Genotype-Dependent Sensitivity of Uveal Melanoma Cell Lines to Inhibition of B-Raf, MEK, and Akt Kinases: Rationale for Personalized Therapy

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PURPOSE. Inhibitors of B-Raf and MEK kinases hold promise for the management of cutaneous melanomas harboring *BRAF* mutations. *BRAF* mutations are rare in uveal melanomas (UMs), but somatic mutations in the G protein α subunits Gαq and Gα11 (encoded by *GNAQ* and *GNA11*, respectively) occur in a mutually exclusive pattern in ~80% of UMs. The impact of B-Raf and MEK inhibitors on Gα-mutant UMs remains unknown.

METHODS. The impact of the B-Raf inhibitor PLX4720, the MEK inhibitor AZD6244, and the Akt inhibitor MK2206 on UM cell lines was assessed with the use of cell viability, proliferation, and apoptosis assays and immunoblot analysis.

RESULTS. *BRAF*-mutant UM cells were sensitive to both PLX4720 and AZD6244, undergoing cell cycle arrest but not apoptosis. UM cells with a Gα-protein mutation (*GNAQ* or *GNA11*) were mildly sensitive to AZD6244 but completely resistant to PLX4720. In fact, PLX4720 paradoxically increased ERK phosphorylation in Gα-mutant UM cells. The combination of AZD6244 with PLX4720 had synergistic anticancer activity in *BRAF*-mutant cells but not in Gα-mutant cells. The Akt inhibitor MK2206 sensitized *BRAF*-mutant cells to both PLX4720 and AZD6244 and sensitized Gα-mutant cells to AZD6244 but did not overcome the resistance of the Gα-mutant cells to PLX4720.

CONCLUSIONS. The response of UM cells to inhibition of B-Raf, MEK, and Akt depends on their genotype. Future use of such targeted therapies in clinical trials of UM patients will require careful design and patient selection based on genotype to provide personalized and effective therapy. (*Invest Ophthalmol Vis Sci.* 2011;52:7248–7255) DOI:10.1167/iovs.11-7398

Somatic activating mutations in the RAS/RAF/MEK/ERK signaling pathway are frequent in cutaneous melanomas (CMs), with 50% to 70% of them harboring *BRAF* mutations (usually the V600E substitution).[1] Mutations in *RAS* genes occur in approximately another 20% to 30% of CMs (most frequently in *NRAS*) and are usually mutually exclusive with *BRAF*[V600E].[2–6] *BRAF*[V600E], the most frequent oncogenic protein kinase mutation known, activates the MEK/ERK cascade and represents a promising therapeutic target for melanomas and for thyroid, colon, and ovarian carcinomas and other malignancies harboring this mutation.[1,7–10] Kinase inhibitors targeting B-Raf (in particular the V600E mutant), including PLX4720 (Plexxikon Inc., Berkeley, CA)[11] and the related PLX4032 (vemurafenib, RG7204), are in clinical development.[12] Preclinical and clinical evidence suggests that these B-Raf inhibitors suppress ERK phosphorylation and induce cell cycle arrest and apoptosis in *BRAF*[V600E]-bearing CM cells, whereas in *RAS*-mutant/*BRAF* wild-type CM cells they can paradoxically enhance ERK phosphorylation and promote cell proliferation through a cRaf-mediated mechanism.[11,13–18] In phase 1 and 2 clinical trials of PLX4032 in patients with metastatic CM, complete or partial tumor regression was observed in the majority of patients with a *BRAF*[V600E] tumor.[12,19] In a phase 3 trial of patients with advanced-stage CM with *BRAF*[V600E] mutations who were randomly assigned to PLX4032 or dacarbazine, the hazard ratios for overall survival and progression-free survival were 0.37 and 0.26, respectively, both favoring PLX4032.[20] Therefore, B-Raf inhibitors are very promising targeted therapeutics specifically for *BRAF*[V600E] CMs and careful patient selection is crucial.[12,20] However, it should be emphasized that, even in *BRAF*[V600E] CMs, clinical responses with BRAF inhibitors are usually short-lived because of the emergence of compensatory oncogenic signaling pathways.[21–24]

Furthermore, preclinical evidence suggests that *BRAF*[V600E] CMs are highly sensitive to MEK inhibition, whereas CMs with a wild-type *BRAF*/mutant *NRAS* status exhibit variable and usually lower sensitivity and those that are wild-type for both *BRAF* and *NRAS* are uniformly resistant to MEK inhibition.[25] These data again confirm the "oncogenic addiction" of BRAF-mutant CM cells to this activated pathway and provide another therapeutic method for targeting it in patients with metastatic CMs. Clinical trials of MEK inhibitors such as AZD6244 (AstraZeneca, Wilmington, DE) in *BRAF*[V600E] tumors are ongoing.

However, in uveal melanomas (UMs), *BRAF*[V600E] mutations are rare.[26–28] Instead, somatic mutations in the G protein α subunits Gαq and Gα11 (encoded by *GNAQ* and *GNA11*, respectively)[29] are present in a mutually exclusive pattern in ~80% of UMs. Gαq and Gα11 are 90% homologous and transmit signals between G-protein–coupled receptors and downstream effectors. Their mutations occur most commonly in exon 5, affecting codon Q209 (for both proteins)[30,31] in their

Ras-like domain, abolishing their GTPase activity in a manner similar to that for the $NRAS^{Q61R}$ mutation, and resulting in a constitutively active Gα protein that functions as a bona fide oncogene.[30,31] A second hot spot for mutations has been discovered in exon 4, affecting codon R183 (for both proteins).[31] The presence of these mutations in tumors at all stages of malignant progression suggests that they are early events in UM.[32] However, the sensitivity of Gα-mutant UM cells to the B-Raf and MEK inhibitors currently undergoing clinical development for the management of CM remains unknown.

