Co-targeting HGF/cMET Signaling with MEK Inhibitors in Metastatic Uveal Melanoma

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Abstract

Patients with metastatic uveal melanoma usually die within 1 year of diagnosis, emphasizing an urgent need to develop new treatment strategies. The liver is the most common site of metastasis. Mitogen-activated protein kinase kinase (MEK) inhibitors improve survival in V600 BRAF-mutated cutaneous melanoma patients but have limited efficacy in patients with uveal melanoma. Our previous work showed that hepatocyte growth factor (HGF) signaling elicits resistance to MEK inhibitors in metastatic uveal melanoma. In this study, we demonstrate that expression of two BH3-only family proteins, Bim-EL and Bmf, contributes to HGF-mediated resistance to MEK inhibitors. Targeting HGF/cMET signaling with LY2875358, a neutralizing and internalizing anti-cMET bivalent antibody, and LY2801653, a dual cMET/RON inhibitor, overcomes resistance to trametinib provided by exogenous HGF and by conditioned medium from primary hepatic stellate cells. We further determined that activation of PI3Kα/γ/β isoforms mediates the resistance to MEK inhibitors by HGF. Combination of LY2801653 with trametinib decreases AKT phosphorylation and promotes proapoptotic PARP cleavage in metastatic uveal melanoma explants. Together, our data support the notion that selectively blocking cMET signaling or PI3K isoforms in metastatic uveal melanoma may break the intrinsic resistance to MEK inhibitors provided by factors from stromal cells in the liver. Mol Cancer Ther; 16(3); 516–28. ©2017 AACR.

Introduction

Uveal melanoma is the most common intraocular malignant tumor in adults and comprises approximately 5% of all melanomas (1). Even after treatment of the primary tumor, 20% to 50% of patients succumb to metastatic disease. The liver is the predominant organ of metastasis. Standard chemotherapies and immune checkpoint blockers rarely induce clinical responses in patients with macrometastasis and their 1-year survival rate is less than 30% (2). This knowledge emphasizes an urgent unmet need for effective therapeutic strategies for advanced uveal melanoma.

Activating mutations in GNAQ and GNA11 (typically Q209 and less commonly R183), which encode alpha subunits of the heterotrimeric G proteins, Gqα and G11α, are found in 80% to 90% of uveal melanoma (3–6). Silencing GNAQ induces apoptosis in mutant but not wild-type uveal melanoma cells (4, 7). Mutant Gqα and G11α activate phospholipase C-β (PLC-β), which regulates several pathways including MEK-ERK1/2 and protein kinase C (PKC) signaling (8). In addition, PLC-β-independent activation of Trio, a guanine nucleotide exchange factor, transduces signaling downstream from Gqα to activate Yes-associated protein 1 (YAP) and promote uveal melanoma tumorigenesis (9–11). In uveal melanoma, GNAQ and GNA11 mutations occur early in disease progression; however, additional alterations are required (5, 12). Mutations in the tumor suppressor BRCA1-associated protein 1 (BAP1) on chromosome 3 are found in 32% to 50% of primary melanomas (13). BAP1 mutations associate with aggressive disease and higher likelihood of metastasis. Silencing BAP1 in primary uveal melanoma cell lines results in a gain of stem-like properties with little/no effect on proliferation and invasion (14). Additional genes mutated in uveal melanoma are SF3B1 and EIF1AX, which associate with a favorable prognosis (15–17), PLCB4 (18), and CYSLTR2 (19).

A major effector pathway downstream of mutant Gqα and G11α is RAF–MEK1/2–ERK1/2 signaling. Inhibition of MEK1/2 with trametinib or selumetinib induces either cell-cycle arrest or apoptosis in uveal melanoma cell lines (7, 20); however, clinical studies in advanced-stage patients with uveal melanoma indicate that MEK inhibitors have limited clinical benefit. Trametinib was ineffective in a phase I trial cohort of 16 metastatic patients with uveal melanoma (21). A phase II trial with selumetinib in 120 patients showed a 9-week improvement in progression-free survival compared with standard chemotherapy but no improvement in overall survival (22). In the most recent phase III trial with 129 patients (23), a combination of selumetinib and standard chemotherapy dacarbazine failed to improve progression-free survival compared with chemotherapy alone. Thus, while MEK inhibitor may form part of a therapeutic approach for advanced-stage uveal melanoma, further investigation is required to identify inhibitors to act in combination.

