Combination Small Molecule MEK and PI3K Inhibition Enhances Uveal Melanoma Cell Death in a Mutant GNAQ and GNA11 Dependent Manner.

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Running Title: GNAQ/11 mutation-dependent sensitivity to MEK and PI3K inhibition

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Abbreviations: phosphorylated MAPK (P-MAPK), phosphorylated AKT (P-AKT), MEKi = GSK1120212, PI3Ki = GSK2126458
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Abstract

Purpose: Activating Q209L/P mutations in GNAQ or GNA11 (GNAQ/11) are present in ~80% of uveal melanomas (UM). Mutant GNAQ/11 are not currently therapeutically targetable. Inhibiting key downstream effectors of GNAQ/11 represents a rational therapeutic approach for UM that harbor these mutations. The MEK/MAPK and PI3K/AKT pathways are activated in UM. In this study, we test the effect of the clinically relevant small molecule inhibitors GSK1120212 (MEK inhibitor) and GSK2126458 (pan class I PI3K inhibitor) on UM cells with different GNAQ/11 mutation backgrounds.

Experimental Design: We use the largest set of genetically annotated uveal melanoma cell lines to-date to perform in vitro cellular signaling, cell cycle regulation, growth and apoptosis analyses. RNA interference and small molecule MEK and/or PI3K inhibitor treatment were employed to determine the dependency of uveal melanoma cells with different GNAQ/11 mutation backgrounds on MEK/MAPK and/or PI3K/AKT signaling. Proteomic network analysis was performed to unveil signaling alterations in response to MEK and/or PI3K small molecule inhibition.

Results: GNAQ/11 mutation status was not a determinant of whether cells would undergo cell cycle arrest or growth inhibition to MEK and/or PI3K inhibition. A reverse
correlation was observed between MAPK and AKT phosphorylation after MEK or PI3K inhibition, respectively. Neither MEK nor PI3K inhibition alone was sufficient to induce apoptosis in the majority of cell lines; however, the combination of MEK + PI3K inhibitor treatment resulted in the marked induction of apoptosis in a GNAQ/11 mutant-dependent manner.

**Conclusions:** MEK + PI3K inhibition may be an effective combination therapy in uveal melanoma given the inherent reciprocal activation of these pathways within these cells.

**Translational Relevance:**

Uveal melanoma is the most common intraocular tumor in adults. Approximately half of primary uveal melanoma tumors metastasize. Metastatic uveal melanoma is essentially recalcitrant to chemotherapeutic and immunological therapies and is fatal in nearly all cases. In this study, we show that the combination of clinically relevant MEK and PI3K small molecule inhibitors potentiates induction of apoptosis in uveal melanoma cells. Importantly, this effect was most pronounced in GNAQ/11 mutant cells, and not significant in GNAQ/11 wild-type uveal melanoma cells. These results offer pre-clinical evidence for the use of combination MEK + PI3K inhibition in metastatic uveal melanoma and suggest that tumors with specific mutations may show variable response to this combination therapy.
Introduction:

Uveal melanoma is the most common intraocular tumor in adults. Approximately half of primary uveal melanoma tumors metastasize. There are no effective therapies for metastatic uveal melanoma which is fatal in nearly all cases(1). Rational approaches for combination therapy based on activated signal transduction networks may avail new opportunities to effectively treat this disease.

Activating-Q209L/P mutations in GNAQ and GNA11 have been identified in approximately 80% of uveal melanoma tumors. GNAQ and GNA11 are very homologous members of the guanine nucleotide-binding G-protein subunit family(2). Under normal conditions, G-proteins are activated by G-protein coupled receptors, and mediate multiple downstream effectors. When mutated on residues R183 or Q209, which disable the intrinsic GTPase enzyme necessary to inhibit G-protein activity, these G-proteins become constitutively activated and oncogenic. To-date, there are no direct inhibitors of GNAQ and GNA11.

The MEK/MAPK and PI3K/AKT pathways are highly activated in uveal melanoma tumors(3-6). However, uveal melanomas are not observed to harbor activating mutations found in other types of melanoma (e.g., BRAF, NRAS, or KIT) that can stimulate these pathways(7-11). The presence of activating-Q209L/P mutations in GNAQ or GNA11 in approximately 80% of uveal melanoma tumors suggests a potential mechanism for the activation of the MEK/MAPK and PI3K/AKT pathways in uveal melanoma(12, 13).
Given the activation of the MEK/MAPK and PI3K/AKT pathways in many cancer types, combination treatment with small molecule inhibitors that target each of these pathways represents a rational therapeutic strategy(14). GSK1120212 is an orally available selective allosteric inhibitor of the MEK1 and MEK2 (MEK1/2) enzymes(15). GSK1120212 has potent \textit{in vitro} and \textit{in vivo} growth inhibitory effects in cells harboring RAS or BRAF mutations for which MEK1/2 is a downstream effector. GSK1120212 has a low $C_{\text{max}}$ to $C_{\text{trough}}$ ratio and a long half-life, and early phase trials show clinical activity(16). GSK2126458 is an orally available selective inhibitor of the class I phosphoinositide 3-kinase (PI3K) enzymes and the mammalian target of rapamycin (MTOR1/2) complexes(17). Biochemical studies show GSK2126458 to have $K_i$ values in the picomolar range for each of the class I PI3K isoforms and MTOR1/2 complexes. GSK2126458 has potent \textit{in vitro} and \textit{in vivo} growth inhibitory effects on cancer cells. The sustained pharmacodynamic effect at very low circulating drug levels in pre-clinical models make GSK2126458 a promising clinical therapeutic candidate. Early phase clinical trials with GSK2126458 are ongoing(18).

