest selectively in melanoma cell lines carrying GNAQ or GNA11 mutations. In contrast, treatment with two different MEK inhibitors, PD0325901 and MEK162, inhibited the proliferation of melanoma cell lines irrespective of their mutation status, indicating that in the context of GNAQ or GNA11 mutation MAPK activation can be attributed to activated PKC. AEB071 significantly slowed the growth of tumors in an allograft model of $GNAQ^{Q209L}$-transduced melanocytes, but did not induce tumor shrinkage. *In vivo* and *in vitro* studies showed that PKC inhibitors alone were unable to induce sustained suppression of MAP-kinase signaling. However, combinations of PKC and MEK inhibition, using either PD0325901or MEK162, led to sustained MAP-kinase pathway inhibition and showed a strong synergistic effect in halting proliferation and in inducing apoptosis *in vitro*. Furthermore, combining PKC and MEK inhibition was efficacious *in vivo*, causing marked tumor regression in a UM xenograft model. Our data identify PKC as a rational therapeutic target for melanoma patients with GNAQ or GNA11 mutations and demonstrate that combined MEK and PKC inhibition is synergistic, with superior efficacy compared to treatment with either approach alone.

*Oncogene* (2014) **33,** 4724–4734; doi:10.1038/onc.2013.418; published online 21 October 2013

**Keywords:** GNAQ/GNA11; MAPK signaling; protein kinase C; combined PKC and MEK inhibition; melanoma; PKC inhibitor

## INTRODUCTION

Uveal melanoma (UM) is a genetically and biologically distinct type of melanoma that arises from choroidal melanocytes, that is, melanocytes of the choroidal plexus, ciliary body and iris of the eye. UM is the most common intraocular malignancy in adults and accounts for about 5% of all melanomas.[1–3] Currently, there are no effective treatment options for patients with metastatic uveal melanoma, and the median survival for a UM patient after diagnosis with metastasis is less than 6 months.[1,4] Different from melanomas originating from the skin, UM does not harbor mutations in BRAF, NRAS or KIT, but instead shows mutations in GNAQ or GNA11. Over 80% of uveal melanomas harbor mutations in these genes in a mutually exclusive pattern.[5–7] The two genes encode closely-related large GTPases of the Gαq family, which are (together with β and γ subunits) components of heterotrimeric G proteins that transfer signaling through certain types of G-protein coupled receptors to downstream effector proteins.[8,9] In the absence of agonist binding to the G-protein coupled receptors, the α subunit is bound to GDP and in an inactive configuration. Agonist binding to the G-protein coupled receptors results in a conformational change of the receptor leading the α subunit to exchange GDP to GTP. The GTP-bound α subunit becomes activated and dissociates from βγ subunits to interact with specific effector proteins. The intrinsic GTPase activity determines the half-life of the activated GTP-bound α subunit. GNAQ and GNA11 mutations in melanoma affect codons 209 (approximately 95%) or 183 (5%) and result in complete or partial loss of GTPase activity, respectively, thereby leading to constitutive activation of downstream effector pathways.[10,11] Downstream effectors of $G\alpha_q$ family members include PLC-β isoforms, which hydrolyze PI(4,5)P2 to release inositol trisphosphate (IP3) and diacylglycerol (DAG) from membrane phospholipids. Both compounds act as second messengers that relay and amplify the signaling to downstream components such as release of calcium (IP3) and activation of DAG-responsive proteins.

It has been shown that mutant GNAQ and GNA11 activate the MAP-kinase pathway.[5,6] However, the specific nature of the oncogenic signaling that results from constitutively activated GNAQ and GNA11 remains incompletely understood. The canonical signaling pathway downstream of Gαq family members includes activation of protein kinase C (PKC).[9,12] Both DAG and calcium activate members of the PKC family, which is considered a critical hub in distributing signaling to downstream pathways that regulate differentiation, cell proliferation, apoptosis and angiogenesis.[9,12,13,14] The PKC family consists of at least 10 serine/threonine kinases, which are subdivided into classic, novel and atypical isoforms.[14] The classical PKCs (α, βI, βII and γ) are DAG and calcium-dependent enzymes, while the novel PKCs

[1]Human Oncology and Pathogenesis Program, Memorial Sloan Kettering Cancer Center, New York, NY, USA; [2]Departments of Dermatology and Pathology, and Helen Diller Family Comprehensive Cancer Center, University of California, San Francisco, CA, USA; [3]Novartis Institutes for BioMedical Research, Novartis, Cambridge, MA, USA and [4]Department of Ophthalmology, Leiden University Medical Center, Leiden, The Netherlands. Correspondence: Dr C Emery, Novartis Institutes for BioMedical Research, Cambridge, MA 02139, USA or Dr BC Bastian, Departments of Dermatology and Pathology, and Helen Diller Family Comprehensive Cancer Center, University of California, San Francisco, CA 94143, USA.
E-mail: caroline.emery@novartis.com or Boris.Bastian@ucsf.edu
Received 2 July 2013; revised 14 August 2013; accepted 15 August 2013; published online 21 October 2013

(δ, ε, θ and η) require only DAG for activation. By contrast, the atypical PKCs (ζ, ι/λ) are not responsive to activation by DAG or calcium, but are activated by other lipid-derived second messengers.

PKCs are involved in regulating a variety of cell functions, including differentiation, cell proliferation, apoptosis and angiogenesis.[13,14] The role of PKC in tumorigenesis was first established when they were identified as the cellular target of phorbol esters. Phorbol esters, most prominently 12-O-tetradecanoylphorbol-13-acetate (TPA), are molecular mimics of DAG, which are more potent and not metabolized rapidly.[15–17] While aberrant PKC activity and expression have been reported in multiple cancers,[14,18,19] no specific genetic alterations leading to constitutive activation of the PKC pathway have been found, and clinical trials with PKC inhibitors in cancer have not shown impressive results. The mechanisms by which PKCs contribute to malignancy, however, are still not clear, in part due to the complexities of PKC.

Previous studies have shown that UM cell lines with GNAQ mutations have an increased sensitivity to PKC inhibition *in vitro*.[20,21] However, the relationship between PKC and MAP-kinase pathway activation in the setting of GNAQ or GNA11 mutations is not fully resolved. It also is not clear whether inhibition of PKC can be translated into meaningful anti-tumor responses *in vivo*.

Here we demonstrate that the activation of PKC is a direct consequence of activating mutations in GNAQ and GNA11 and confirm that inhibition of PKC leads to selective growth arrest in UM cell lines with mutations in GNAQ and demonstrate that these findings extend to cell lines with mutant GNA11. We further show that MAP-kinase pathway activation occurs as a consequence of PKC activation and can be partially or temporarily suppressed by PKC inhibitors, slowing tumor growth. However, a significantly improved response can be obtained by combined PKC and MEK inhibition, which synergistically result in sustained growth inhibition and apoptosis *in vitro* and a markedly enhanced anti-tumoral response *in vivo*. We propose combinatorial inhibition of PKC and MAP-kinase signaling as a rational therapeutic approach for treating melanomas with GNAQ or GNA11 mutations.

## RESULTS

### Oncogenic GNAQ and GNA11 activate PKC and MAPK signaling in both mouse and human melanocytes

To explore the role of PKC as a mediator of the oncogenic output downstream of mutant GNAQ or GNA11, we examined PKC signaling in a panel of melanoma cell lines, with or without activating mutations in GNAQ or GNA11. We performed western blots using an antibody against phosphorylated myristolated alanine-rich C kinase substrate (p-MARCKS), a substrate of PKC.[22–25] All six melanoma cell lines with oncogenic GNAQ or GNA11 mutations (GNA11: UPMD-1; OMM-GN11; GNAQ: Mel270; OMM1.3; 92–1 and Mel202) expressed p-MARCKS levels, whereas three of four UM cell lines without mutations in GNAQ and GNA11 (Mel290, MUM2C and Mel285) showed no expression and the fourth (C918) expressed only trace levels. Two of three cutaneous melanoma cell lines (Sk-Mel-28:$BRAF^{V600E}$, MM485:$NRAS^{Q61L}$ and MM415:$NRAS^{Q61L}$) that also had no mutations in GNAQ and GNA11 expressed p-MARCKS (Figure 1a). We performed the following experiments to determine whether the PKC activation in the UM cell lines with oncogenic GNAQ or GNA11 was a consequence of these mutations. First, we introduced Glu–Glu epitope-tagged $GNAQ^{Q209L}$ or $GNA11^{Q209L}$ into three different cell types, 293T cells, immortalized mouse (melan-a) and immortalized human melanocytes (IHM), and determined the effects on p-MARCKS compared to control cells expressing either GFP, Glu-Glu tagged $GNAQ^{wt}$, Glu-Glu tagged $GNA11^{wt}$, $BRAF^{V600E}$ or myc-tagged $NRAS^{G12V}$. As shown in Figure 1b, expression of $GNAQ^{Q209L}$ or $GNA11^{Q209L}$ consistently resulted in increased p-MARCKS levels in all three cell types. By contrast, wild-type GNAQ or GNA11 had weak effects, whereas mutant BRAF or NRAS or the GFP control had no effect on p-MARCKS. We ruled out that these differences were due to variation in the expression levels of the transfection constructs using antibodies against the Glu–Glu tag of GNA11 or GNAQ protein, respectively. As shown in Figure 1b, the expression levels of $GNAQ^{Q209L}$ and $GNA11^{Q209L}$ were consistently similar or lower compared to the expression levels of the wild-type GNAQ and GNA11 proteins in all three different cell types. To confirm that p-MARCKS induction indeed reflected PKC activation by mutant GNAQ or GNA11, we also used an antibody that detects specific phosphorylation motifs of PKC (Arg/Lys–X–$Ser^{phos}$–Hyd–Arg/Lys, where Hyd represents a hydrophobic residue). This experiment revealed an increase in the phosphorylation level of several proteins in cells transduced with $GNAQ^{Q209L}$ and $GNA11^{Q209L}$ compared to controls, in a pattern consistent throughout the three cell types (Supplementary Figure 1, arrows). Mutant BRAF or NRAS also led to the appearance of certain bands, but their pattern was distinct and not consistent between the three cells. Taken together, these experiments indicate consistent activation of PKC in UM cell lines, and that this activation is due to mutations in GNAQ and GNA11.