We investigated the impact of B-Raf and MEK inhibition on UM cell lines using the small molecule inhibitors PLX4720 and AZD6244, respectively, either as monotherapy or in combination with each other and the Akt inhibitor MK2206 (Merck, North Wales, PA).[33–35] We found that the *BRAF*-mutant UM cells behave similarly to their cutaneous counterparts, with high sensitivity to inhibition of either B-Raf or MEK that can be further enhanced by concurrent Akt inhibition. However, Gα-mutant UM cells are less sensitive to MEK inhibition (but can be further sensitized by concurrent Akt inhibition) and completely resistant to B-Raf inhibition (even in the presence of the Akt inhibitor). In fact, the B-Raf inhibitor PLX4720 paradoxically increased ERK phosphorylation in Gα-mutant UM cells. Our data demonstrate that the response of UM cells to the inhibition of B-Raf and MEK is genotype dependent. Future use of targeted therapies in clinical trials of UM patients will require careful design and patient selection based on genotype to provide personalized and effective therapy.

MATERIALS AND METHODS

Cell Lines and Tissue Culture

The genotype of OMM1.3 and Mel202 UM cells (both *GNAQ*-mutant at *Q209* and *BRAF*-wt) has been previously reported.[30] The OMM1 UM cells carry a *GNA11* Q209 mutation and are wild-type for *BRAF* and *GNAQ*. The OCM3 ($BRAF^{V600E}$/*GNAQ*-wt/*GNA11*-wt) UM cell line and the A375 and M14 (both $BRAF^{V600E}$/*GNAQ*-wt/*GNA11*-wt) CM cell lines were also used. All cell lines examined in this study were carefully genotyped by Sanger sequencing using a cycle sequencing kit (BigDye Terminator v1.1; Applied Biosystems, Foster City, CA) and a genetic analyzer (3130; Applied Biosystems) for *GNAQ* exons 4 and 5, *GNA11* exons 4 and 5, and *BRAF* exon 15, and their mutation status was confirmed (Supplementary Fig. S1, http://www.iovs.org/lookup/ suppl/doi:10.1167/iovs.11-7398/-/DCSupplemental). Overall, *BRAF*, *GNAQ*, and *GNA11* mutations are mutually exclusive. All cells were grown in DMEM/F12 (Invitrogen, Carlsbad, CA) with 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% FBS (Invitrogen).

MTT, Proliferation, and Apoptosis Assays

The B-Raf inhibitor PLX4720, the MEK inhibitor AZD6244, and the Akt inhibitor MK2206 were reconstituted in dimethyl sulfoxide (DMSO) and were stored at −20°C until use. Cells were plated in 24-well plates in medium containing 10% FBS. Drugs were added 24 hours later, and the cells were incubated for 96 more hours. Cell numbers were quantified by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, MO) as described previously,[8] and were expressed as a percentage of the value of control wells. In parallel experiments, the proliferation rate in cells treated with AZD6244 or PLX4720 was quantified by measurement of the amount of BrdU incorporated into DNA (BrdU Cell Proliferation Assay; Calbiochem, San Diego, CA) according to the instructions of the manufacturer and as previously performed.[36]

Apoptosis was detected in UM cells treated with AZD6244 or PLX4720 for 72 hours using the terminal dUTP nick-end labeling (TUNEL) method with a cell death detection kit (In Situ Cell Death Kit, Fluorescein; Roche Applied Science, Indianapolis, IN), in accordance with the instructions of the manufacturer, and were run on a flow

cytometer (BD FACSCanto II; BD Biosciences, San Jose, CA). Flow cytometry software (BD FACSDiva; BD Biosciences) was used to analyze the data.

As a positive control for the induction of apoptosis, treatment with bortezomib (100 nM for 72 hours) was used. The pan-caspase inhibitor ZVAD-FMK was purchased from Calbiochem and used at 20 μM.

Immunoblot Analysis

Immunoblot analysis was performed as previously described.[8] The following antibodies were purchased from Cell Signaling (Danvers, MA): phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) rabbit monoclonal antibody; p27 Kip1 (SX53G8.5) mouse monoclonal antibody; cyclin D1 (92G2) rabbit monoclonal antibody; phospho-Rb (Ser780) rabbit monoclonal antibody; phospho-Akt (Ser473) (D9E) rabbit monoclonal antibody; phospho-Akt (Thr308) (C31E5E) rabbit monoclonal antibody; and Akt (pan) (C67E7) rabbit monoclonal antibody. The anti–β-actin clone AC-15 mouse monoclonal antibody was from Sigma-Aldrich.

Statistical Analysis

To evaluate the differences across various experimental conditions in the viability experiments, one-way analysis of variance was performed, and post hoc tests (Duncan and Dunnett's T3 tests) served to evaluate differences between pairs of experimental conditions (e.g., vehicle-treated cells vs. cells treated with each concentration of each agent). The additive or synergistic nature of the interaction between agents used in combination was evaluated by isobologram analysis using dose-effect analyzer software (Calcusyn; Biosoft, Ferguson, MO). In all analyses, $P < 0.05$ was considered statistically significant.

RESULTS

Impact of MEK Inhibition on UM Cell Lines Depends on Their Genotype

We tested the impact of the MEK inhibitor AZD6244 on UM cell lines using MTT assay and immunoblotting with the *BRAF*-mutant CM cell lines A375 and M14 serving as controls. We found that the *BRAF*-mutant OCM3 cells were very sensitive to AZD6244, similar to their CM counterparts (Fig. 1A). The Gα-mutant UM cell lines were less sensitive (higher IC_{50}) than the *BRAF*-mutant cells but still responded with growth arrest (Fig. 1A).