A previous report has shown that exogenous expression of either mutant GNAQ or GNA11 Q209L in immortalized melanocytes injected into the flank of immunodeficient mice results in highly pigmented xenograft lesions(12, 13). In addition, transfection of GNAQ or GNA11 Q209L into melanocytes results in the elevation of MAPK phosphorylation, consistent with the notion that mutant GNAQ or GNA11 activate the MEK/MAPK pathway in uveal melanoma tumors. Like the MEK/MAPK pathway, the PI3K/AKT pathway is highly active in the majority of uveal melanoma tumors, and elevated phosphorylation levels of AKT are associated with a higher risk of metastatic
However, the effect of activated GNAQ or GNA11 on signaling through the PI3K/AKT pathway appears to be cell-type specific, and has not been determined in uveal melanoma(19, 20).

In this study, we use very selective small molecule inhibitors of MEK1/2 and/or PI3K that are currently in multiple clinical trials to inhibit the MEK/MAPK and/or PI3K/AKT pathways in uveal melanoma cell lines with a GNAQ/11 mutation or wild-type background. We employ assays to measure cell growth, cell cycle regulation, apoptotic induction, and perform network analysis of proteomic data to determine the relative effects on cellular signaling pathways following treatment with MEK1/2 and PI3K inhibitors alone and in combination.

Results:

Effect of GNAQ or GNA11 knockdown on MAPK and AKT signaling and growth in uveal melanoma cells  To examine the dependency on activated mutant GNAQ or GNA11 for growth in uveal melanoma cells that harbor either mutation, siRNAs specific to GNAQ or GNA11 were employed. Figure 1A shows the growth inhibitory effect of GNAQ knockdown in uveal melanoma cells with an activating GNAQ (Q209L/P) or GNA11 (Q209L) mutation (hereafter referred to as either GNAQ or GNA11 mutant) or without either a GNAQ or GNA11 mutation (hereafter referred to as ‘wild-type’). siRNA knockdown of GNAQ or GNA11 resulted in a marked inhibition of growth (on par with the siRNA positive control) in GNAQ or GNA11 mutant uveal melanoma cells, respectively, relative to untreated or control (lipid transfectant alone or non-targeting
siRNA) treated cells. Neither GNAQ nor GNA11 knock-down in wild-type uveal melanoma cells resulted in a growth inhibitory effect similar to the siRNA positive control.

In order to determine the effect of mutant GNAQ or GNA11 on the MEK/MAPK and PI3K/AKT signaling pathways, western blot analysis of phosphorylated MAPK (threonine 202, tyrosine 204) and phosphorylated AKT (serine 473) was performed following the siRNA knockdown of GNAQ or GNA11 in GNAQ or GNA11 mutant uveal melanoma cells, respectively. Figure 1B shows that siRNA knock-down of GNAQ or GNA11 using two unique siRNAs in GNAQ or GNA11 mutant uveal melanoma cells, respectively, results in the loss of MAPK phosphorylation, with no significant change in the phosphorylation of AKT. No significant change in the phosphorylation status of MAPK or AKT was observed after GNAQ or GNA11 knockdown in wild-type uveal melanoma cells. Total levels of MAPK, AKT, or PTEN (a negative regulator of PI3K/AKT signaling) were unchanged after GNAQ or GNA11 knockdown in either GNAQ mutant, GNA11 mutant or wild-type cells. Of note, we have previously demonstrated that the uveal melanoma cell lines used in this study do not harbor genetic alterations previously identified in other types of melanoma (e.g., NRAS, BRAF, MEK, AKT, or PI3K) that signal through the MEK/MAPK or PI3K/AKT pathways(21).

Concentration-dependent effect of MEKi or PI3Ki on immediate downstream effectors and uveal melanoma cell growth

All the uveal melanoma cell lines used in this study demonstrate phosphorylation of MAPK and phosphorylation AKT at baseline (Supplementary Fig. 1). Wild-type cell
lines showed a greater phospho-MAPK/total MAPK ratio than the GNAQ/11 mutant cell lines. In addition, phospho-AKT levels were moderately less in all the GNA11 mutant cell lines.

Western blot analysis was used to assess the concentration dependent effect of MEKi (0-1 uM) or PI3Ki (0-1uM) treatment on the phosphorylation status of their respective downstream effectors, MAPK or AKT. Figure 2A shows that MEKi or PI3Ki treatment of either GNAQ mutant, GNA11 mutant or wild-type cells results in the loss of phosphorylation of their respective downstream targets at low nanomolar concentrations (also see Supplementary Fig. 2). No change in total MAPK or AKT was observed with either treatment in any of the cell lines.