The experiments in Figure 1b also showed a concomitant increase in pERK and p-p90RSK levels in all three cell lines expressing $GNAQ^{Q209L}$and $GNA11^{Q209L}$, indicative of MAPK pathway activation in response to GNAQ or GNA11 mutation, consistent with prior reports.[5,6] As expected, $BRAF^{V600E}$ and $NRAS^{G12V}$ also resulted in an increase in pERK and p-p90RSK, but did not affect the levels of p-MARCKS. Similar results were also obtained with melan-a cell lines generated to stably express GFP, $GNAQ^{WT}$, $GNA11^{WT}$, $GNAQ^{Q209L}$, $GNA11^{Q209L}$ or $BRAF^{V600E}$, respectively (Supplementary Figures 2A and B). This constellation raised the possibility that in the context of mutant GNAQ and GNA11 the MAP-kinase pathway activation is significantly mediated by PKC activation. To confirm this notion and to further corroborate the role of PKC signaling as an effector of mutant GNAQ or GNA11, we performed a series of experiments in UM cell lines that harbored GNAQ or GNA11 mutations.

### GNAQ knockdown suppressed PKC and MAPK signaling in melanoma cells harboring GNAQ activating mutations

We knocked down the expression of GNAQ with small interfering RNA (siRNAs) in three different melanoma cells (OMM1.3, 92-1 and Mel202) with GNAQ mutation and found PKC and MAPK signaling to be suppressed 72 h after knockdown. As shown in Figure 1c, knockdown of GNAQ inhibited the expression of pMEK, pERK, pMARCKS and p-(ser) PKC substrate compared to non-target siRNA control in all three cell lines. Similar results were also observed with shRNA-mediated knockdown of GNAQ. 92–1 cells were stably transduced with lentiviruses expressing one of three different GNAQ shRNAs or GFP as a control to determine the effect of GNAQ knockdown. As shown in Supplementary Figure 3 (left panel), shRNA1 and shRNA3, which knocked down GNAQ protein most efficiently, also had the strongest effect in reducing the expression levels of p-MARCKS, pERK and the p-(ser)PKC substrate. In contrast, GNAQ shRNA2, which did not deplete GNAQ protein, had no effect on the levels of pMARCKS, p-ERK and p-(ser)PKC substrate. Similar results were obtained when melan-a cells, stably expressing $GNAQ^{Q209L}$, were transduced with lentiviruses encoding GNAQ shRNA 1 and GNAQ shRNA3 (Supplementary Figure 3, right panel).

### The PKC inhibitors AEB071 and AHT956 selectively inhibit the growth of GNAQ or GNA11 mutant melanoma cell lines

To investigate whether PKC inhibition could inhibit growth in melanoma cell lines with GNAQ or GNA11 mutations, we evaluated the antiproliferative effect of two distinct ATP-competitive PKC

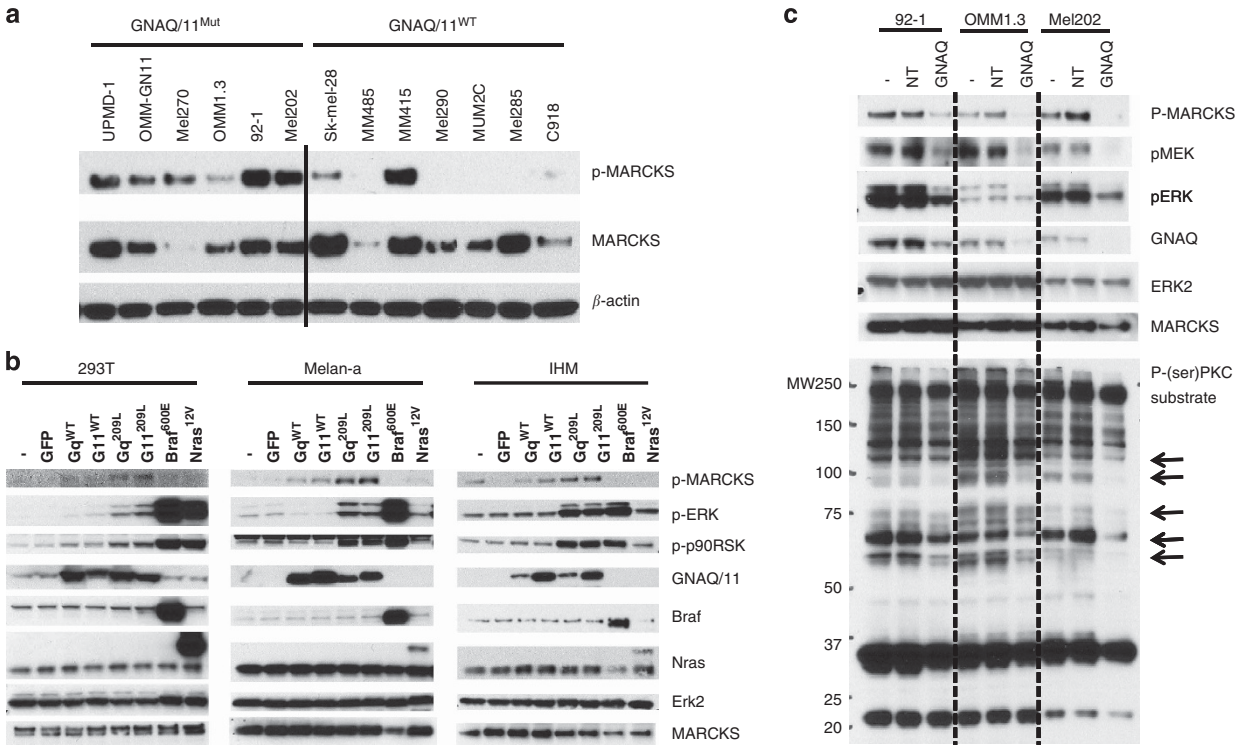


**Figure 1.** GNAQ$^{Q209L}$ or GNA11$^{Q209L}$ induces activation of PKC and MAPK signaling in melanocytes. (**a**) Increased phosphorylation of the PKC substrate MARCKS in human melanoma cell lines with GNAQ (Mel270, OMM1.3, 92-1, Mel202) or GNA11 (UPMD-1, OMM-GN11) mutations, compared to cell lines with mutations other than GNAQ or GNA11. (**b**) GNAQ$^{Q209L}$ or GNA11$^{Q209L}$ compared to wild-type GNAQ and GNA11 induces activation of the PKC pathway read out by p-MARCKS and MAP-kinase pathways read out by p-ERK and p-p90RSK in three different cells: immortalized human embryonic kidney cells (293T), mouse melanocytes (melan-a) and immortalized human melanocytes. In comparison, mutant BRAF and NRAS only activate the MAP-kinase pathway. 293T cells were transfected with the respective plasmids for 24 h, whereas melanocytes were infected with lentiviruses for 48 h. Expression levels of GNAQ and GNA11 were monitored using an antibody detecting the Glu-Glu tag. The NRAS antibody detects both endogenous and the larger myc-tagged NRAS proteins (upper bands). (**c**) Depletion of GNAQ reduces PKC and MAPK signaling. Western blots of three human uveal melanoma cell lines with GNAQ mutation were transfected with non-targeting siRNAs (NT), siRNAs against GNAQ or mock for 72 h and lysed. Note decreased expression of p-MARCKS, p-ERK, p-MEK and certain bands detected by the p-(ser) PKC substrate motif antibody (arrows) with only siRNAs against GNAQ.

inhibitors, AEB071[26,27] and AHT956. We used two panels of six human melanoma cell lines with and without GNAQ or GNA11 mutations, respectively, and three mouse melan-a cell lines stably expressing GNAQ$^{Q209L}$, GNA11$^{Q209L}$ or BRAF$^{V600E}$. Without exception, human and mouse melanoma cell lines carrying mutations of GNAQ or GNA11 expressed a significantly higher sensitivity to both AEB071 (Figure 2a and Supplementary Figures 4A and B, top panel) and AHT956 (Figure 2b and Supplementary Figures 4A and B, lower panel), compared with melanoma cell lines without these mutations. The $IC_{50}$ ranged from 56 to 467 nM for AEB071 (Figure 2a) and from 4 nM to 123 nM for AHT956 (Figure 2b) in the mutant lines. By contrast, the cell lines without GNAQ or GNA11 mutations invariably were insensitive to both compounds at doses up to 1 μM. The selective effect of AEB071 and AHT956 on cells with GNAQ or GNA11 mutations was also reflected in the growth curves of the cell lines, with GNAQ-mutant cells revealing a dose-dependent growth inhibition compared to cell lines without GNAQ or GNA11 mutations (Figure 2a right panel and Figure 2b right panel).