Immunoblot analysis revealed that AZD6244 decreased levels of phosphorylated ERK, decreased levels of cyclin D1, increased levels of the cyclin-dependent kinase inhibitor p27, and decreased levels of phosphorylated Rb in both *BRAF*-mutant (OCM3) and Gα-mutant (OMM1.3) UM cell lines (Fig. 1B). All these changes are consistent with and probably mediate the growth-suppressive effect of AZD6244, as has been previously reported for *BRAF*-mutant CM cell lines.[25]

Impact of B-Raf Inhibition on UM Cell Lines Depends on Their Genotype

We tested the impact of the B-Raf inhibitor PLX4720 on UM cell lines using cell survival assays and immunoblot analysis with the *BRAF*-mutant CM cell lines A375 and M14 serving as controls. We found that the *BRAF*-mutant OCM3 cells were sensitive to PLX4720, similar to their CM counterparts (Fig. 2A). However, the Gα-mutant UM cell lines were resistant to the anticancer effect of PLX4720, and, in fact, OMM1.3 cells even exhibited a paradoxical mild increase in proliferation (Fig. 2A). This stimulatory effect of PLX4720 occurred both in the presence of 10% FBS and in serum-free conditions.

Immunoblot analysis revealed that PLX4720 decreased levels of phosphorylated ERK, decreased levels of cyclin D1, increased levels of p27, and decreased levels of phosphory-
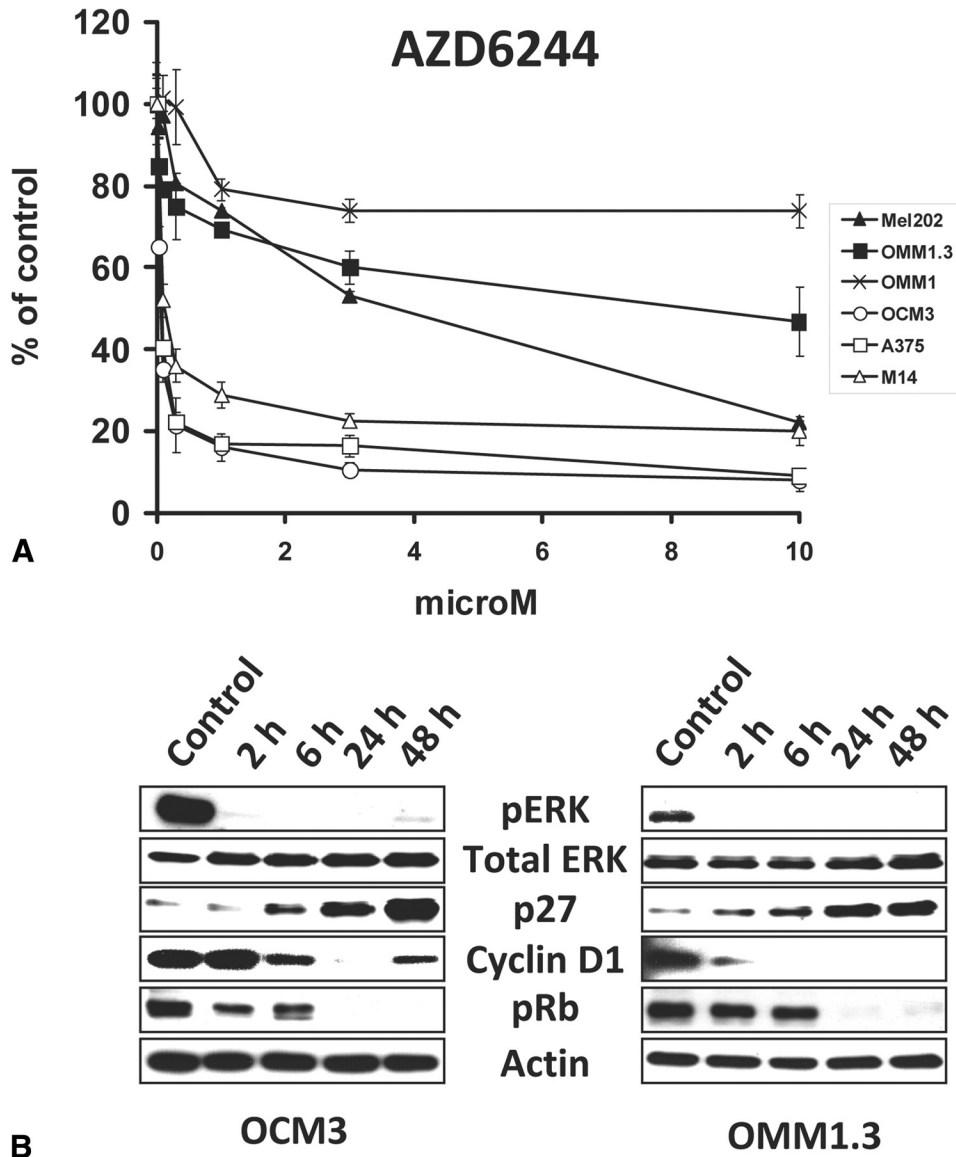
A

B OCM3 OMM1.3

FIGURE 1. The MEK inhibitor AZD6244 suppresses the growth of *BRAF*-mutant and Gα-mutant UM cell lines. (**A**) The *BRAF*-mutant OCM3 cells, the *GNAQ*-mutant OMM1.3 and Mel202 cells, and the *GNA11*-mutant *OMM1* cells were treated with AZD6244 at the indicated concentrations for 96 hours in medium containing 10% FBS. Cell number was quantified with the MTT assay and expressed as percentage of control (DMSO) wells (average ± SD). The *BRAF*-mutant CM cell lines A375 and M14 served as controls. The *BRAF*-mutant OCM3 cells were very sensitive to AZD6244, similar to their CM counterparts. The Gα-mutant UM cell lines were less sensitive than the *BRAF*-mutant cells but still responded with growth arrest. (**B**) Immunoblot analysis revealed that AZD6244 (1 μM) decreased levels of phosphorylated ERK, decreased levels of cyclin D1, increased levels of p27, and decreased levels of phosphorylated Rb in both *BRAF*-mutant (OCM3) and Gα-mutant (OMM1.3) UM cell lines.

lated Rb only in *BRAF*-mutant (OCM3) cells. On the contrary, in Gα-mutant (OMM1.3) UM cells, PLX4720 induced a paradoxical increase in phosphorylated ERK and an early increase in cyclin D1 and pRb levels and did not stimulate p27 levels (Fig. 2B).