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The majority of patients with uveal melanoma with overt metastasis show primary resistance to MEK inhibitors, which may be mediated by factors from the tumor microenvironment. In uveal melanoma cell monocultures, hepatocyte growth factor (HGF) provides resistance to MEK inhibitors (20). HGF is secreted by quiescent hepatic stellate cells. Consistent with the presence of HGF in tumor microenvironment, the majority of uveal melanoma live metastases express phosphorylated/activated cMET (20). Together, these results suggest that MEK inhibitors in combination with cMET targeting agents may have utility in advanced uveal melanoma. In this study, we explored the molecular mechanism of HGF-mediated resistance to MEK inhibitors in uveal melanoma cells and preclinically evaluated the efficacy of cotargeting cMET with MEK inhibitor in metastatic cell lines and ex vivo explants. Our data show that downregulation in the BH3-only proteins, Bim-EL and Bmf, contribute to HGF-mediated protective effect in metastatic uveal melanoma cells. Clinical grade cMET targeting agents effectively overcome the resistance provided by exogenous HGF as well as factors derived from hepatic stellate cells. Combined inhibition of cMET and MEK1/2 enhances apoptotic signal in cell lines and an ex vivo explant model of metastatic uveal melanoma. Together, these results suggest that MEK inhibitors in combination with cMET targeting agents may have utility in advanced-stage patients with uveal melanoma.

**Materials and Methods**

**Cell culture**

UM001 and UM004 cells were derived from liver and orbital metastases of human uveal melanoma, respectively; both harbor GNAQ Q209L mutations (20, 24). UM001 cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 10% nonessential amino acids, 2 mmol/L l-glutamine, and 10 mmol/L HEPES buffer. UM004 cells were maintained in MEM containing 10% heat-inactivated FBS and 2 mmol/L l-glutamine. LX-2 human hepatic stellate cell line was purchased from Lonza and cultured in DMEM containing 2% FBS and 2 mmol/L l-glutamine according to manufacturer’s protocol. Human hepatic stellate cells (HHSteC) were cultured in basal medium, 2% FBS, and 1% stellate cell growth supplement according to manufacturer’s protocol (SceintCell Research Laboratories). HHSteC conditioned medium is collected from HHSteC cultures immediately before subculture. Medium conditioned by early passage (<6 passages). HHSteC culture was used for functional coculture experiments with UM001 and UM004 cells.

**Cell line validation**

UM001 and UM004 cells were confirmed as harboring GNAQ mutations as determined by Sanger DNA sequencing. UM001 and UM004 cells were analyzed by STR analysis on January 15, 2015. The UM001 and UM004 profiles were unique, although the latter had a 94% match with 3 changed alleles to MDA-MB-330 cells on the DMSZ resource.

**Inhibitors, growth factors, and function-blocking antibodies**

Trametinib (GSK1120212), MK2206 [PubMed compound database (CID, 24964624)], GDC0033 (25), TGX221 (26), BYL719 (25), and IPI145 (25) were purchased from Selleck Chemicals. Recombinant human HGF was purchased from PeproTech and used at 10 ng/mL on the basis of our previous studies (20). The neutralizing and internalizing anti-cMET antibody, LY2873358, and the cMET/RTK inhibitor, LY2801653 (27), were provided by Eli Lilly and Company.

**siRNA and transfection**

UM004 cells (3 x 10⁶) were seeded in 6-well plates overnight before transfection with chemically synthesized siRNAs at a final concentration of 25 nmol/L using Lipofectamine RNAiMAX (Invitrogen) as previously described (28). Bim-EL-specific siRNAs (GAGCGAGAAGGUACAAUUGTT and CAAGUUCUGUCUUCGGUUCCTT) were purchased from Cell Signaling Technology. Bmf-specific siRNA (GAGUUAACGAGAUAACGAAHUUA) was purchased from Dharmacon Inc. A nontargeting siRNA (UAGCGAUAACACACAUCC) was used as a control.