In contrast to the selective effect of PKC inhibitors on GNAQ/11 mutant melanoma cell lines, the MEK inhibitor PD0325901 inhibited melanoma cell lines independent of mutation status (Supplementary Figure 5 and Figure 2c). To explore the mechanism of the selective growth-inhibitory effects in response to PKC inhibition, we treated GNAQ mutant UM cell lines with 500 nM AEB071 or 100 nM AHT956 and analyzed the effect on cell proliferation and death. The doses were selected to be above their respective $IC_{50}$ values. In the GNAQ-mutant 92-1 and OMM1.3 cells, DNA synthesis was significantly suppressed by AEB071 and AHT956 but was unaffected in the two GNAQ wild-type cells (Supplementary Figure 6A). This coincided with a G1 arrest in the GNAQ mutant but not wild-type cell lines, as evidenced by an increase in the G1 population and concomitant decrease in the fraction of cells in S phase (Supplementary Figure 6B). The G1 arrest in the GNAQ-mutant cell lines was accompanied by a decrease of phospho- and total RB, downregulation of cyclinD1 and induction of p27, all supporting G1 cell cycle arrest in response to treatment, whereas the level of these proteins remained unaffected in the GNAQ wild-type cell lines (Supplementary Figure 6C). By contrast, the cell cycle effects of the MEK inhibitor PD0325901 are independent of the mutation status of GNAQ (Supplementary Figure 6D).

### In melanoma cell lines with GNAQ or GNA11 mutation MAPK activation occurs as a consequence of PKC activation

The results above indicate that mutations in GNAQ and GNA11 may result in a selective dependency on PKC activation, which could be of therapeutic relevance to treat cancers with these mutations. To confirm that effects of the compounds reflected an on-target effect, we investigated whether their effects on cell growth were paralleled by corresponding effects on the pathways downstream of oncogenic GNAQ or GNA11 (Figure 1). We performed dose–response studies of two panels of four melanoma

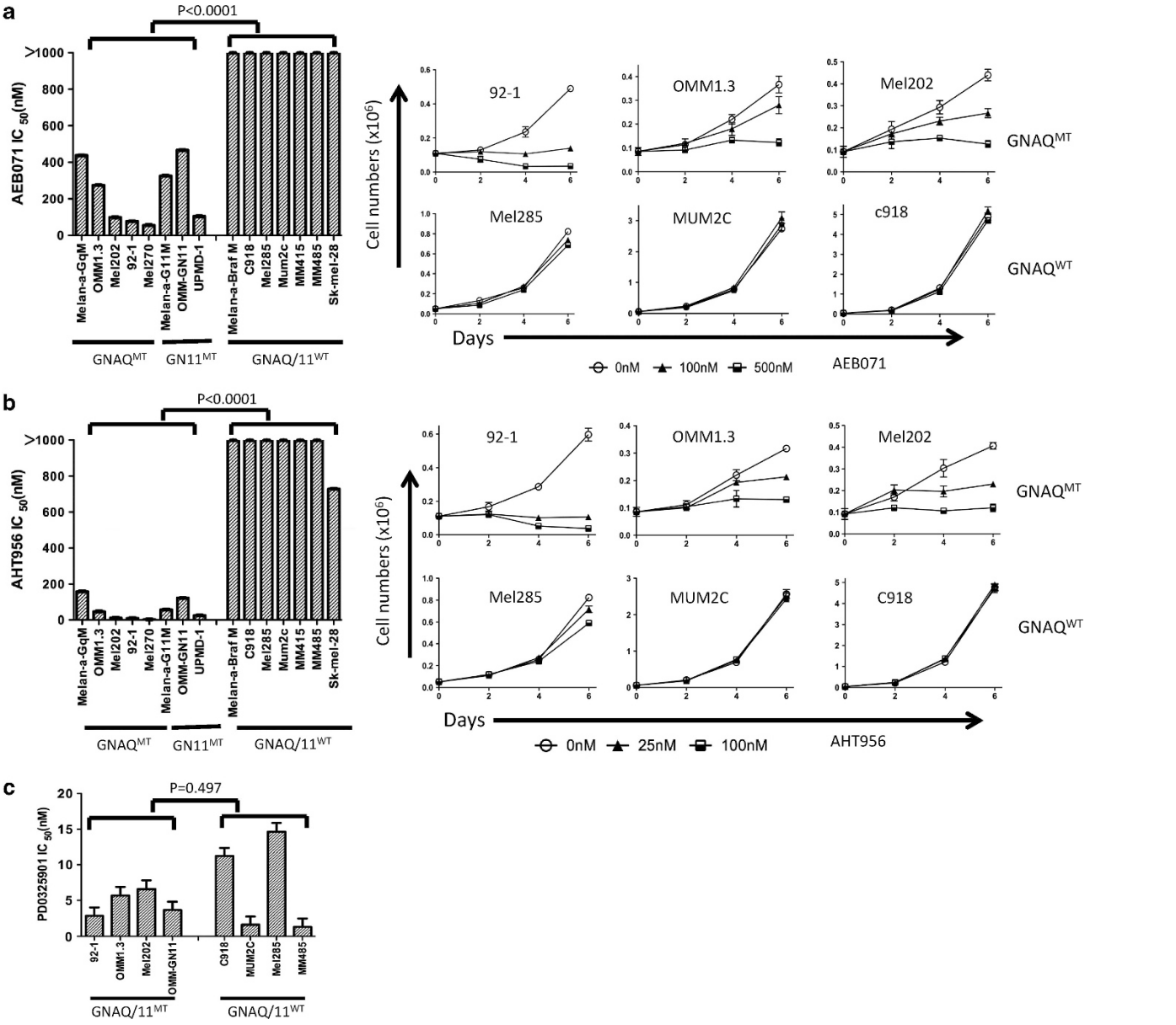


**Figure 2.** The PKC inhibitors AEB071 and AHT956, but not the MEK inhibitor PD0325901, selectively inhibit proliferation of melanoma cell lines with GNAQ or GNA11 mutations. (**a**) The left panel shows the $IC_{50}$ of the cell lines treated for 4 days with AEB071 sorted by mutation status. The right panel shows the growth kinetics of three representative cell lines with or without GNAQ mutations, respectively, with two different concentrations of AEB071 (100 nM, 500 nM) compared to vehicle (DMSO) over 6 days. Cells were counted at day 0, 2, 4 and 6. (**b**) The panels show a similar experiment as in (**a**) using the PKC inhibitor AHT956. (**c**) $IC_{50}$ values for the melanoma cell lines treated with the MEK inhibitor PD0325901. Data represent the mean ± s.e.m.

cell lines with and without GNAQ or GNA11 mutation by exposing them to increasing doses (0–1 μM) of AEB071 or AHT956 for 24 h. Both compounds, in a dose-dependent manner, abrogated PKC and MAPK signaling, as determined by suppressed levels of p-MARCKS, p-ERK and p-p90RSK in all four GNAQ/11 mutant lines (Figures 3a and b, top panel). In contrast, the compounds had no effect on MAPK signaling in the four cell lines without GNAQ/11 mutations, although they reduced p-MARCKS at higher concentrations in two (AEB071) or three (AHT956) of the four cell lines (Figures 3a and b, lower panels). When we treated two of the GNAQ mutant cells (mel202 and 92–1) with the MEK inhibitor PD0325901, we observed the expected dose-dependent inhibition of MAPK signaling, but no effect on p-MARCKS levels, even at higher concentrations (Figure 3c). In aggregate, our findings indicate that in melanoma cell lines with GNAQ or GNA11 mutations, MAPK pathway activation occurs downstream of PKC.

### AEB071 inhibits $GNAQ^{Q209L}$-mediated tumor growth *in vivo*

Our data establish PKC activation by oncogenic GNAQ/11 as a critical effector required for melanoma cell growth *in vitro*. To determine its effect on GNAQ-mediated cell growth *in vivo*, we used an allograft model with melan-a cells stably expressing $GNAQ^{Q209L}$ injected subcutaneously into c57BL/6 mice. Melan-a cells were originally derived from c57BL/6 mice and $GNAQ^{Q209L}$-transduced melan-a cells cause rapidly growing tumors in syngeneic mice with histopathological features recapitulating human melanocytic neoplasms with GNAQ mutations.[5] As shown