To determine the growth dependency of GNAQ or GNA11 mutant or wild-type uveal melanoma cells on activation of the MEK/MAPK or PI3K/AKT pathways, cells were treated with increasing concentrations of either MEKi or PI3Ki. Figure 2B shows GNAQ and GNA11 mutant cell growth to be more sensitive to MEKi treatment than wild-type cells, reaching 50% of half-maximal growth inhibition at ~1 log less nanomolar concentration. Inversely, wild-type cells reached 50% of half-maximal growth at lower PI3Ki concentrations.

Given the limitations inherent in redox-dye assays to assess growth inhibition, real-time impedance measurements were also performed. Cell impedance is affected by three factors: cell growth, cell spreading and loss of attachment. Impedance measurements were taken over time in GNAQ mutant and wild-type cells with increasing concentrations of MEKi (0-500 nM) or PI3Ki (0-1000 nM) compared to no treatment (Supplementary Fig. 3). MEKi treatment resulted in an initial inflection in
impedance that corresponded with the morphological spreading of the cell lines. However, a marked concentration-dependent reduction in impedance in GNAQ mutant cells was observed over time which was not observed in wild-type cells. PI3Ki treatment resulted in a similar reduction in impedance in both GNAQ mutant and wild-type cells. A differential loss of cells from the plate surface was not observed in either mutant or wild-type cells.

**Effect of MEKi and/or PI3Ki treatment on MAPK and AKT signaling pathways, cell cycle and apoptosis mediators in uveal melanoma cells**

Reverse Phase Protein Array was used to assess the effect of MEKi alone, PI3Ki alone, or combination MEKi + PI3Ki treatment on the MEK/MAPK and PI3K/AKT signaling pathways, the Rb cell cycle regulator, and apoptosis mediators in uveal melanoma cells.

Figure 3A shows that both mutant and wild-type uveal melanoma cells demonstrate a marked reduction in MAPK phosphorylation after MEKi treatment. PI3Ki treatment resulted in the reduction of phosphorylation in AKT notably in GNAQ wild-type cells. A reciprocal increase in AKT phosphorylation after MEKi treatment and an increase MAPK phosphorylation after PI3Ki treatment was also noted. However, these effects appeared dependent on the relative expression of phosphorylated MAPK or AKT at baseline. If MAPK or AKT phosphorylation levels were relatively high at baseline, a drug-induced increase was not observed. Combination MEKi + PI3Ki treatment resulted in a decrease in phosphorylation in both MAPK and AKT in both GNAQ mutant and wild-type cells without a reciprocal increase in AKT or MAPK phosphorylation, respectively.
MEKi alone treatment resulted in the relative decrease in phosphorylation of p70S6K (threonine 389), with little change in 4EBP1 (serine 65, threonine 37) or S6 phosphorylation (serine 236/244) in GNAQ mutant and wild-type cells. PI3Ki alone treatment resulted in decreased phosphorylation of 4EBP1, S6, and p70S6K in GNAQ mutant and wild-type cells, although the complete extinguishment of S6 phosphorylation required combination MEKi + PI3Ki treatment in mutant cells.

MEKi alone treatment resulted in a stark reduction in myc transcription factor levels after 12 hours with a corresponding reduction in the phosphorylation of Rb (serine 807/811) in GNAQ mutant cells. PI3Ki alone treatment resulted in decreased Rb phosphorylation in both GNAQ mutant and wild-type cells, but failed to have any effect on myc levels. Combination MEKi + PI3Ki treatment resulted in no greater reductions in myc or Rb phosphorylation than MEKi treatment alone.

Molecular mediators of apoptosis were assessed after 24 hrs of MEKi alone, PI3Ki alone, or MEKi + PI3Ki combination treatment. Neither MEKi nor PI3Ki treatment alone resulted in significant elevation in cleaved caspase 3 or 7 in mutant or wild-type cells. However, combination MEKi + PI3Ki treatment resulted in a marked increase in cleaved caspase 3 and 7 levels in GNAQ mutant cells, not observed in wild-type cells. No clear trend in Bcl-2 family member protein expression was observed after MEKi and/or PI3Ki treatment in any of the cell lines, with the exception of BIM, which was elevated after MEKi alone and combination MEKi + PI3Ki treatment in both mutant and wild-type cells.

To determine whether elevated levels of BIM following MEKi + PI3Ki treatment mediated cleavage of caspase 3 and 7 in GNAQ mutant cells, two distinct siRNAs
targeting BIM were employed. Cells were transfected with BIM siRNAs, then treated with MEKi + PI3Ki. Targeting BIM was unable to rescue the apoptotic effect of MEKi + PI3Ki treatment (Supplementary Fig. 4).