**Western blotting**

Cells were washed in cold PBS and lysed directly in Laemmli sample buffer. Lysates were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Membranes were blocked with 1% BSA and incubated with indicated primary antibodies overnight at 4°C. Proteins were detected using the horseradish peroxidase-conjugated secondary antibodies followed by development using chemiluminescence substrate (Pierce). The following primary antibodies were used: ERK2 (D-2), cyclin A, and Noxa from Santa Cruz Biotech. Inc.; Bcl-2, Bcl-xL, Mcl-1, Bax, Bad, Bid, Puma, cleaved caspase-3, cleaved PARP, RB, phospho-RB (S780), cMET, phospho-cMET Y1234/1235 (D26), phospho-cMET Y1349, AKT, phospho-AKT T308 (C31ESE), phospho-AKT S473 (D9E), and phospho-ERK1/2 (D13.14.4E). Stat3, phospho-Stat3 Y705, p110α-P13K, p110β-P13K, and p110δ-P13K from Cell Signaling Tech; cyclin D1 and Bcl-w from BD Pharmingen, p110δ-P13K, α-SMA, and FAP from Abcam; Bim-EL and Bmf from Enzo Life Sciences; actin from Sigma-Aldrich; and Hsp90 (clone 4F3.E8) from StressMarq Biosciences. Chemiluminescence was visualized on a ChemiDoc Imaging System and quantitated using Image Lab 4.1 software (Bio-Rad).

**EdU incorporation assay**

UM001 and UM004 cells were treated with DMSO, 50 nmol/L trametinib, 10 ng/mL HGF or trametinib plus HGF for 32 hours before the addition of 10 nmol/L EdU for another 16 hours. Cells were then processed using the Click-IT Plus EdU Alexa Fluor 647 Flow Cytometry Assay Kit (Invitrogen) according to the manufacturer's protocol. Experiments were performed in triplicate, and statistical analysis was performed using a 2-tailed t test assuming unequal variance with error bars representing SD.

**Annexin V/propidium iodide staining**

Cells were trypsinized, washed with cold PBS, and resuspended in 0.1-mL binding buffer. Cells were then stained with 5 μL of Annexin V-APC and 2 μL of propidium iodide (PI) for 30 minutes at room temperature. Staining was then analyzed by flow cytometry on a BD FACSCalibur flow cytometer (BD Biosciences). Data were analyzed by FlowJo software (Tree Star, Inc.). Experiments were performed in triplicate with statistical analysis as in EdU incorporation assay.

**Crystal violet staining**

Exponentially growing UM001 and UM004 cells were plated in 6-well or 12-well dishes and treated as described in figure.
Statistical analysis
Statistical analyses were performed using SPSS v22.0 (IBM). One-way ANOVA analyses were performed on normalized data from groups of equal sizes. No outliers were identified during inspection of boxplots. All of the groups were determined to be normally distributed using Shapiro–Wilk test (P > 0.05). There was homogeneity of variances among all groups, as determined by Levene test of equality of variances (P > 0.05). Dunnett one-tailed multiple comparison post-hoc tests were performed to determine statistical significance.

Results
HGF promotes G1/S cell-cycle progression and decreases cytotoxicity of trametinib-treated cells
We determined whether HGF-mediated resistance to MEK inhibitors in uveal melanoma cells was associated with effects on S-phase entry (EdU incorporation) and/or apoptosis (Annexin V/PI staining). HGF was used at 10 ng/mL on the basis of our previous studies (20). In UM001 and UM004 cells, trametinib treatment resulted in more than 90% decrease in EdU incorporation (Fig. 1A) and 45% to 60% increase in Annexin V staining (Fig. 1B). Treatment with HGF in the absence of MEK inhibition elicited minimal effect on S-phase entry and apoptosis. In contrast in trametinib-treated UM001 and UM004 cells, HGF restored S-phase entry by 70% and 40%, respectively, compared with DMSO controls (Fig. 1A). In addition, HGF significantly reduced the Annexin V population in trametinib-treated UM001 and UM004 cells (Fig. 1B; Supplementary Fig. S1).