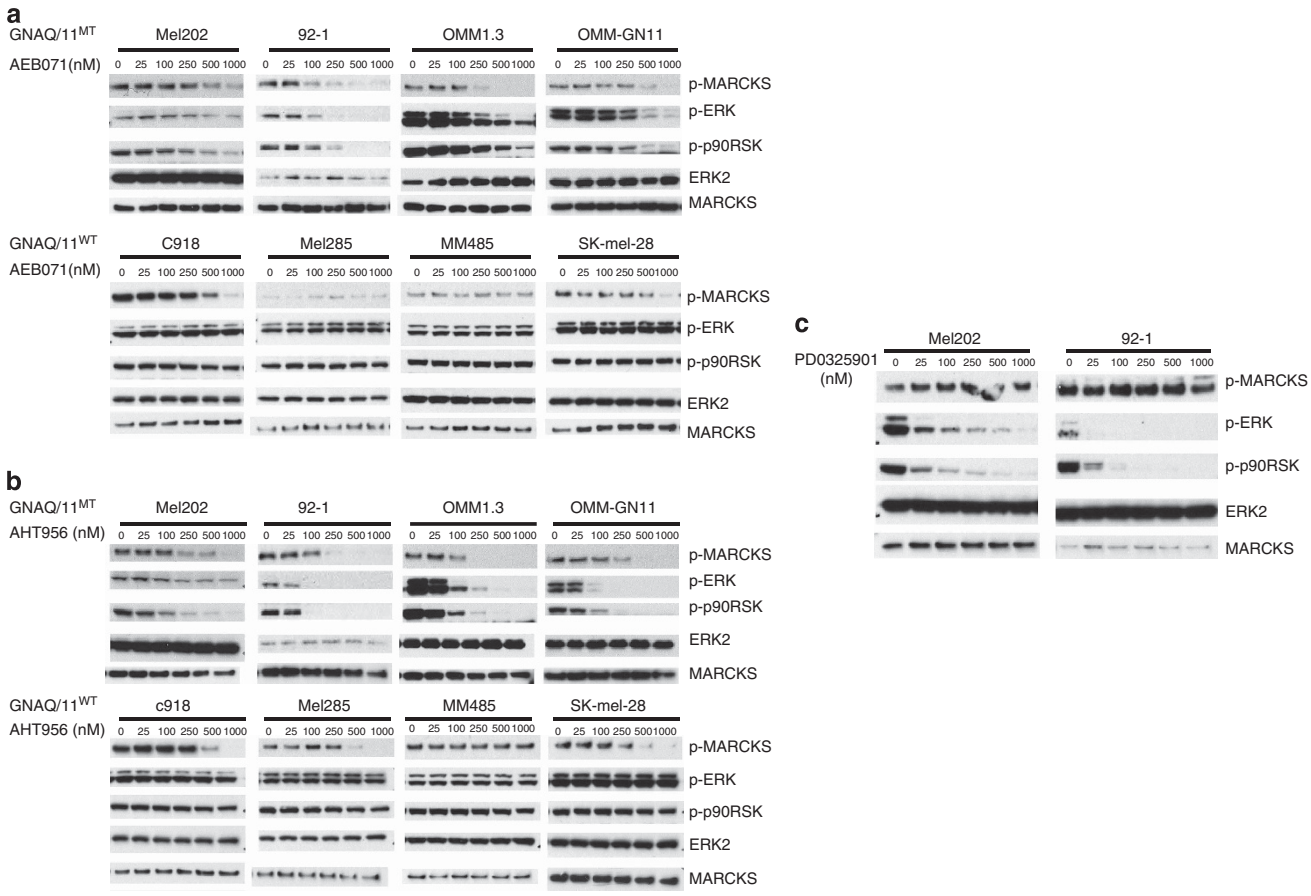


**Figure 3.** PKC inhibition suppresses MAP-kinase signaling in melanoma cells with GNAQ or GNA11 mutations but not vice versa, indicating that in the context of GNAQ and GNA11 mutations the MAPK resides downstream of PKC. In GNAQ or GNA11 mutant melanoma cell lines AEB071 (**a**) and AHT956 (**b**) blocked PKC and MAPK signaling in a concentration-dependent manner. Western blots of four GNAQ or GNA11 mutant cell lines (top panel) and cell lines without GNAQ or GNA11 mutations (lower panel) treated with increasing concentrations of AEB071 for 24 h probed. High-sensitivity detection reagents were used to visualize the significantly weaker pMARCKS levels in MM485, c918 and Mel285 cells. (**c**) By contrast, PD0325901 effectively abrogates MAPK signaling but has no effects on PKC signaling in melanoma cell lines with GNAQ mutation.

in Figure 4a, oral treatment of tumor-bearing mice with 120 mg/kg AEB071 three times daily elicited a significant growth retardation (58%: $P<0.05$) but no tumor regression compared to vehicle-treated controls. There was no apparent toxicity or change in body weight in the treatment compared to the control arm (data not shown). At the end of the experiment, tumors were excised 2 h after the last treatment, and tumor cell lysates were examined by western blot. There was a substantial reduction in PKC signaling, as evidenced by a decrease in p-MARCKS (Figure 4b, left panel) and p-(ser) PKC substrate levels (Figure 4b, right panel) in tumors from the treatment group compared to vehicle-treated controls. By contrast, no substantial reduction of pMEK and pERK levels (Figure 4b) or evidence of apoptosis was noted in AEB071-treated tumors (data not shown). These results indicate that AEB071 inhibits PKC signaling *in vivo*, but that compensatory mechanisms prevent the suppression of the MAP-kinase pathway and that the treatment is not sufficient to completely stop cell proliferation or kill cells.

### Incomplete suppression of MAPK signaling after long-term treatment with AEB071 in GNAQ-mutant human melanoma cell lines

Incomplete suppression or reactivation of the MAPK pathway under treatment is a well-studied phenomenon in melanomas with BRAF mutations following administration of BRAF inhibitors and can occur by several mechanisms linked to resistance.[28–30] In order to follow MAPK pathway output under continued treatment with AEB071, we performed a time-course experiment with AEB071 in GNAQ-mutant cell lines. As shown in Figure 5a, treatment of both 92-1 and OMM1.3 cells with 250 nM AEB071 resulted in a sustained inhibition of pMARCKS over the 72 h of the experiment. By contrast, there was no complete extinction of MAPK signaling as indicated by sustained expression of pERK and pMEK. Maximal inhibition was observed at about 24 h, with a subsequent subtle increase starting 48 h after treatment in both cell lines. Similar results were observed at twice the concentration of AEB071 (Figure 5b).

### Combined PKC and MEK inhibition synergistically kills GNAQ/11 mutant melanoma cells by induction of apoptosis

Combination of an RAF inhibitor with a MEK inhibitor is a successful strategy to increase pathway inhibition and treatment response in patients with BRAF-mutant melanomas.[31] Considering incomplete suppression of MAPK signaling with PKC inhibition alone, we attempted to improve treatment efficacy and inhibition of MAPK signaling using a combination of PKC and MEK inhibition. We exposed two GNAQ mutants, one GNA11 mutant and two melanoma cell lines without either mutation to combinations of AEB071 and PD0325901 at doses ranging from 0 to 5 μM and 0 to 0.5 μM, respectively, and determined the effect on

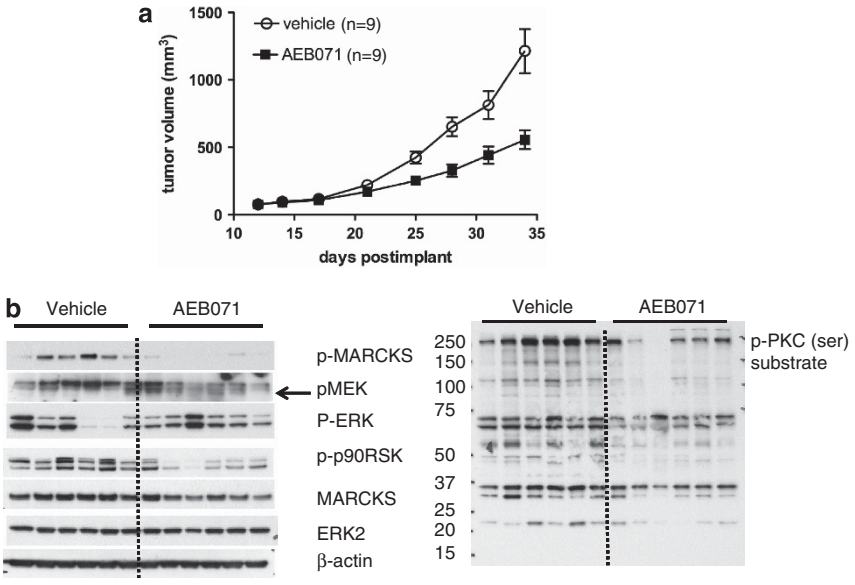


**Figure 4.** Treatment with the PKC inhibitor AEB071 significantly inhibits $GNAQ^{Q209L}$-mediated tumor growth *in vivo*. (**a**) Melan-a cells stably expressing $GNAQ^{Q209L}$ were implanted into the flanks of c57Bl/6 mice and allowed to reach a tumor volume of about 100 $mm^3$. Mice were then treated by oral gavage with AEB071 (120 mg/kg, TID) or vehicle control ($n = 9$ per group). Data represent the tumor volume mean ± s.e.m. (**b**) Western blot of tumor lysates from mice treated with vehicle ($n = 6$) or AEB071 ($n = 6$) revealed reduced PKC signaling read out by p-MARCKS (left panel) or p-PKC(ser)substrate (right panel) in the treatment arm with no effect on pMEK and pERK expression levels.

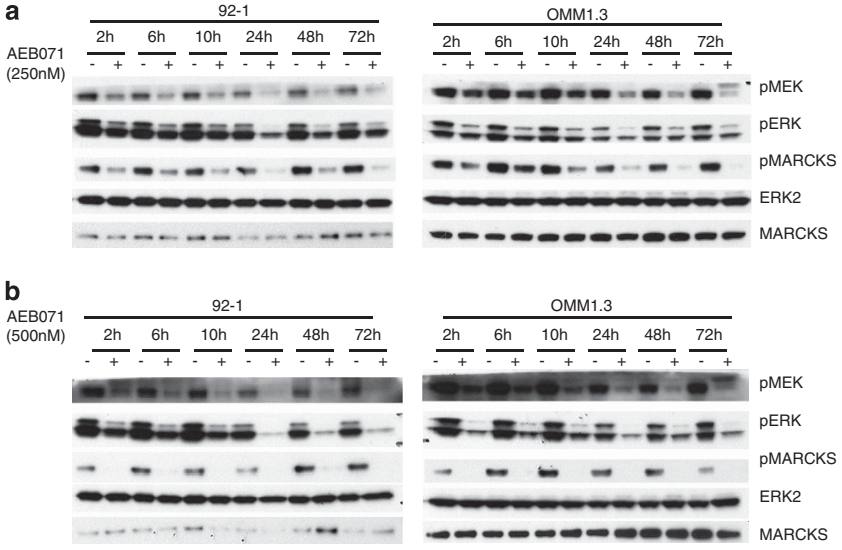


**Figure 5.** Incomplete suppression of MAP-kinase signaling under long-term PKC inhibition. (**a**) 92-1 or OMM1.3 cells were incubated with DMSO or 250 nM AEB071 for the times indicated. p-ERK levels were maximally suppressed at 24 h treatment with AEB071 in both cell lines, with a subsequent slight increase of pERK levels at later time points. By contrast, pMARCKS levels remained suppressed, indicating ongoing inhibition of PKC signaling. (**b**) Repeat experiment with 500 nM of AEB071 showing an identical pattern.

cell viability after 4 days. The 64 different combinations for each cell are depicted in a dose matrix (Figure 6a) where growth inhibition relative to non-treated cells is visualized using a color scale. We observed an improved growth inhibition of the combinations in all three melanoma cells with GNAQ/11 mutation (92-1, OMM1.3 and OMM-GN11) compared to treatments with either compound alone. Isobologram analysis[32] (Figure 6b) and Combination Index (Supplementary Figure 7) revealed that the effects of AEB071 and PD0325901 were strongly synergistic. By contrast, no improved effect of the combination was seen in melanoma cells without GNAQ/11 mutations (MUM2C and Mel285).