Collectively, our findings suggest that the B-Raf inhibitor PLX4720 does not exert anticancer activity against Gα-mutant melanoma cell lines (in fact, it can stimulate ERK signaling).

BrdU Incorporation

Using the BrdU cell proliferation assay, we studied the impact of AZD6244 and PLX4720 on UM DNA synthesis (S phase). Overall, we obtained parallel results to our MTT data. We found that the *BRAF*-mutant OCM3 cells were very sensitive to AZD6244, whereas OMM1.3 cells still responded with decreased DNA synthesis (but to a lesser degree; Fig. 3A). We also found that OCM3 cells were sensitive to PLX4720. However, the Gα-mutant OMM1.3 cells were resistant to the anticancer effect of PLX4720 (Fig. 3A).

TUNEL Assay

UM cells treated with AZD6244 or PLX4720 for 72 hours were tested for apoptosis using the terminal dUTP nick-end labeling

(TUNEL) assay. We did not detect any increase in TUNEL labeling in OCM3 or OMM1.3 cells treated with AZD6244 and PLX4720, suggesting lack of apoptosis (Fig. 3B). The proteasome inhibitor bortezomib (100 nM for 72 hours) served as a positive control for the induction of apoptosis.

Collectively, these data suggest that the mechanism of the observed activity is by growth arrest, not apoptosis. In support, we did not detect any cleavage of caspase-3 or PARP in our immunoblotting analysis of UM cells treated with AZD6244 or PLX4720 for 72 hours (not shown), and the anticancer activity of AZD6244 and PLX4720 was not attenuated by pretreatment with the pan-caspase inhibitor ZVAD-FMK, indicating that apoptosis is not substantially involved in their effects (Supplementary Fig. S2, http://www.iovs.org/lookup/suppl/doi:10. 1167/iovs.11-7398/-/DCSupplemental).

Impact of Combined B-Raf and MEK Inhibition on BRAF-Mutant and Gα-Mutant UM Cells

We next assessed whether combined B-Raf and MEK inhibition would achieve an enhanced anticancer activity. We found that, in *BRAF*-mutant OCM3 cells, the combination of sublethal concentrations of PLX4720 and AZD6244 resulted in synergistic anticancer activity (Fig. 4A). However, in Gα-mutant

FIGURE 2. The B-Raf inhibitor PLX4720 suppressed the growth of *BRAF*-mutant but not Gα-mutant UM cell lines. (**A**) The *BRAF*-mutant OCM3 cells, the *GNAQ*-mutant OMM1.3 and Mel202 cells, and the *GNA11*-mutant *OMM1* cells were treated with PLX4720 at the indicated concentrations for 96 hours in medium containing 10% FBS. Cell number was quantified with the MTT assay and expressed as percentage of control (DMSO) wells (average ± SD). The *BRAF*-mutant CM cell lines A375 and M14 served as controls. The $BRAF^{V600E}$ OCM3 cells were sensitive to PLX4720, similar to their CM counterparts. The Gα-mutant UM cell lines were resistant to the anticancer effect of PLX4720 and, in fact, exhibited a paradoxical mild increase in proliferation. (**B**) Immunoblot analysis revealed that PLX4720 (3 μM) decreased levels of phosphorylated ERK, decreased levels of cyclin D1, increased levels of p27, and decreased levels of phosphorylated Rb in *BRAF*-mutant (OCM3) cells. On the contrary, in Gα-mutant (OMM1.3) UM cells, PLX4720 induced a paradoxical increase in phosphorylated ERK and Rb levels and an early increase in cyclin D1 levels but did not stimulate p27 levels.
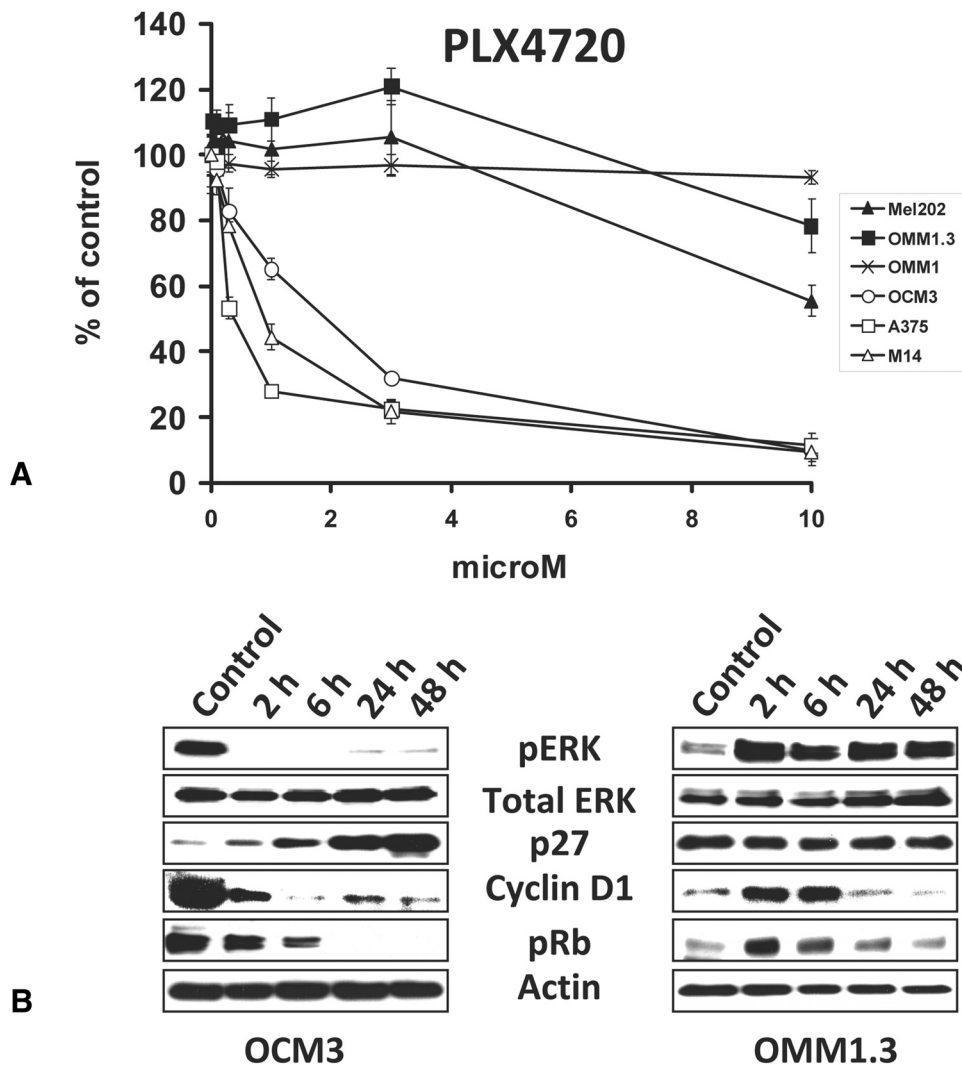
OMM1.3 cells, the addition of AZD6244 could not overcome their resistance to PLX4720 (Fig. 4B). Therefore, the combination of B-Raf and MEK inhibition appears to be a promising approach only for *BRAF*-mutant cells.