In order to characterize the nature of signaling molecule interactions induced by MEKi alone, PI3Ki alone or combination MEKi + PI3Ki treatment, network-based analysis was performed on the GNAQ (Q209L) mutant cell line 92.1. NetWalk is a random-walk-based algorithm that scores the relevance of protein-protein interactions (represented as “edge flux” values) based on protein expression level values in the context of neighboring nodes (proteins) and local network connectivity. Figure 3B shows the highest scoring networks following MEKi alone, PI3Ki alone, or combination MEKi + PI3Ki treatment compared to no treatment. Following MEKi alone treatment, among the highest scoring interactants within the signaling network was AKT phosphorylated on serine 473. Conversely, network analysis following PI3Ki alone treatment demonstrated phosphorylated MAPK (threonine 202, tyrosine 204) to be among the highest scoring interactants within the signaling network. Combination MEKi + PI3Ki treatment revealed a distinct signaling network pattern in which neither phosphorylated MAPK nor AKT were relevant, rather, the transcription factor c-jun (pS73) became central to the network with multiple interactions with other signaling molecules (e.g., JNK, STAT3, NfκB (RelA)). Similar data was observed with the GNAQ mutant MEL 202 (not shown).

Given the central role of phosphorylated c-jun (p-cjun) indicated by network analysis following combination MEKi + PI3Ki treatment, protein levels of p-cjun at baseline and following MEKi + PI3Ki treatment was evaluated. The p-cjun protein
showed a significant increase following combination MEKi + PI3Ki treatment (Figure 4A and Supplementary Fig. 5). To gain greater insight into the potential functional implication of elevated p-c-jun in this context, two distinct siRNAs targeting p-c-jun were employed. Figure 4B shows a marked loss of c-jun and p-c-jun expression following siRNA knockdown of c-jun. A growth assay demonstrates knock-down of c-jun to have a modest effect on uveal melanoma cell growth at baseline, however, knockdown of c-jun significantly enhanced the growth inhibitory effect of MEKi + PI3Ki treatment (Fig. 4C).

Effect of MEKi and/or PI3Ki treatment on cell cycle regulation in uveal melanoma cells

To determine the effect of MEKi and/or PI3Ki treatment on cell cycle regulation in uveal melanoma cells at the single cell level, we performed flow cytometric analysis of DNA content in GNAQ mutant, GNA11 mutant, or wild-type cells treated with MEKi alone, PI3Ki alone, or the combination. The percent of cells in the S/G2/M phases was determined relative to untreated cells (Fig. 5). MEKi treatment resulted in a significant decrease in cycling cells in the majority of uveal melanoma cell lines (3 of 3 GNAQ mutant, 2 of 3 GNA11 mutant, 0 of 2 wild-type). Similarly, uveal melanoma cell lines showed a significant decrease in cycling cells after PI3Ki treatment alone (2 of 3 GNAQ mutant, 2 of 3 GNA11 mutant, 1 of 2 wild-type). The combination of MEKi + PI3Ki treatment further decreased the number of cycling cells in the majority of cell lines compared to MEKi or PI3Ki alone treatment (3 of 3 GNAQ mutant, 3 of 3 GNA11 mutant, 1 of 2 wild-type).
Effect of MEKi and/or PI3Ki treatment on apoptosis induction in uveal melanoma cells

To examine the capacity of MEKi and/or PI3Ki treatment to induce apoptosis in uveal melanoma cells, flow cytometry assessment of annexin V and propidium iodide staining was performed. Analysis of the induction of apoptosis over 3 days in response to treatment with MEKi alone, PI3Ki alone, or the combination of MEKi + PI3Ki indicated that the 48 hour response allows for the reliable detection of early apoptotic cells. After 72 hours of treatment with the combination of MEKi + PI3Ki, few cells are available to assay in drug sensitive lines as shown in Figure 6A.

GNAQ mutant, GNA11 mutant, or wild-type cells were treated with MEKi alone, PI3Ki alone, or the combination of MEKi + PI3Ki. Figure 6B shows MEKi treatment alone resulted in little to no induction of early apoptosis in all the uveal melanoma cell lines tested, with the exception of the GNAQ mutant MEL270 cell lines in which 55% of cells (relative to baseline) were observed to have an early apoptotic response. PI3Ki alone treatment also resulted in a modest induction of early apoptosis in all the uveal melanoma cell lines tested with the exception of the GNAQ mutant MEL 270 cell line (38%, relative to baseline). A clearer mutant genotype-dependent apoptotic response was observed with MEKi + PI3Ki combination treatment. GNAQ mutant cells demonstrated a marked induction of early apoptosis after MEKi + PI3Ki combination treatment (70% in MEL270, 55% in 92.1, and 44% in MEL202). GNA11 mutant and wild-type cells displayed a variable apoptotic response to MEKi + PI3Ki combination treatment (GNA11 = 20% in UPMD2, 20% in OMM1, 4% in UPMD1; wild-type = 5% in MEL285 and 7% in MEL290).
Prior studies have demonstrated that drug treatments that show efficacy in two-dimensional cultures can fail when cells are grown in three-dimensional tumor-like structures(22). Thus, to examine the effect of MEKi + PI3Ki treatment in a three-dimensional cellular system we generated uveal melanoma cell spheroids (Fig. 6C). Spheroids were treated with MEKi (0, 3.1, or 12.5 nM) and/or PI3Ki (0, 6.25, or 100 nM) and assessed for caspase 3/7 activity using a fluorescent substrate reporter by high throughput confocal microscopy (Fig. 6D). The cells making up spheroids showed negligible caspase 3/7 activity at baseline. Increasing doses of either MEKi or PI3Ki resulted in a modest increase in caspase 3/7 activity within spheroid cells (Fig. 6E). However, combination of MEKi + PI3Ki treatment resulted in markedly elevated caspase 3/7 activity within spheroid cells. Single cells that were unincorporated into spheroids tended to demonstrate caspase 3/7 activity under all treatment conditions.