We next analyzed the cell-cycle profiles of UM001 and UM004 cells treated with trametinib, HGF, or the combination of HGF plus trametinib. Trametinib treatment was associated with changes in G1–S regulators including lower expression of cyclin A2 and cyclin D1 and reduced retinoblastoma (RB) phosphorylation (Fig. 1C). Downregulation of total RB expression following trametinib treatment was also detected, an effect previously observed in breast cancer cell lines following inhibition of cell-cycle progression with CDK4/6 inhibitors (30, 31). MEK inhibition increased expression of the apoptotic markers, cleaved PARP and cleaved caspase-3. Notably, trametinib-treated cells treated with HGF showed a partial recovery of cyclin A2, cyclin D1, and phospho-RB levels. HGF also modestly increased levels of phospho-ERK1/2 in trametinib-treated cells. In addition, the induction of cleaved PARP and cleaved caspase-3 was mitigated by HGF in trametinib-treated cells (Fig. 1C). Together, these data indicate that HGF promotes the growth of trametinib-treated cells through restoration of cell-cycle progression and inhibition of apoptosis.

Downregulation of Bim-EL and Bmf contributes to HGF-mediated resistance to trametinib in uveal melanoma cells
To molecularly understand how HGF counteracts trametinib-mediated apoptosis, we compared the levels of Bcl-2 family proteins in uveal melanoma cells treated with HGF, trametinib, or the combination of both. We also treated cells with MK2206
Figure 1.
HGF promotes cell-cycle progression of trametinib-treated uveal melanoma cells. A, HGF promotes S-phase entry in trametinib-treated cells. A total of $3.0 \times 10^5$ UM001 cells and UM004 cells were seeded in triplicates and treated with DMSO, 50 nmol/L trametinib, 10 ng/mL HGF, and a combination of 50 nmol/L trametinib and HGF, respectively, for a total of 32 hours. A final concentration of 10 µmol/L EdU was allowed to incorporate for 16 hours before processing. S-phase entry is normalized to DMSO condition, and data points are indicative of 3 experimental repeats. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. B, HGF inhibits trametinib-induced apoptosis in uveal melanoma cells. UM001 and UM004 cells were treated with DMSO, 10 ng/mL HGF, 50 nmol/L trametinib, a combination of HGF and trametinib, respectively, for 48 hours. Cells were then subjected to Annexin V/PI staining. The percentage of Annexin V–positive cells is graphed. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. C, HGF modulates expression of cell-cycle regulators in trametinib-treated cells. UM001 cells (left) and UM004 cells (right) were treated with 50 nmol/L trametinib, 10 ng/mL HGF, or combinations as indicated for 48 hours. Cell lysates were probed for levels of cell-cycle regulators cyclin A2, cyclin D1, phospho-RB, RB, cleaved PARP, and cleaved caspase 3, respectively. Levels of pAKT and pERK were also determined by Western blotting.

HGF promotes cell-cycle progression of trametinib-treated uveal melanoma cells. A, HGF promotes S-phase entry in trametinib-treated cells. A total of $3.0 \times 10^5$ UM001 cells and UM004 cells were seeded in triplicates and treated with DMSO, 50 nmol/L trametinib, 10 ng/mL HGF, and a combination of 50 nmol/L trametinib and HGF, respectively, for a total of 32 hours. A final concentration of 10 µmol/L EdU was allowed to incorporate for 16 hours before processing. S-phase entry is normalized to DMSO condition, and data points are indicative of 3 experimental repeats. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. B, HGF inhibits trametinib-induced apoptosis in uveal melanoma cells. UM001 and UM004 cells were treated with DMSO, 10 ng/mL HGF, 50 nmol/L trametinib, a combination of HGF and trametinib, respectively, for 48 hours. Cells were then subjected to Annexin V/PI staining. The percentage of Annexin V–positive cells is graphed. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. C, HGF modulates expression of cell-cycle regulators in trametinib-treated cells. UM001 cells (left) and UM004 cells (right) were treated with 50 nmol/L trametinib, 10 ng/mL HGF, or combinations as indicated for 48 hours. Cell lysates were probed for levels of cell-cycle regulators cyclin A2, cyclin D1, phospho-RB, RB, cleaved PARP, and cleaved caspase 3, respectively. Levels of pAKT and pERK were also determined by Western blotting.
to evaluate the role of AKT activity. HGF promoted the phosphorylation of AKT in the presence of trametinib, an effect that was diminished by MK2206 (Fig. 2A). Trametinib treatment did not alter expression of antiapoptotic proteins, Bcl-w and Bcl-xl, multidomain proapoptotic proteins, Bak and Bax, or BH3-only proteins, Bad, Bid, and Noxa, in UM001 and UM004 cells (Fig. 2A). In contrast, the BH3-only proapoptotic proteins, Bcl-2–interacting mediator of cell death extra large (Bim-EL), and Bcl-2 modifying factors (Bmf) were upregulated in trametinib-treated cells. The induction of Bim-EL and Bmf was diminished or markedly reduced when HGF was supplemented to trametinib-treated uveal melanoma cells (Fig. 2A). Notably, Bim-EL and Bmf levels were reinduced in cells treated with a combination of trametinib, HGF, and MK2206, suggesting that HGF activation of AKT mediates the resistance to trametinib.