Impact of B-Raf and MEK Inhibition on the Akt Pathway in BRAF-Mutant and Gα-Mutant UM Cells

We next investigated the impact of B-Raf and MEK inhibition on the Akt pathway in $BRAF^{V600E}$ and Gα-mutant UM cells. We found that both AZD6244 and PLX4720 induced an early (within 2–6 hours of exposure) increase in the levels of phosphorylated Akt (both Ser473 and Thr308) in both *BRAF*-mutant (OCM3) and Gα-mutant (OMM1.3) cells (Fig. 5A). Eventually, and with prolonged drug exposure, the phosphorylation of Akt returned to baseline in Gα-mutant OMM1.3 cells and decreased even below baseline levels in *BRAF*-mutant OCM3 cells (Fig. 5A).

We hypothesized that the early upregulation of pAkt caused by B-Raf or MEK inhibition might attenuate the anticancer activity of AZD6244, PLX4720, or both, and, therefore, concurrent inhibition of the Akt pathway might enhance the anticancer activity of B-Raf and MEK inhibition. To address this hypothesis, we evaluated the effect of the Akt inhibitor MK2206 in combination with AZD6244 or PLX4720. We found that the Akt inhibitor MK2206 potently enhanced the anticancer activity of the MEK inhibitor (Fig. 5B) and the B-Raf inhib-

itor (not shown) against the *BRAF*-mutant OCM3 cells. MK2206 also enhanced the anticancer activity of the MEK inhibitor against the Gα-mutant UM cells but did not overcome the resistance of the Gα-mutant UM cells to the B-Raf inhibitor (Figs. 6A, 6B).