A combination of 250 nM AEB071 and 20 nM PD0325901 inhibited proliferation of 92-1 and OMM1.3 to a greater extent, compared to the individual compounds at these concentrations (Figure 6c). In 92-1 cells and less pronounced in OMM1.3 cells, a reduction of cell numbers was also observed, indicating that the combination caused cell death (Figure 6c). As shown in Figure 6d, the combination of AEB071 and PD0325901 resulted in a significant increase in cleaved PARP level, supporting this notion. The combination treatment resulted in near-complete extinction of pMEK and pERK expression in all three melanoma cells with GNAQ or GNA11 mutation, compared to treatment with the individual inhibitors alone. In contrast, the combination did not increase levels of cleaved PARP or reduce pMEK and pERK levels in the three control cell lines without GNAQ and GNA11 mutations. Similar synergistic results were also observed with a combination

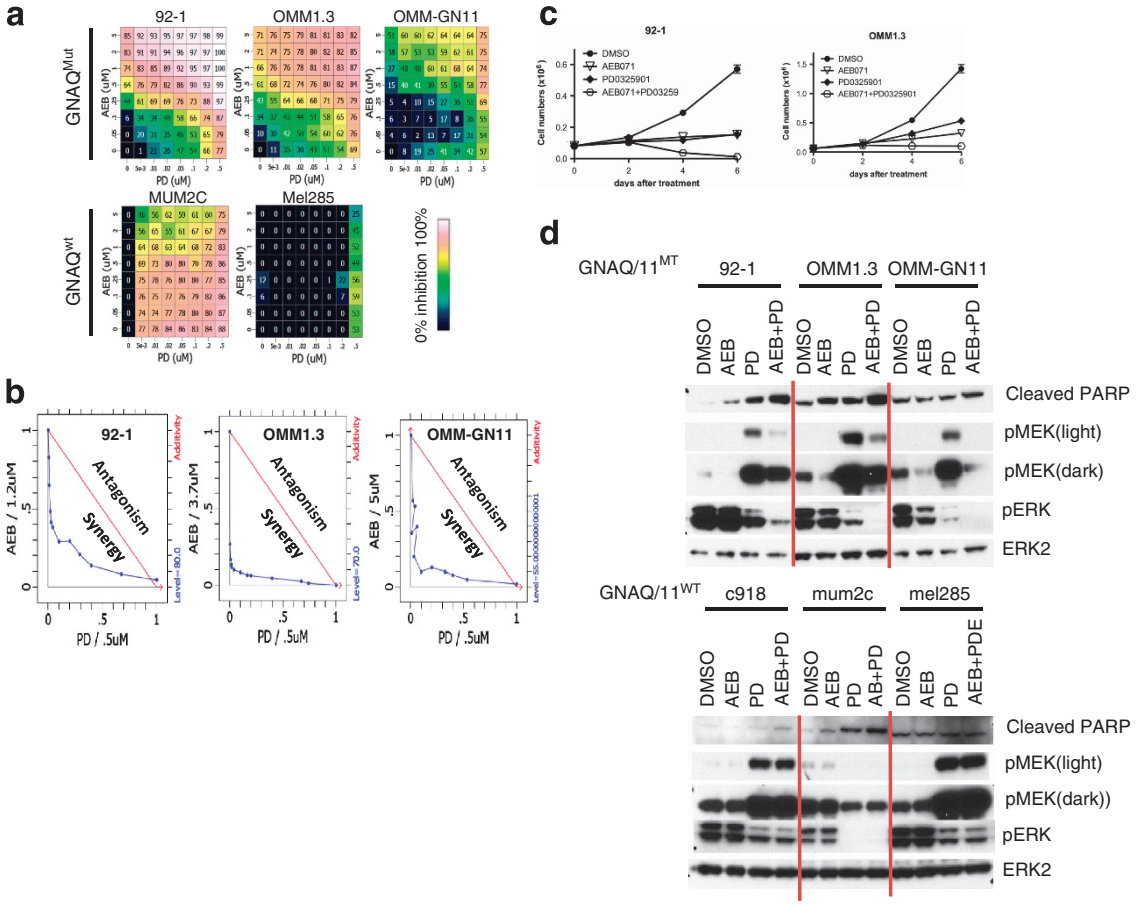


**Figure 6.** Combined PKC and MEK inhibition leads to sustained blockade of MAP-kinase signaling and induction of apoptosis. (**a**) Drug dose matrix data of three different melanoma cell lines with GNAQ or GNA11 mutation and two without either mutation. The numbers in the individual cells indicate the percentage of growth inhibition of cells treated for 96 h with the corresponding compound combination relative to vehicle control-treated cells. The data were visualized over matrix using a color scale. (**b**) Isobologram analysis of the data in (**a**) indicates strong synergy for the combination in GNAQ or GNA11 mutant cell lines. The effect level for the isobologram display and combination index calculations on the y-axis is chosen to display the maximum level. (**c**) Growth kinetics of cell lines in the presence of a combination of PKC and MEK inhibitor compared to single agent alone. The indicated cell lines were incubated with AEB071 (250 nM), PD0325901 (20 nM), combination of 250 nM AEB071 and 20 nM PD0325901 or vehicle (DMSO). Cells were counted on days 0, 2, 4 and 6. (**d**) A combination of AEB071 and PD0325901 led to sustained suppression of pMEK and pERK levels and induction of apoptosis as evidenced by increased levels of cleaved PARP in melanoma cells with GNAQ or GNA11 mutations. Cells were incubated with DMSO, AEB071, PD0325901 or AEB071 plus PD0325901. Media were changed after 24 h and cells were lysed after treatment for 48 h. For 92-1 and OMM1.3 cells, the drug concentrations used were AEB071 250 nM and PD0325901 100 nM. For OMM-GN11, c918, MUM2C, Mel285, the drug concentrations used were AEB071 1000 nM and PD0325901 100 nM.

of AEB071 and another MEK inhibitor MEK162 (Supplementary Figure 8A and B). Only the combination of AEB071 and MEK162 yielded sustained suppression of pMEK and pERK in UM cell lines with GNAQ or GNA11 mutations (Supplementary Figure 9). Interestingly, we observed that single-agent treatment with one of two different MEK inhibitors (PD0325901 or MEK162) resulted in the accumulation of pMEK in GNAQ and GNA11 mutant melanoma cells due to relieved negative feedback on the MAPK pathway (Figure 6d and Supplementary Figure 9). This observation is comparable and consistent with the findings in BRAF wild-type melanomas treated by MEK inhibitors.[33] However, this increase in pMEK could be suppressed by concomitant administration of AEB071 in the GNAQ and GNA11 mutant cells, whereas it had no effect in cell lines without GNAQ and GNA11 mutations (c918 and mel285) (Figure 6d and Supplementary Figure 9).

Taken together, these results suggest that a combination of PKC and MEK inhibition leads to sustained blockade of MAP-kinase signaling and induction of cell death in melanoma cell lines with GNAQ or GNA11 mutations. To determine whether the synergistic effects of combined MEK and PKC inhibition translate into improved *in vivo* efficacy, we used a mouse xenograft model in which subcutaneous tumors are generated by injecting the GNAQ-mutant human UM cell line 92–1 into nude mice. After 12 days, the tumors had grown to 108–256 $mm^3$, and the mice were divided into eight groups. Treatment with AEB071 and MEK162 alone or combination with different doses was initiated for 21 days. As shown in Figure 7, AEB071 monotherapy resulted in slow growth of tumors compared to vehicle control in a dosage-dependent manner. AEB071 monotherapy at the highest dosage (TID at 80 mg/kg) inhibited tumor growth by 82% ($P<0.05$). By contrast, MEK162 monotherapy administered twice daily at 3.5 mg/kg inhibited tumor growth by about 44% compared to vehicle control. The combination treatment of AEB071 and MEK162 led to a significant reduction of tumor growth compared to monotherapy with either compound in a dose-dependent manner. At higher doses, the combination led to significant (*P*-values $<0.001$) tumor shrinkage (12% tumor regression at 40 mg/kg TID of AEB071 and 3.5 mg/kg BID MEK162 and 52% regression at 80 mg/kg TID of AEB071 and 3.5 mg/kg BID MEK162 compared to initial tumor volume before treatment). Those results

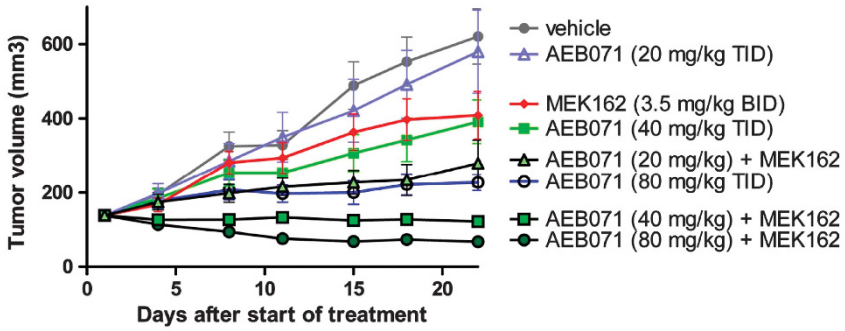


**Figure 7.** Combined PKC and MEK inhibition synergistically induced tumor regression in the 92-1 UM xenografts mouse model. The UM cell line 92-1 ($GNAQ^{Q209L}$) was implanted into the flanks of nude mice and allowed to grow tumors for 12 days. Mice were then divided into eight groups with a mean tumor volume of about 130–140 $mm^3$ per group and received vehicle control ($n = 10$), AEB071 (20 mg/kg, TID) ($n = 10$), AEB071 (40 mg/kg, TID) ($n = 10$), AEB071 (80 mg/kg, TID) ($n = 10$), MEK162 (3.5 mg/kg, BID) ($n = 10$), AEB071 (20 mg/kg, TID) + MEK162(3.5 mg/kg, BID) ($n = 10$), AEB071 (40 mg/kg, TID) + MEK162 (3.5 mg/kg, BID) ($n = 10$) and AEB071 (80 mg/kg, TID) + MEK162(3.5 mg/kg, BID) ($n = 9$) for 21 days by oral gavage. Tumor volumes were measured twice per week. Data represent the tumor volume mean ± s.e.m.

are consistent with *in vitro* data (Figure 6), suggesting that the combination of PKC and MEK inhibition has a synergistic effect on melanomas with GNAQ mutation.