Discussion

Functional studies in uveal melanoma research have been hampered by the paucity of cell lines available for analysis. Of cell lines noted to be of uveal origin, many have been identified to have an activating V600E mutation in BRAF despite BRAF mutations not being identified in uveal melanoma tissues using standard techniques(5, 8-10, 23, 24). Given that approximately 80% of uveal melanoma tumors harbor mutually exclusive Q209 mutations in GNAQ or GNA11, use of uveal melanoma cell lines with these mutations are a powerful tool to assess the efficacy of targeted therapeutic approaches. This study uses the largest number of GNAQ mutant, GNA11 mutant, or...
wild-type uveal melanoma cell lines to-date in order to determine the relative efficacy of employing very selective MEK and/or PI3K inhibitors in uveal melanoma.

Activation of the MEK/MAPK and PI3K/AKT pathways in all types of melanoma, including uveal melanoma tumors, has been extensively documented(3, 4, 6, 25). Activating mutations in BRAF, NRAS, KIT, AKT, PI3K and loss of PTEN are mechanisms by which the MEK/MAPK and PI3K/AKT pathways are activated in cutaneous melanoma. We have previously determined that the uveal melanoma cell lines examined in this study lack common mutations in BRAF, NRAS, KIT, AKT, PI3K or loss of PTEN, and consistent with their uveal melanoma tissue counterparts, harbor mutually exclusive activating Q209L/P mutations in GNAQ or GNA11(21). Activated GNAQ/11 have been proposed to be both activators and inhibitors of the MEK/MAPK and PI3K/AKT pathways in different biochemical and non-cancer cell studies(19, 20).

Thus we investigated the relative contribution of mutation activated GNAQ or GNA11 on MEK/MAPK and/or PI3K/AKT signaling in uveal melanoma cells. Knockdown of GNAQ or GNA11 resulted in diminished MAPK phosphorylation in uveal melanoma cell lines with GNAQ or GNA11 mutations, respectively, but not in wild-type cells, confirming that activated GNAQ or GNA11 signal through the MEK/MAPK pathway in GNAQ or GNA11 mutant uveal melanoma, respectively. However, loss of mutant GNAQ or GNA11 had no significant effect on AKT phosphorylation in either GNAQ or GNA11 mutant or wild-type uveal melanoma cell lines. We show that inhibition of MEK, and therefore MAPK signaling results in the reciprocal activation of AKT activity in uveal melanoma cell lines regardless of GNAQ/11 mutant status.
It has been proposed that tumors with activating mutations in the RAS/RAF axis depend more on MAPK signaling whereas activating mutations in receptor tyrosine kinases depend more on PI3K signaling(26). Data presented in this paper suggest that the activating mutations in GNAQ and GNA11 are akin to mutations in the RAS/RAF axis given their greater sensitivity to growth inhibition following treatment with a selective MEKi and loss of MAPK phosphorylation with GNAQ or GNA11 loss, respectively. This conclusion is further supported by previous data that shows 4EBP1 to be a redundant downstream mediator in tumors with coexistent mutational activation of the MEK/MAPK and PI3K/AKT pathways (e.g., colon carcinomas with both KRAS and PI3KCA mutations)(27). The mutant uveal melanoma cells examined in the current study demonstrated marked loss of 4EBP1 phosphorylation only after PI3Ki treatment, with no significant additional loss with combination MEKi + PI3Ki treatment. The fact that the PI3Ki treatment effects on cell growth, cell cycle regulation and apoptosis induction were similar in all uveal melanoma cells tested, suggests that non-GNAQ/11 mutant-driven mechanisms may drive the PI3K/AKT pathway in uveal melanoma.

Given the clear activation of the MEK/MAPK pathway by mutant GNAQ or GNA11, inhibition of MEK would appear to be a rational therapeutic approach in patients with GNAQ or GNA11 mutant tumors. In a phase I clinical trial Adjei et al., reported one patient with both metastatic uveal melanoma and renal cell carcinoma to have stable disease for 22 cycles on the AZD6244 MEK inhibitor(28). A review of three completed trials in which 20 patients with metastatic uveal melanoma were treated either upfront with AZD6244 or following progression on temozolomide suggested an improvement in progression free survival in favor of MEK inhibitor treatment, although
too few patients were treated to definitively make this conclusion(29). Finally, a phase I clinical trial has recently been completed which used the GSK1120212 MEK inhibitor. Seventy-two melanoma patients, including uveal melanoma patients, were enrolled, but the outcomes have yet to be published(30). None of the aforementioned studies systematically evaluated the GNAQ or GNA11 mutation status of tumors to determine if efficacy correlated with GNAQ mutation, GNA11 mutation or wild-type status. A clinical trial powered to determine the efficacy of MEK inhibition in GNAQ/11 mutant uveal melanoma is ongoing(31).