DISCUSSION

Recent advances in our understanding of the molecular pathophysiology of cancer have allowed for rational development of targeted therapies designed to interrupt molecular pathways critical for cell growth and survival.[37] Kinases represent such "druggable" targets and an area of very active clinical research in oncology. Various malignancies that exhibit "oncogenic addiction" to select kinase pathways respond clinically to treatment with respective kinase inhibitors (e.g., imatinib in chronic myelogenous leukemia and gastrointestinal stromal tumors, erlotinib in EGFR-mutant non–small cell lung carcinoma, and others).[38–41] Because of the biological heterogeneity and interindividual variation in human cancers, molecular profiling of each patient's tumor is necessary to guide selection of the appropriate targeted therapy for the patient most likely to benefit from it.[37] The goal of this personalized approach is to avoid exposing patients to drugs from which they are unlikely to benefit, thus sparing them unnecessary toxicity and cost. For example, responses of non–small cell lung carcino-
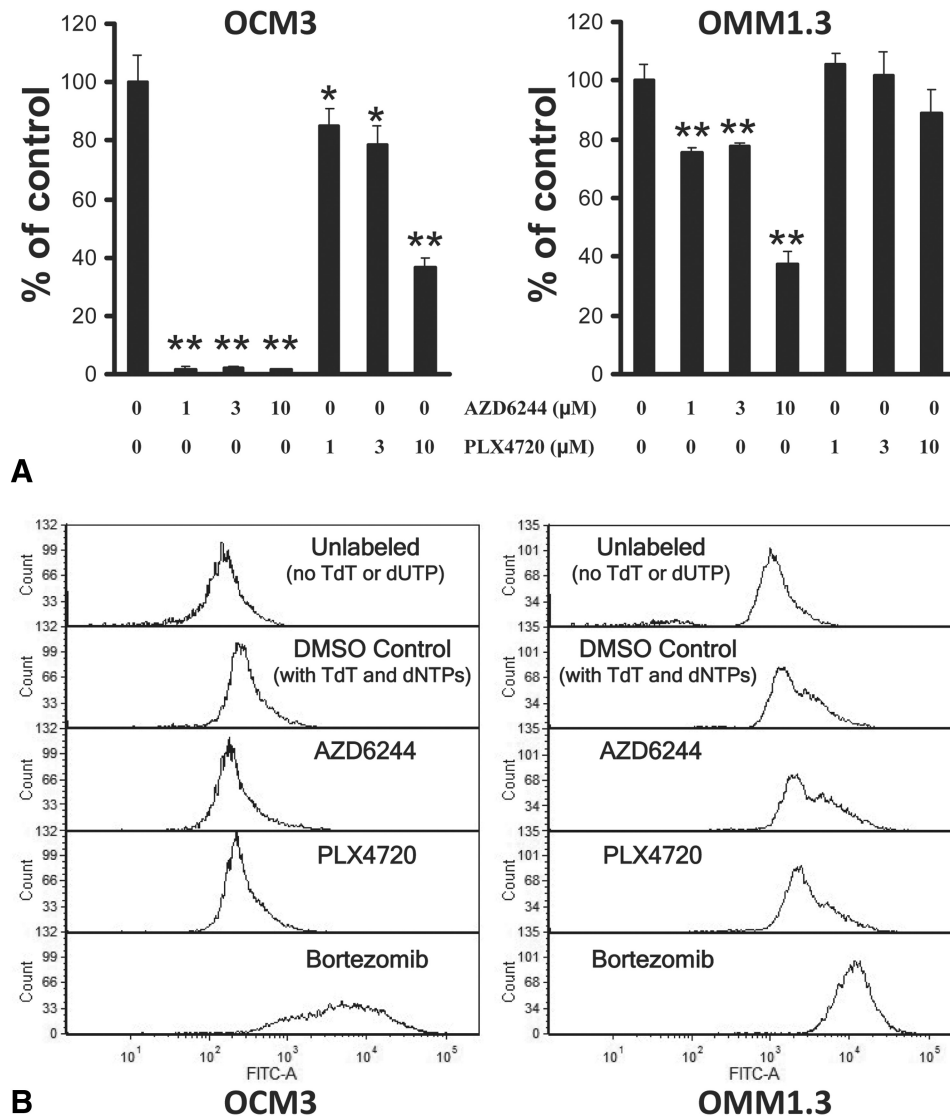
FIGURE 3. Growth arrest without apoptosis in UM cell lines treated with AZD6244 or PLX4720 monotherapy. (A) Using the BrdU cell proliferation assay, we studied the impact of AZD6244 and PLX4720 on UM DNA synthesis (S phase). The *BRAF*-mutant OCM3 cells and the Gα-mutant OMM1.3 cells were treated with AZD6244 or PLX4720 (or DMSO) at the indicated concentrations in 24-well plates for 48 hours. Subsequently, BrdU was added to the wells for an additional 24 hours, and the amount of incorporation into DNA was quantified according to the instructions of the manufacturer and was expressed as percentage of control (DMSO) wells (average ± SD). We found that the *BRAF*-mutant OCM3 cells were sensitive to AZD6244 and PLX4720, whereas Gα-mutant OMM1.3 cells still responded with decreased DNA synthesis to AZD6244 (but to a lesser degree than OCM3 cells) though they were resistant to the anticancer effect of PLX4720 (*$P < 0.05$, **$P < 0.001$ vs. respective DMSO controls). (B) Using the TUNEL assay, we studied the impact of AZD6244 and PLX4720 on UM cell apoptosis. UM cells were treated with AZD6244 or PLX4720 at 3 μM (or DMSO control) for 72 hours and then labeled for apoptosis using the enzyme terminal deoxynucleotidyl transferase (TdT) and the fluorescein-dUTP. We did not detect any shift in fluorescence (TUNEL labeling) in OCM3 or OMM1.3 cells treated with AZD6244 or PLX4720 monotherapy, suggesting a lack of apoptosis. The proteasome inhibitor bortezomib (100 nM for 72 hours) served as a positive control for the induction of apoptosis. Cells incubated without TdT or fluorescein-dUTP served as a negative control.

mas to EGFR small molecule inhibitors (such as erlotinib) are generally limited to tumors harboring somatic mutations in the EGFR tyrosine kinase domain.[39–41] Moreover, colorectal carcinomas harboring certain activating somatic mutations in *KRAS* are resistant to EGFR-targeting monoclonal antibodies, and use of such agents should be avoided in these particular carcinomas.[42] In the present study, we investigated the activity of

small molecule inhibitors of MEK, B-Raf and Akt against UM cells in vitro. Our results demonstrate that the sensitivity of UM cells to these inhibitors is genotype dependent and make a strong case for a personalized approach in the management of UMs with targeted therapies.

CMs frequently harbor mutations in *BRAF* (usually *BRAF^{V600E}*)[1] or *NRAS*[2–6] that cause constitutive activation of
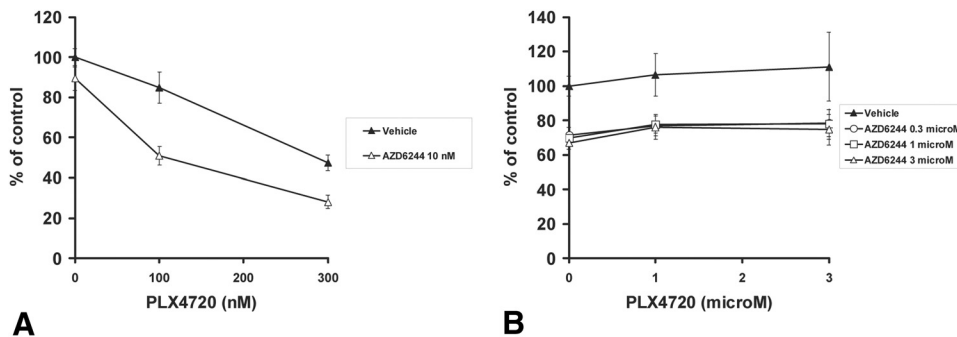