## DISCUSSION

Our study highlights PKC signaling as a critical oncogenic effector pathway in melanomas with oncogenic GNAQ and GNA11 mutations and as a potential therapeutic target for UM. The importance of PKC is indicated by several lines of evidence: (1) we found a consistent increase in PKC activation across a panel of UM cell lines with GNAQ or GNA11 mutations; (2) depletion of mutant GNAQ in these lines abrogates PKC activation, whereas (3) introduction of mutant GNAQ or GNA11 into human and murine melanocytes activated PKC.

Activation of PKC has been reported in a broad range of cancer types. However, the therapeutic efficacy of PKC inhibitors in the treatment of cancer patients has been disappointing.[34–36] Our results indicate that melanoma cells with GNAQ or GNA11 mutations are selectively sensitive to PKC inhibition, consistent with the findings from other groups.[20,21] We show here that these findings extend to melanoma cell lines with GNA11 mutations, but that neither cells do express selective sensitivity to MEK inhibitors, compared to melanoma cell lines without these mutations. Using two different PKC inhibitors across a panel of six different UM cell lines with GNAQ or GNA11 mutations and two melanocyte lines stably expressing GNAQ or GNA11, the inhibitors reduced growth at $IC_{50}$s ranging from 56 to 467 nM for AEB071 and from 4 to 159 nM for AHT956, whereas melanoma cell lines with other mutations, irrespective of whether they were derived from uveal or cutaneous melanoma, were not sensitive. Interestingly, two of the latter cell lines also showed PKC activation as evidenced by expression of phospho-MARCKS (Figure 1). This indicates that in contrast to other cell lines GNAQ or GNA11 mutant cell lines are dependent on PKC activation to initiate critical growth pathways. The role of PKC activation in melanocyte proliferation is well established. The synthetic DAG analogue TPA is a potent mitogen for melanocytes, including the melan-a cells used in our study, which were derived from normal epidermal melanoblasts from embryos of C57BL/6 mice.[37] In the absence of TPA, melan-a cells do not proliferate, even when grown in full serum[37] (Supplementary Figure 10). The precise mechanism of TPA action is not clear, but it has been demonstrated to activate PKC in human melanocytes[38] and mouse melanocytes (Supplementary Figure 11A and B). In contrast to normal melan-a cells, melan-a cell lines stably expressing $GNAQ^{Q209L}$ or $GNA11^{Q209L}$ proliferate independently of TPA (Supplementary Figure 10). The observation that oncogenic GNAQ and GNA11 are able to substitute for addition of TPA to the growth medium further supports the notion that these oncogenes exert parts of their proliferation-promoting effect through activation of PKC.

Our results and published data indicate that MAP-kinase pathway activation is a critical component of oncogenic signaling in the context of mutant GNAQ or GNA11 and occurs downstream of PKC. Prior studies have shown that $G\alpha_q^{Q209L}$ can stimulate MAPK pathway via PLC-DAG-PKC, presumably by PKC-mediated phosphorylation of Raf-1.[39–41] MAP-kinase pathway activation has also been demonstrated via PLC-IP3-Ca2 + -Pyk2-Src-Ras signaling,[12] and the precise conduit of signaling in the context of mutant GNAQ or GNA11 remains to be determined. In our study with two different PKC inhibitors, PKC inhibition attenuated MAPK phosphorylation in the context of GNAQ or GNA11 mutation, whereas MEK inhibition only blocked MAPK phosphorylation, with no effect on PKC activation in GNAQ or GNA11 mutant cells (Figure 3). The experiments using the PKC substrate motif antibody revealed that introduction of mutant BRAF or NRAS also results in PKC activation, but with a different spectrum of phosphorylation targets than $G\alpha_q^{Q209L}$ (Supplementary Figure 1). As BRAF signals predominantly through the MAP-kinase pathway, in melanoma cells with BRAF mutations PKC most likely becomes activated as a consequence of MAP-kinase pathway activation, contrary to the context of mutant GNAQ or GNA11 where the MAP-kinase pathway operates downstream of PKC.

MAP-kinase pathway activation via PKC appears to represent a major proliferative stimulus in the context of GNAQ or GNA11 mutations, as both PKC or MEK inhibition led to G1 arrest with loss of phospho-RB, reduction of cyclin D1 and upregulation of the negative cell cycle regulator p27kip1. The findings are consistent with the effects of MEK inhibition in BRAF mutant melanoma cells.[42] PKC inhibition with either PKC inhibitor alone did not result in cell death in UM cell lines with GNAQ or GNA11 mutations at the doses used in our study. Wu *et al.*[20] found that AEB071 at high doses (5 μM) induced apoptosis in 92-1, mel202 and omm1.3 UM cell lines, which harbor GNAQ mutations. In the allograft experiment with melan-a cells transduced with $GNAQ^{Q209L}$, AEB071 slowed tumor growth at the maximally tolerated dose level, but did not result in tumor regression or apoptosis. These findings may have implications for the clinical use of PKC inhibitors for the treatment of patients with UM. A clinical trial with AEB071 is currently underway in patients with UM with GNAQ or GNA11 mutations. While the results of this trial are not yet available, our data indicate that AEB071 monotherapy may primarily slow tumor growth rather than induce significant tumor shrinkage. The finding that the treated tumors in our allograft

experiment continued to express pERK while PKC output continued to be suppressed points to additional complexity in the activation of the MAP-kinase pathway in the context of GNAQ or GNA11 mutation. The incomplete pERK suppression was already visible *in vitro*, where under continuous PKC inhibition the MAP-kinase pathway was only transiently suppressed. The rapid nature of the rebound makes a genetic mechanism less likely, and instead points to relieve of feedback inhibition as a potential mechanism.[43] A similar phenomenon has been observed with the use of BRAF inhibitors in melanoma cells with BRAF mutations, in which the MAP-kinase pathway becomes re-activated after 24 h of treatment. In this setting, the addition of an MEK inhibitor overcomes the rebound and leads to sustained pathway suppression.[44] The intriguing synergy between compounds targeting the same pathway in a seemingly redundant fashion has now been shown to have clinical benefits in patients with BRAF mutant melanomas.[45] These findings motivated us to investigate a combination of PKC inhibition and MEK inhibition, and we also found a strong synergistic effect of the two approaches both *in vitro* and *in vivo* (Figures 6 and 7 and Supplementary Figures 8 and 9) associated with nearly full suppression of pMEK and pERK levels in melanomas with GNAQ and GNA11 mutation. Melanomas with activated MAPK signaling mediated by oncogenes such as BRAF[46] and GNAQ/11(Figure 2c and Supplementary Figures 5 and 6D) were sensitive to MEK inhibition. However, MEK inhibitors inhibit ERK signaling in all cells including normal tissues, resulting in a reduced therapeutic index.[47] The accumulation of pMEK induced by MEK inhibition in GNAQ and GNA11 mutant cells is consistent with the findings in BRAF wild-type melanoma cells[33,48] and has been explained by the abrogation of negative feedback pathways as a consequence of MEK inhibition. Recent studies show that pMEK accumulation induced by MEK inhibitors indicates the sensitivity of cells to MEK inhibitors, indicating residual MEK activity in the presence of the inhibitor.[48] Using two different MEK inhibitors, we found that pMEK accumulation in response to MEK inhibition can be abrogated by the addition of the PKC inhibitor AEB071 (Figure 6d and Supplementary Figure 9) in the GNAQ/11 mutant cells, but not melanoma cell lines without GNAQ/11 mutations. While this could point to a role of PKC in the negative feedback loop, a simpler explanation is that PKC inhibition reduces some of the pathway flux upstream of MEK, coming from mutant GNAQ or GNA11. Although we used both classical allograft and xenograft mouse model to perform *in vivo* study for convenient and economic purpose, a liver metastatic mouse model is worth exploring in the future. We conclude that in the setting of GNAQ or GNA11 mutation the combined treatment with PKC and MEK inhibitors may improve treatment responses in patients with metastatic UM compared to either of the two compounds alone. The data provided indicate that the combination may allow reducing the doses of the individual compounds, which is expected to improve the tolerability of the drugs.

## MATERIALS AND METHODS

### Plasmids and reagents

The GNAQ and GNA11 cDNA constructs were obtained from Missouri S&T cDNA Resource Center. They are internally Glu-Glu tagged, with altered residues at positions 171–176 from AYLPTQ(GNAQ) or GYLPTQ (GNA11) to EYMPTE and were cloned into Plenti6 lentiviral vectors (Invitrogen, Grand Island, NY, USA). All constructs were sequenced for confirmation. AEB071,AHT956 and MEK162 were synthesized at Novartis Pharma AG (East Hanover, NJ, USA). PD0325901 was obtained from Chemie Tek (Indianapolis, IN, USA). G06976 was purchased from Calbiochem (La Jolla, CA, USA).