Data presented in this paper suggests that MEK inhibition alone is able to achieve cell cycle arrest and reduced growth in most uveal melanoma cells, but only results in modest apoptotic cell death in the majority of uveal melanoma cells. Likewise, PI3K inhibition alone can cause cell cycle arrest and reduced growth, but is insufficient to induce substantive apoptotic death. However, the combination of MEK + PI3K inhibition results in a strong induction of apoptotic death, most pronounced in GNAQ mutant cells, but also evident in the majority of GNA11 mutant cells. Proteomic network analysis reveals a homeostatic relationship between the MEK/MAPK and PI3K/AKT pathways in uveal melanoma cells -- inhibition of MEK resulted in a relative increase in AKT phosphorylation, whereas, an increase in the phosphorylation of MAPK was elicited after inhibition of PI3K. Similar feedback regulatory mechanisms have been observed in cancers driven by activating RAS or RAF mutations in lung, breast and cutaneous melanoma(32-34). In addition, signaling network analysis of proteomic data revealed that inhibition of both the MEK/MAPK and PI3K/AKT pathways increases the activation of the transcription factor c-jun. Knockdown of c-jun in significantly enhanced
the growth inhibitory effects of combination MEKi + PI3Ki therapy, suggesting that c-jun may play a potential compensatory role in cell growth after treatment.

The pre-clinical data presented in this paper support the MEK/MAPK pathway as an important effector of GNAQ/11 mutant signaling, and MEK inhibition as a therapeutic strategy to mitigate cell growth. Combinatorial targeted therapies, such as the one employed in this study, using MEK inhibitors as a “back-bone” may offer greater therapeutic effect by enhancing uveal melanoma cell death.

Materials and Methods:

Cell culture

Cells were cultured in RPMI 1640 supplemented with 5% fetal bovine serum at 37°C in a humidified 5% CO2 atmosphere. The MEK inhibitor, GSK1120212 (MEKi), and PI3K inhibitor, GSK2126458 (PI3Ki), were kindly provided by GlaxoSmithKline (Middlesex, UK). siRNAs were purchased from Life Technologies (Grand Island, NY) for GNAQ#1 (s5886: UUGUGCAUGAGCCUUAUUGtg), GNAQ#2 (s5887: UCGUCUAUCAUGCAUUCCtg), GNA11#1 (s5862: AAAGGGUACUCGAUGAUGCg), GNA11#2 (s5864: UUCUGGUAGACGCUUGGtg), BIM#1 (s19474), BIM#2 (s223065), c-JUN#1(s7658), c-JUN#2 (s7659), PLK1 (positive control) (s450), and Silencer® Select Negative Control No. 1 siRNA (negative control). Oligonucleotides were transfected at 10nM with Lipofectamine RNAiMax per manufacturer’s instructions.

Western Blotting
Cells were lysed using RIPA based lysis buffer supplemented with Na3VO4, PMSF, and Protease Cocktail Inhibitors (Invitrogen, Carlsbad, CA). Antibodies against GNAQ, GNA11, pMAPK, MAPK1/2, pAKT, AKT, p-c-jun, c-jun, GAPDH and PTEN were obtained from Cell Signaling Technology (Beverly, MA). Antibodies against MAPK2 were obtained from Millipore (Billerica, MA). Antibodies against BIM were obtained from BioVision (Milpitas, CA).

Reverse Phase Protein Array (RPPA)

The methodological basis for RPPA has been previously explicated in detail(35, 36). Cells were treated with MEKi (10nM) alone, PI3Ki (100nM) alone or the combination of MEKi + PI3Ki for 4, 12, or 24 hrs. After cellular lysis, protein isolation, quantification, and denaturing, proteins are spotted on nitrocellulose-coated glass slides in serial dilutions. To detect proteins, slides were blocked, then incubated with primary and secondary antibodies and signal amplified by a DakoCytomation catalyzed detection system (Carpinteria, CA). Stained slides were scanned, analyzed, and quantitated using Microvigene software (VigeneTech Inc., North Billerica, MA) to generate a serial dilution-signal intensity “supercurve” for all samples on the slide. Each sample was then fitted to this “supercurve” to generate logarithmic values representative of relative signal intensity. Differences in protein loading are determined using the median expression level for each sample across all antibodies used; protein values were divided by this factor. Corrected values were employed to generate heat maps using Treeview and Xcluster software.
Network Analysis

Network analysis was performed on the RPPA data to identify reactive signaling networks following treatment with MEKi alone, PI3Ki alone or both. Data points collected in RPPA experiments were analyzed with Netwalker, as described previously (30, 31). Briefly, protein-signal averages were analyzed for the triplicate conditions in ratio (MEKi, PI3Ki and MEKi + PI3Ki) relative to the untreated cell RPPA signal. For the analysis of proximal alterations in signaling (4 hours) in the GNAQ mutant cell line 92.1, Netwalker analysis was performed for each condition, and the top 40 highest interactions based on EF values were composed in a network diagram. Color-scale corresponds to the log^2 transformed signal data for the indicated sample comparison. Nodes that were not directly measured in the RPPA analysis were excluded for clarity of presentation. Interacting edges represent protein interactions (PPI).