FIGURE 4. Impact of combined B-Raf and MEK inhibition on *BRAF*-mutant and Gα-mutant UM cells. The *BRAF*-mutant OCM3 cells (A) and the Gα-mutant OMM1.3 (B) and Mel202 cells were treated with the combination of PLX4720 and AZD6244 at the indicated concentrations for 96 hours in medium containing 10% FBS. Cell number was quantified with the MTT assay and expressed as a percentage of control wells (average ± SD). In *BRAF*-mutant OCM3 cells, the combination of sublethal concentrations of PLX4720 and AZD6244 resulted in synergistic anticancer activity (A), whereas in Gα-mutant OMM1.3 cells (B) and Mel202 cells (not shown), the addition of AZD6244 could not overcome their resistance to PLX4720.
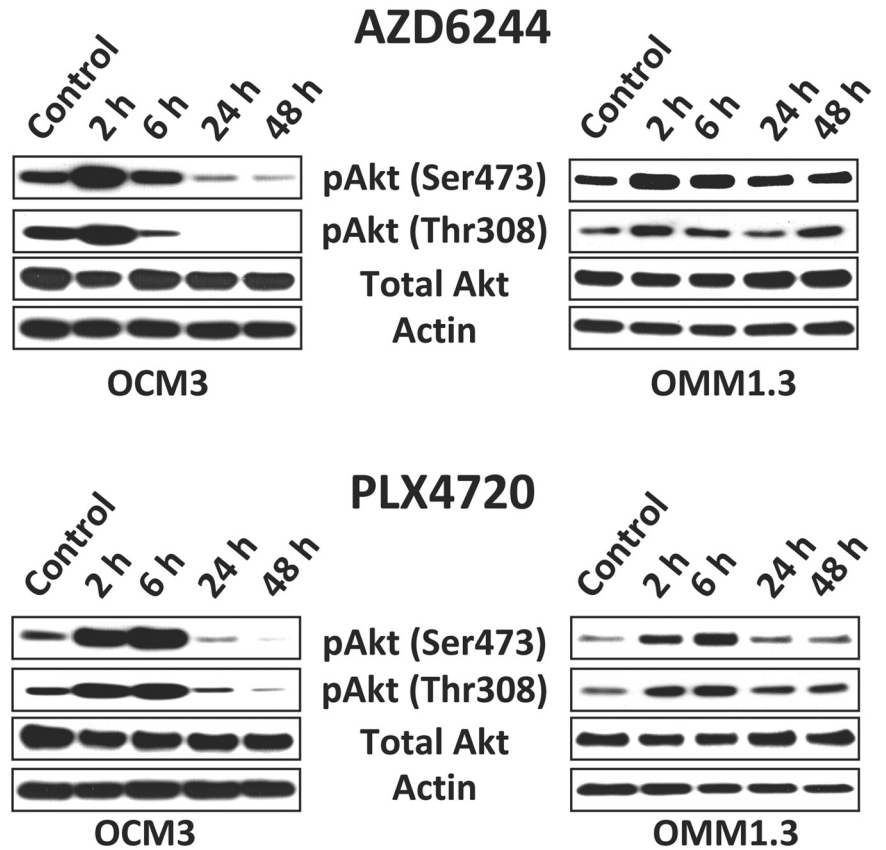
FIGURE 5. Impact of B-Raf and MEK inhibition on the Akt pathway in *BRAF*-mutant and Gα-mutant UM cells. (**A**) Immunoblot analysis revealed that both AZD6244 and PLX4720 induced an early (within 2–6 hours of exposure) increase in the levels of phosphorylated Akt (at residues Ser473 and Thr308) in both *BRAF*-mutant OCM3 and Gα-mutant OMM1.3 cells. Eventually, and with prolonged drug exposure, the phosphorylation of Akt returned to baseline in Gα-mutant OMM1.3 cells and decreased even below baseline levels in *BRAF*-mutant OCM3 cells. (**B**) The Akt inhibitor MK2206 potently enhanced the anticancer activity of the MEK inhibitor AZD6244 against the *BRAF*-mutant OCM3 cells. The cells were treated with drugs at the indicated concentrations for 96 hours in medium containing 10% FBS. Cell number was quantified with the MTT assay and expressed as a percentage of control wells (average ± SD).
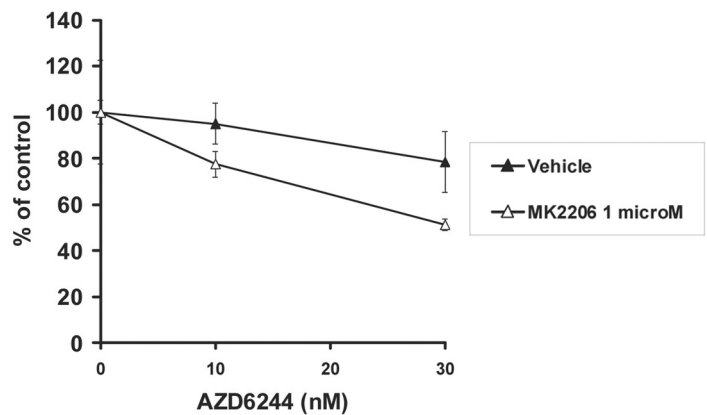
the MEK/ERK pathway and cell proliferation. CMs harboring *BRAF*[V600E] mutations exhibit increased sensitivity to inhibitors of MEK[25] and B-Raf.[13–20] We found similar sensitivity to the MEK and B-Raf inhibitors in *BRAF*[V600E] UM cells, suggesting that such targeted therapies may represent a promising option for this subset of UM patients as well. In agreement with their CM counterparts,[13,25] *BRAF*[V600E] UM cells treated with AZD6244 or PLX4720 exhibited decreased levels of phosphorylated ERK, decreased levels of cyclin D1, increased levels of the cyclin-dependent kinase inhibitor p27, and decreased levels of phosphorylated Rb. All these changes are consistent with and probably mediate the growth-suppressive effect (inhibition of cell proliferation) of MEK or B-Raf inhibition.