### Cell culture and cell line generation

The sources of the melanoma cell lines have been previously described.[49] Melanoma cell lines were maintained in RPMI 1640 with 10% FBS. 293T cells were cultured in DMEM with 10%FBS. Melan-a cells (a gift from Dr Dorothy Bennett, St George University, London, UK) were maintained in RPMI with 10% FBS and 200 nM TPA TPA (Sigma, St Louis, MO, USA). Immortalized human melanocytes (a gift from Dr David Fisher, Dana Farber Cancer Institute, Boston, MA, USA) were cultured in 254 media supplemented by human melanocyte growth supplements (HMGS; Invitrogen).

For generation of stable melan-a cells expressing various proteins, melan-a cells were transduced with lentiviruses expressing GFP, GNAQ $^{wt}$, GNA11$^{wt}$, GNAQ$^{Q209L}$, GNA11$^{Q209L}$ or Braf $^{V600E}$, respectively. Lentiviral transductions were performed as previously described.[6] Two hundred nanomolar TPA was added to the media for cells transduced with lentivirus expressing GFP, GNAQ$^{wt}$ or GNA11$^{wt}$, but not for cells infected with lentivirus expressing GNAQ$^{Q209L}$, GNA11$^{Q209L}$ or Braf$^{V600E}$. Forty-eight hours after lentivirus infection, cells were selected with blasticidin (10 μg/ml) for 2 weeks.

### Transient transfection, lentiviral transduction and knockdown of GNAQ by siRNA

For transient transfection, 293T cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. Cells were lysed 24 h after transfection. For lentiviral transduction, cells were infected with the respective lentiviruses and media were changed 24 h after infection to TPA-free media supplemented with 10% FBS for melan-a cells or 254 media without HMGS for IHM cells. Cells were lysed 48 h post infection.

The human GNAQ ON-TARGET plus SMARTpool siRNAs (L-008562-00-0005) and non-targeting siRNA pool (D-001810-10-05) were from Thermo Scientific (Rockford, IL, USA). Cells were transfected with 30 nM siRNAs with RNAiMAX (Invitrogen) following the manufacturer's instruction and were lysed 72 h later.

### Western blot analysis

Cells were harvested in Pierce RIPA buffer (Thermo Scientific, Waltham, MA, USA), supplemented with protease and phosphatase inhibitor cocktails (Thermo Scientific). The protein concentration of the lysates was determined using the BCA protein assay (Pierce Biotechnology, Rockford, IL, USA). 10–30 μg of protein was separated by 4–12% Bis–Tris gradient gels (Invitrogen) and transferred to Immobilon-P membrane (Millipore, Billerica, MA, USA). Primary antibodies used were as follows: p-MARCKS (#2741), p-PKC (ser)-substrate (#2261), RB (#9309), p-RB (#9307), pERK (#4370), p-MEK (9121), cleaved PARP (9541) from Cell Signaling Technology (Danvers, MA, USA); MARCKS (1944–1), p-p90RSK (#2006–1), all from Epitomics (Burlingame, CA, USA); MARCKS (M-20), BRAF(C-19), NRAS(F155), ERK2 (c-14), GNAQ (E-17), Cyclin D1 (M-20), p27 (C-19)all from Santa Cruz (Santa Cruz, CA, USA); β-actin (#A1978) from Sigma; Glu-Glu (MMS-115P) from Covance Inc. (Princeton, NJ, USA).

### Proliferation assays

Cells were plated in triplicate into six-well plates at $5 \times 10^4$ cells per well, and the following day were treated with or without the indicated concentrations of AEB071 or AHT956 or PD0325901, with media changes every day for AEB071 and AHT956, every 2 days for PD0325901. Cells were collected by trypsinization and counted in a Vi-Cell cell viability analyzer (Beckman Coulter, Brea, CA, USA) or TC10 Automated Cell Counter ( Bio-Rad, Hercules, CA, USA). $IC_{50}$ values were calculated using Prism software (La Jolla, CA, USA).

### Cell cycle analysis and BRDU labeling

Cells were seeded in six-well plates with $1.5 \times 10^5$ cells per well 1 d before treatment with AEB071 or AHT956 for 72 h. Before fixation, cells were incubated with 40 μM BRDU for 4 h and labeled with an anti-BRDU FITC antibody and 7-AAD for cell cycle assays using the FITC BRDU Flow Kit ( BD Pharmingen, San Jose, CA, USA) according to the manufacturer's instructions. Fluorescence-activated cell sorting was carried out to determine cell cycle distribution and the percentage of BRDU-positive cells using a Cell Lab Quanta flow cytometer (Beckman Coulter).

### *In vivo* allograft studies

$1 \times 10^6$ melan-a cells stably expressing GNAQ$^{Q209L}$ were injected into the flanks of c57/Bl6 mouse and allowed 10–14 days to reach a tumor volume of about 100 mm$^3$. Subsequently, the animals received 120 mg/kg AEB071 ($n = 9$) or vehicle control ($n = 9$) three times per day for 3 weeks by oral

gavage. Tumor volume was recorded twice per week. After 3 weeks of treatment, animals were euthanized and tumors were harvested and analyzed by western blotting. All animal experiments were performed in accordance with a protocol approved by the Institutional Animal Care and Use Committee of the Memorial Sloan-Kettering Cancer Center.

### Synergy analysis of drug combinations

Cells were plated in triplicate into 96-well tissue culture plates at 1000–3000 cells per well, depending on doubling times. On the next day, mixtures of inhibitors were added to the cells according to the planned dose matrices. Media were changed after 2-day treatment. Cell viability was analyzed 96 h later by using CellTiter 96 Aqueous One solution (Promega Corporation, Madison, MI, USA) according to the manufacturer's instructions. Plates were read in a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA, USA).

Isobologram analysis was performed using the Chalice Multi-target Discover Software (Zalicus, Cambridge, MA, USA). The CompuSyn software (ComboSyn, Paramus, NJ, USA) was used to calculate the CI value to indicate additive or synergistic effects.[50] A CI of 1 was interpreted as additive and CI $<1$ as synergistic.

### *In vivo* drug combination analysis

The 92-1 UM cells were harvested during exponential growth and resuspended in cold PBS with 50% Matrigel (BD Biosciences, San Jose, CA, USA). Each 9-week- old female athymic nude mouse (Crl:NU(Ncr)-Foxn1nu, Charles River, Hollister, CA, USA) was inoculated subcutaneously in the right flank with $5 \times 10^6$ cells (0.2 ml of cell suspension). Tumors were calipered in two dimensions to monitor growth as their mean volume approached the desired 100–150 $mm^3$ range. Tumor size, in $mm^3$, was calculated from: tumor volume $= (w^2 \times l)/2$, where $w$ is the width and $l$ the $=$ length, in mm, of the tumor. Twelve days after tumor cell implantation, on Day 1 of the study, animals with individual tumor volumes of 108–256 $mm^3$ were sorted into eight groups ($n = 10$/group) with group mean tumor volumes of 130–140 $mm^3$. Subsequently, the animals received different treatment for 21 days by oral gavage. Tumor volumes were measured twice per week till the end of the study. The animal program at DRS-NC is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International, which assures compliance with accepted standards for the care and use of laboratory animals.

## CONFLICT OF INTEREST

Carrie Emery, Dale Porter and Lujian Tan are employees of Novartis. The other authors declare no conflict of interest.

## ACKNOWLEDGEMENTS

This work was supported by grant CA142873 from the National Cancer Institute and a Melanoma Research Alliance Team Science Award.