Cell Growth Assay

Cells (2.5 E4) were plated in 96-well plates and treated the following day with increasing concentrations of drug or equamolar dimethyl sulfoxide (DMSO) in triplicate. After 72 hours, redox-dye conversion for each treatment was determined using the Cell Titer-Blue Assay (Promega, Madison, WI) relative to DMSO alone treatment, by flurometry per the manufacturer’s instructions. DMSO vehicle at equimolar concentrations had no significant effect on cell viability in all lines.

Flow Cytometry
For cell cycle analysis, culture supernatants were collected, and combined with cultured cells removed by brief trypsin treatment. Cells were washed twice with PBS and fixed with 70% Ethanol. Cells were stored overnight at -20°C. Cells were then washed twice with PBS, and reconstituted in RNAse A 100ug/mL and 20 ug/mL Propidium Iodide, both purchased from Sigma Aldrich Chemicals (St. Louis, MO), and stored at 4°C prior to analysis. For apoptosis measurements, cells were similarly collected and washed cells were stained with Annexin V, washed once and re-suspended in 20 ug/mL Propidium Iodide. Cells were analyzed on a FACs Canto and data analyzed using FlowJo (Treestar Ashland, OR). Cell index was determined as the percent of cells in S/G2/M phase normalized to untreated baseline cultures. Means of triplicate experiments were compared with repeated measures one-way ANOVA and Bonferroni’s Multiple Comparison Test using Graphpad Prism. Significance indicates a p-value of less than 0.05 given a Confidence Interval of 95% of difference.

**Spheroids Apoptosis Assay**

Spheroids were generated as previously described(37). Uveal melanoma spheroids plated in 384-well imaging plates in the presence of the indicated concentrations of MEKi or PI3Ki. Spheroids were treated in triplicate wells and after 44 hours the CellEvent ™ Caspase-3/7 Green Detection Reagent was added to each well and incubated for 4 hours prior to imaging. Caspase active cells were identified as described in manufacturers instruction. Each well was imaged using a BD Biosciences Pathway bioimaging system and analyzed with Image J. Drug effect is quantified as
caspase 3/7 active cells/spheroid area. Between 4-17 spheroids were analyzed per condition.
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Figure Legends:

Figure 1. Effect of GNAQ or GNA11 knockdown on cell growth and MAPK/AKT signaling in GNAQ mutant, GNA11 mutant or wild-type uveal melanoma cells. A, GNAQ mutant [Q209L (92.1 and MEL202), Q209P (MEL 270)] or GNA11 mutant [Q209L (OMM1, UPMD1, and UPMD2)] or wild-type (MEL285, MEL290) uveal melanoma cells were transfected with two unique siRNAs specific to GNAQ or GNA11. Cell growth was determined 72 hrs after transfection relative to cells that were untreated (untransfected), or transfected with lipid alone, non-targeting siRNA (negative control), or siRNA targeting PIK1 (positive control, siRNA-P). Combined results of two independent experiments, bars represent 1 SEM. B, Western-blot analysis of GNAQ, GNA11, P-MAPK (threonine 202, tyrosine 204), MAPK, P-AKT (serine 473), AKT and PTEN expression in GNAQ mutant (Q209L (92.1)), GNA11 mutant (Q209L (UPMD2)),
versus GNAQ/11 wild-type (MEL285) uveal melanoma cells following transfection with two unique GNAQ or GNA11 siRNAs relative to non-targeting control.

Figure 2. Effect of small molecule MEK or PI3K inhibitors on GNAQ mutant, GNA11 mutant, or wild-type uveal melanoma cell growth and MAPK/AKT signaling. GNAQ mutant [Q209L (92.1, MEL202)] or GNA11 mutant [Q209L (OMM1, UPMD2)] or GNAQ/11 wild-type (MEL285, MEL 290) uveal melanoma cells were used for the following experiments: A, uveal melanoma cells were treated with increasing doses of GSK1120212 (MEK inhibitor = MEKi) or GSK2126458 (PI3K inhibitor = PI3Ki) for 3 hrs and expression of P-MAPK, MAPK, P-AKT, and AKT determined by western-blot analysis. B, uveal melanoma cells were treated with increasing doses of MEKi or PI3Ki for 72 hrs and percent of maximal cell growth inhibition determined. (representative experiments, bars represent SD).

Figure 3. Effect of small molecule MEK and/or PI3K inhibitors on signaling pathways in GNAQ mutant versus wild-type uveal melanoma. A, Mutant GNAQ [Q209L (92.1, MEL202)] or wild-type (MEL285, MEL290) uveal melanoma cells were treated with MEKi alone (10nM), PI3Ki alone (100nM), or the combination of MEKi + PI3Ki for 4, 12 or 24 hrs. Reverse Phase Protein Array was performed to assess the expression of proteins in the MEK/MAPK, PI3K/AKT, cell cycle and apoptosis pathways. The heatmap represents the mean centered log$_2$ expression value for each protein after the indicated time of treatment (red = greater, green = lesser). Each treatment was performed in triplicate; therefore there are three distinct heatmap boxes per treatment.
condition.  