However, *BRAF*[V600E] mutations are rare in UMs.[26–28] Instead, an equivalent oncogenic event is frequently present in the form of mutually exclusive somatic mutations in the het-

erotrimeric G protein α-subunit *GNAQ* (~40%-50% of primary UMs)[30,32,43,44] or its related *GNA11* (~30% of primary UMs).[31] These mutations apparently occur early in UM carcinogenesis,[32] contrary to other genetic events associated with increased metastatic potential such as the recently described BRCA1-associated protein 1 (BAP1) mutations.[45] The *GNAQ*[Q209] and *GNA11*[Q209] mutations affect the Ras-like domain of these G proteins, specifically corresponding to *NRAS*[Q61R], abolishing GTPase activity, and resulting in constitutive activation. In this study, we examined the sensitivity of *GNAQ*-mutant and *GNA11*-mutant UM cell lines to MEK and B-Raf inhibition. We found that Gα-mutant UM cells are sensitive to MEK inhibition (exhibiting decreased levels of pERK, cyclin D1, and pRb, increased levels of p27, and decreased proliferation) but to a lower degree (higher IC$_{50}$) than *BRAF*-mutant UM and CM cells. In direct contrast to *BRAF*-mutant
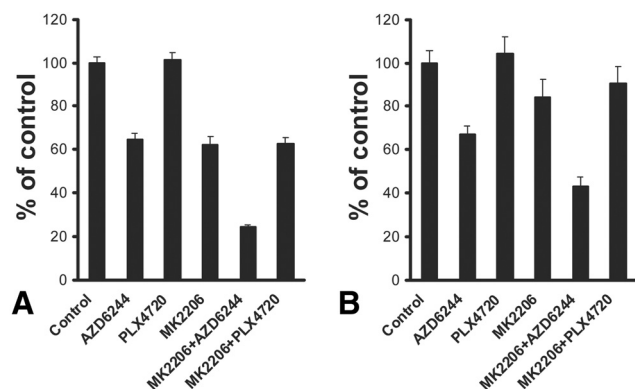
FIGURE 6. Akt inhibition enhances the anticancer activity of MEK, but not B-Raf, inhibition in Gα-mutant UM cells. A sublethal concentration of the Akt inhibitor MK2206 (1 μM) potently enhanced the anticancer activity of the MEK inhibitor AZD6244 (1 μM) against the Gα-mutant Mel202 cells (**A**) and OMM1.3 cells (**B**). However, MK2206 (1 μM) did not sensitize them to PLX4720 (1 μM). The cells were treated with drugs for 96 hours in medium containing 10% FBS. Cell number was quantified with the MTT assay and expressed as a percentage of control wells (average ± SD).

UM and CM cells, Gα-mutant UM cells were resistant to the anticancer effect of the B-Raf inhibitor PLX4720 and, in fact, exhibited a paradoxical increase in pERK signaling.

These findings emphasize the need for a personalized approach to the use of targeted therapies in patients with UM because the Gα-mutant activates the ERK pathway in a manner that cannot be targeted, thus far, by PLX4720, at least when used as a monotherapy. Therefore, new therapeutic approaches are needed for Gα-mutant tumors. The paradoxical activation of the ERK pathway in Gα-mutant UM cells by PLX4720 parallels the similar behavior of *NRAS*-mutant and other *BRAF* wild-type CM cell lines.[13-16,18] Collectively, these data highlight the importance of choosing the appropriate, personalized targeted therapy for each patient, not only to avoid unnecessary toxicity to normal tissues but also because an inappropriate targeted therapy can enhance the proliferation rate and growth of the cancer cells harboring the wrong genotype, with detrimental effects. Tumor genotyping should be a prerequisite for enrollment of UM patients (and probably all patients) in clinical trials of targeted therapies.[18]

In view of the resistance of Gα-mutant UM cells to PLX4720, we investigated whether combination of the B-Raf inhibitor with MEK or Akt inhibition would restore sensitivity. Unfortunately, in Gα-mutant UM cells, the addition of the MEK inhibitor AZD6244 or the Akt inhibitor MK2206 did not restore sensitivity to PLX4720. On the contrary, in *BRAF*-mutant cells, combined B-Raf and MEK inhibition resulted in synergistic anticancer activity. Furthermore, the Akt inhibitor MK2206 enhanced the anticancer activity of both the B-Raf inhibitor and the MEK inhibitor against the *BRAF*-mutant UM cells and the anticancer activity of the MEK inhibitor against the Gα-mutant UM cells. This finding is in agreement with the early upregulation of phosphorylated Akt that we observed on MEK or B-Raf inhibition in our models and with reports of the synergistic activity of AZD6244 in combination with the inhibition of Akt in *BRAF*[V600E] cutaneous melanoma[46] and lung carcinoma[47] cell lines. Moreover, in UM cells, cotreatment with the PI3K inhibitor LY294003, in combination with the MEK inhibitor U0126 or with the B-Raf inhibitor BAY43-9006, has been reported to result in more potent inhibition of cell proliferation.[48] These data highlight the combined inhibition of MEK and Akt pathways as a rationally designed targeted therapeutic

approach for Gα-mutant UMs, that deserves investigation in a clinical trial.

In conclusion, we report that the Gα mutations, present in the majority of UMs, are functionally similar to the *NRAS* mutations seen in CMs in that they are associated with lower sensitivity to MEK inhibition and complete resistance to the anticancer activity of the B-Raf inhibitor PLX4720. Combined inhibition of MEK and Akt results in synergistic anticancer activity and represents a promising targeted therapeutic approach for Gα-mutant UMs. Our findings emphasize the importance of a personalized, genotype-based approach to the use of targeted therapies in clinical trials of patients with UM.

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