## REFERENCES

1 Singh AD, Bergman L, Seregard S. Uveal melanoma: epidemiologic aspects. *Ophthalmol Clin North Am* 2005; **18**: 75–84.
2 Egan KM, Seddon JM, Glynn RJ, Gragoudas ES, Albert DM. Epidemiologic aspects of uveal melanoma. *Surv Ophthalmol* 1988; **32**: 239–251.
3 McLean IW, Saraiva VS, Burnier Jr MN. Pathological and prognostic features of uveal melanomas. *Can J Ophthalmol* 2004; **39**: 343–350.
4 Gragoudas ES, Egan KM, Seddon JM, Glynn RJ, Walsh SM, Finn SM *et al.* Survival of patients with metastases from uveal melanoma. *Ophthalmology* 1991; **98**: 383–389 (discussion 390).
5 Van Raamsdonk CD, Bezrookove V, Green G, Bauer J, Gaugler L, O'Brien JM *et al.* Frequent somatic mutations of GNAQ in uveal melanoma and blue naevi. *Nature* 2009; **457**: 599–602.
6 Van Raamsdonk CD, Griewank KG, Crosby MB, Garrido MC, Vemula S, Wiesner T *et al.* Mutations in GNA11 in uveal melanoma. *N Engl J Med* 2010; **363**: 2191–2199.
7 Lamba S, Felicioni L, Buttitta F, Bleeker FE, Malatesta S, Corbo V *et al.* Mutational profile of GNAQQ209 in human tumors. *PLoS One* 2009; **4**: e6833.
8 Dorsam RT, Gutkind JS. G-protein-coupled receptors and cancer. *Nat Rev Cancer* 2007; **7**: 79–94.
9 Hubbard KB, Hepler JR. Cell signalling diversity of the Gqalpha family of heterotrimeric G proteins. *Cell Signal* 2006; **18**: 135–150.
10 Markby DW, Onrust R, Bourne HR. Separate GTP binding and GTPase activating domains of a G alpha subunit. *Science* 1993; **262**: 1895–1901.
11 Kalinec G, Nazarali AJ, Hermouet S, Xu N, Gutkind JS. Mutated alpha subunit of the Gq protein induces malignant transformation in NIH 3T3 cells. *Mol Cell Biol* 1992; **12**: 4687–4693.
12 Goldsmith ZG, Dhanasekaran DN. G protein regulation of MAPK networks. *Oncogene* 2007; **26**: 3122–3142.
13 Mackay HJ, Twelves CJ. Targeting the protein kinase C family: are we there yet? *Nat Rev Cancer* 2007; **7**: 554–562.
14 Griner EM, Kazanietz MG. Protein kinase C and other diacylglycerol effectors in cancer. *Nat Rev Cancer* 2007; **7**: 281–294.
15 Castagna M, Takai Y, Kaibuchi K, Sano K, Kikkawa U, Nishizuka Y. Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. *J Biol Chem* 1982; **257**: 7847–7851.
16 Kikkawa U, Takai Y, Tanaka Y, Miyake R, Nishizuka Y. Protein kinase C as a possible receptor protein of tumor-promoting phorbol esters. *J Biol Chem* 1983; **258**: 11442–11445.
17 Teicher BA. Protein kinase C as a therapeutic target. *Clin Cancer Res* 2006; **12**: 5336–5345.
18 Bosco R, Melloni E, Celeghini C, Rimondi E, Vaccarezza M, Zauli G. Fine tuning of protein kinase C (PKC) isoforms in cancer: shortening the distance from the laboratory to the bedside. *Mini Rev Med Chem* 2011; **11**: 185–199.
19 Urtreger AJ, Kazanietz MG, Bal de Kier Joffe ED. Contribution of individual PKC isoforms to breast cancer progression. *IUBMB Life* 2012; **64**: 18–26.
20 Wu X, Li J, Zhu M, Fletcher JA, Hodi FS. Protein kinase C inhibitor AEB071 targets ocular melanoma harboring GNAQ mutations via effects on the PKC/Erk1/2 and PKC/NF-kappaB pathways. *Mol Cancer Ther* 2012; **11**: 1905–1914.
21 Wu X, Zhu M, Fletcher JA, Giobbie-Hurder A, Hodi FS. The protein kinase C inhibitor enzastaurin exhibits antitumor activity against uveal melanoma. *PLoS One* 2012; **7**: e29622.
22 Herget T, Oehrlein SA, Pappin DJ, Rozengurt E, Parker PJ. The myristoylated alanine-rich C-kinase substrate (MARCKS) is sequentially phosphorylated by conventional, novel and atypical isotypes of protein kinase C. *Eur J Biochem* 1995; **233**: 448–457.
23 Blackshear PJ. The MARCKS family of cellular protein kinase C substrates. *J Biol Chem* 1993; **268**: 1501–1504.
24 Heemskerk FM, Chen HC, Huang FL. Protein kinase C phosphorylates Ser152, Ser156 and Ser163 but not Ser160 of MARCKS in rat brain. *Biochem Biophys Res Commun* 1993; **190**: 236–241.
25 Thelen M, Rosen A, Nairn AC, Aderem A. Regulation by phosphorylation of reversible association of a myristoylated protein kinase C substrate with the plasma membrane. *Nature* 1991; **351**: 320–322.
26 Wagner J, von Matt P, Sedrani R, Albert R, Cooke N, Ehrhardt C *et al.* Discovery of 3-(1H-indol-3-yl)-4-[2-(4-methylpiperazin-1-yl)quinazolin-4-yl]pyrrole-2,5-dione (AEB071), a potent and selective inhibitor of protein kinase C isotypes. *J Med Chem* 2009; **52**: 6193–6196.
27 Naylor TL, Tang H, Ratsch BA, Enns A, Loo A, Chen L *et al.* Protein kinase C inhibitor sotrastaurin selectively inhibits the growth of CD79 mutant diffuse large B-cell lymphomas. *Cancer Res* 2011; **71**: 2643–2653.
28 Johannessen CM, Boehm JS, Kim SY, Thomas SR, Wardwell L, Johnson LA *et al.* COT drives resistance to RAF inhibition through MAP kinase pathway reactivation. *Nature* 2010; **468**: 968–972.
29 Nazarian R, Shi H, Wang Q, Kong X, Koya RC, Lee H *et al.* Melanomas acquire resistance to B-RAF(V600E) inhibition by RTK or N-RAS upregulation. *Nature* 2010; **468**: 973–977.
30 Montagut C, Sharma SV, Shioda T, McDermott U, Ulman M, Ulkus LE *et al.* Elevated CRAF as a potential mechanism of acquired resistance to BRAF inhibition in melanoma. *Cancer Res* 2008; **68**: 4853–4861.
31 Flaherty KT, Infante JR, Daud A, Gonzalez R, Kefford RF, Sosman J *et al.* Combined BRAF and MEK inhibition in melanoma with BRAF V600 mutations. *N Engl J Med* 2012; **367**: 1694–1703.
32 Lehar J, Krueger AS, Avery W, Heilbut AM, Johansen LM, Price ER *et al.* Synergistic drug combinations tend to improve therapeutically relevant selectivity. *Nat Biotechnol* 2009; **27**: 659–666.
33 Pratilas CA, Taylor BS, Ye Q, Viale A, Sander C, Solit DB *et al.* (V600E)BRAF is associated with disabled feedback inhibition of RAF-MEK signaling and elevated transcriptional output of the pathway. *Proc Natl Acad Sci USA* 2009; **106**: 4519–4524.
34 Millward MJ, House C, Bowtell D, Webster L, Olver IN, Gore M *et al.* The multikinase inhibitor midostaurin (PKC412A) lacks activity in metastatic melanoma: a phase IIA clinical and biologic study. *Br J Cancer* 2006; **95**: 829–834.
35 Gronberg BH, Ciuleanu T, Flotten O, Knuuttila A, Abel E, Langer SW *et al.* A placebo-controlled, randomized phase II study of maintenance enzastaurin

following whole brain radiation therapy in the treatment of brain metastases from lung cancer. *Lung Cancer* 2012; **78**: 63–69.
36 Clement-Duchene C, Natale RB, Jahan T, Krupitskaya Y, Osarogiagbon R, Sanborn RE *et al.* A phase II study of enzastaurin in combination with erlotinib in patients with previously treated advanced non-small cell lung cancer. *Lung Cancer* 2012; **78**: 57–62.
37 Bennett DC, Cooper PJ, Hart IR. A line of non-tumorigenic mouse melanocytes, syngeneic with the B16 melanoma and requiring a tumour promoter for growth. *Int J Cancer* 1987; **39**: 414–418.
38 Arita Y, O'Driscoll KR, Weinstein IB. Growth of human melanocyte cultures supported by 12-O-tetradecanoylphorbol-13-acetate is mediated through protein kinase C activation. *Cancer Res* 1992; **52**: 4514–4521.
39 Kolch W, Heidecker G, Kochs G, Hummel R, Vahidi H, Mischak H *et al.* Protein kinase C alpha activates RAF-1 by direct phosphorylation. *Nature* 1993; **364**: 249–252.
40 Schonwasser DC, Marais RM, Marshall CJ, Parker PJ. Activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase pathway by conventional, novel, and atypical protein kinase C isotypes. *Mol Cell Biol* 1998; **18**: 790–798.
41 Ueda Y, Hirai S, Osada S, Suzuki A, Mizuno K, Ohno S. Protein kinase C activates the MEK-ERK pathway in a manner independent of Ras and dependent on Raf. *J Biol Chem* 1996; **271**: 23512–23519.
42 Solit DB, Garraway LA, Pratilas CA, Sawai A, Getz G, Basso A *et al.* BRAF mutation predicts sensitivity to MEK inhibition. *Nature* 2006; **439**: 358–362.
43 Sturm OE, Orton R, Grindlay J, Birtwistle M, Vyshemirsky V, Gilbert D *et al.* The mammalian MAPK/ERK pathway exhibits properties of a negative feedback amplifier. *Sci Signal* 2010; **3**: ra90.
44 Paraiso KH, Fedorenko IV, Cantini LP, Munko AC, Hall M, Sondak VK *et al.* Recovery of phospho-ERK activity allows melanoma cells to escape from BRAF inhibitor therapy. *Br J Cancer* 2010; **102**: 1724–1730.
45 Flaherty KT, Infante JR, Daud A, Gonzalez R, Kefford RF, Sosman J *et al.* Combined BRAF and MEK inhibition in melanoma with BRAF V600 mutations. *N Engl J Med* 2012; **703**: 1694–1730.
46 Solit DB, Rosen N. Resistance to BRAF inhibition in melanomas. *N Engl J Med* 2011; **364**: 772–774.
47 Kirkwood JM, Bastholt L, Robert C, Sosman J, Larkin J, Hersey P *et al.* Phase II, open-label, randomized trial of the MEK1/2 inhibitor selumetinib as monotherapy versus temozolomide in patients with advanced melanoma. *Clin Cancer Res* 2012; **18**: 555–567.
48 Friday BB, Yu C, Dy GK, Smith PD, Wang L, Thibodeau SN *et al.* BRAF V600E disrupts AZD6244-induced abrogation of negative feedback pathways between extracellular signal-regulated kinase and Raf proteins. *Cancer Res* 2008; **68**: 6145–6153.
49 Griewank KG, Yu X, Khalili J, Sozen MM, Stempke-Hale K, Bernatchez C *et al.* Genetic and molecular characterization of uveal melanoma cell lines. *Pigment Cell Melanoma Res* 2012; **25**: 182–187.
50 Chou TC. Drug combination studies and their synergy quantification using the Chou-Talalay method. *Cancer Res* 2010; **70**: 440–446.

Supplementary Information accompanies this paper on the Oncogene website (http://www.nature.com/onc)