B. The highest scoring network interactions (edge flux values) from the NetWalk analysis of directly measured proteins following 4 hours of MEKi and/or PI3Ki treatment. Nodes represent proteins, edges represent network interactions among nodes, node-coloring represents relative increase in protein level. Asterisks (*) indicate phosphorylated proteins.

Figure 4. Effect of c-jun knockdown on combination MEKi + PI3Ki treatment of uveal melanoma cells.  

A, GNAQ Q209L mutant uveal melanoma cells (92.1) were treated with combination MEKi (10nM) + PI3Ki (100nM) for 4 hours and levels of phosphorylated c-jun determined relative to untreated cells.  

B, Cells were transfected with two distinct siRNAs targeting c-jun, then levels of phosphorylated c-jun, total c-jun or beta-actin were determined by western blot.  

C, Cells were transfected with two distinct siRNAs targeting c-jun, then treated with combination MEKi (10nM) + PI3Ki (100nM). The effect of c-jun knockdown in MEKi + PI3Ki treated cells was compared to the effect of MEKi + PI3Ki treatment alone. Asterisks (*) indicate p value <0.05.

Figure 5. Effect of small molecule MEK and/or PI3K inhibitors on cell cycle regulation in GNAQ mutant, GNA11 mutant or wild-type uveal melanoma cells. The indicated mutant GNAQ mutant GNA11 or wild-type uveal melanoma cells were treated with MEKi alone, PI3Ki alone, or the combination of MEKi + PI3Ki for 24 hrs. Cell cycle regulation was assessed by single cell flow cytometry analysis and the percent of cells in the S/G2/M phases determined. Cell index was determined as the percent of cells in S/G2/M phase normalized to untreated baseline cultures, bars represent 1 SEM. Means of triplicate
experiments were compared with repeated measures one-way ANOVA and Bonferroni’s Multiple Comparison Test using Graphpad Prism. Significance indicates a p-value < 0.05 given a confidence interval of 95% of difference.

Figure 6. Effect of small molecule MEK and/or PI3K inhibitors on apoptosis induction in GNAQ mutant, GNA11 mutant or wild-type uveal melanoma cells. A, Representative flow cytometry experiment assessing levels of annexin V and propidium iodide in GNAQ mutant cells (MEL202) after MEKi alone, PI3Ki alone, or the combination of MEKi + PI3Ki treatment at 24, 48 and 72 hours. B, Mutant GNAQ [Q209L (92.1, MEL202), Q209P (MEL270)], mutant GNA11 [Q209L (OMM1, UPMD1, UPMD2)] or wild-type (MEL285, MEL290) uveal melanoma cells were treated with MEKi alone, PI3Ki alone, or the combination of MEKi + PI3Ki for 48 hrs. Induction of apoptosis was assessed by flow cytometry using Annexin V and Propidium Iodide staining. The percent of cells in early apoptosis was determined by gating the high annexin V/low propidium iodide cell population following treatment. Data represents n=3-6 experiments, bars represent 1 SEM. C, Light microscopy image of uveal melanoma spheroids (bar = 50 um). D, Confocal microscopic imaging of caspase 3/7 activity in cells within uveal melanoma spheroids (UPMD2) following treatment with increasing doses of MEKi and/or PI3Ki. Heatmap represents caspase 3/7 positive cells per spheroid area.
References:


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FIGURE 1

A

B

GNAQ Q209L         GNAQ/11 WT

Control  GNAQ #1  GNAQ #2  Control  GNAQ #1  GNAQ #2

GNAQ  

p-MAPK  

MAPK  

p-AKT  

AKT  

PTEN  

GNA11  

p-MAPK  

MAPK  

p-AKT  

AKT  

PTEN  

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FIGURE 3

A

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Cleaved Caspase 3
Cleaved Caspase 7
Bcl2
BclX
BclXL
Xiap
BAX
BIM

B

MEKi
PI3Ki
MEKi & PI3Ki
FIGURE 6

A

B

C

D

MEKi & PI3Ki

MEKi

PI3Ki

GNAQ mut

GNA wt

GNA11 wt

GNA11 mut

Early Apoptosis (%)

MEL270

MEL202

UPMD2

OMM1

MEL290

92.1

92.1

92.1

92.1

FIGURE 6

C D

MEKi

PI3Ki

0 nM

3.1 nM

12.5 nM

0 nM

PI3Ki

6.25 nM

100 nM

Annexin V

Propidium Iodide

24 Hr

48 Hr

72 Hr

Untreated

MEKi & PI3Ki

MEKi

PI3Ki

maxmin

50 um
Combination Small Molecule MEK and PI3K Inhibition Enhances Uveal Melanoma Cell Death in a Mutant GNAQ and GNA11 Dependent Manner


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