A modest upregulation of the prosurvival protein Bcl-2 and the BH3-only proapoptotic protein, Puma, was detected with trametinib treatment in one (Puma) or both (Bcl-2) cell lines (Fig. 2A).

To determine whether upregulation of Bim-EL and Bmf is required for trametinib-induced inhibition of cell viability, Bim-EL- and/or Bmf-silenced UM004 cells were treated with trametinib (Fig. 2B) and evaluated by crystal violet staining (Fig. 2C). In comparison with controls, trametinib decreased cell
viability by about 60% (Fig. 2C). Individual knockdown of Bim-EL and Bmf each partially rescued cells from trametinib with cell viability inhibited by 32% to 39%; however, simultaneous silencing of Bim-EL and Bmf further restored the viability of trametinib-treated cells with cell growth decreased by about 26% of the control (Fig. 2C). To examine whether Bim-EL and Bmf are sufficient to promote uveal melanoma cell apoptosis, UM001 and UM004 cells were infected with adenoviruses to express Bim-EL, Bmf, and enhanced green fluorescence protein (eGFP), as a control. Ectopic expression of Bim-EL and/or Bmf significantly increased apoptosis in uveal melanoma cells, whereas expression of eGFP showed little effect (Supplementary Fig. S2). These results suggest that Bim-EL and Bmf are sufficient to induce apoptosis and are downregulated in HGF-mediated resistance to MEK inhibitors.

**LY2801653 and LY2875358 abrogate HGF-mediated resistance to trametinib in uveal melanoma cells**

To inhibit HGF-mediated signaling, we utilized 2 cMET targeting agents that are being tested in clinical trials for patients with uveal melanoma with liver metastasis as well as other advanced cancers. Of these 2 agents, LY2801653 is a type II kinase inhibitor with cMET as one of its target and displays antitumor activity in non–small cell lung carcinoma and cholangiocarcinoma preclinical models (32–34). LY2875358 is a neutralizing and internalizing anti-cMET bivalent antibody that showed potent antitumor activity in both HGF-dependent and cMET-amplified preclinical tumor models (35). Initially, UM001 and UM004 cells were treated with increasing doses of LY2801653 and LY2875358 followed by HGF stimulation. Both cMET inhibitors effectively blocked HGF-induced phosphorylation of ERK1/2, AKT, and cMET at tyrosine 1349 (Fig. 3A and 3B). Phosphorylation at tyrosine 1349 in the cMET cytoplasmic domain provides a direct binding site for Gab1 (36), which promotes AKT pathway activation. Of note, LY2875358 had minimal effect on HGF-induced phosphorylation of cMET at tyrosine 1234/5 (Fig. 3A and B), critical sites for kinase activation. We evaluated the ability of these 2 cMET targeting agents in overcoming HGF-mediated resistance to trametinib in uveal melanoma cells. LY2801653 alone did not significantly alter UM001 and UM004 cell growth at 100 nmol/L; however, growth of trametinib-treated uveal melanoma cells which decreased by about 57% compared with the vehicle control was further inhibited when treated with LY2801653 (Fig. 3B). The viability of trametinib/LY2801653 cotreated UM001 and UM004 cells decreased by 81% and 64%, respectively, of the vehicle control. Importantly, HGF-mediated growth protection from trametinib treatment was abrogated by LY2801653 (Fig. 3B). Similarly, LY2875358 alone had little effects on UM001 and UM004 cell growth. Although LY2875358 did not further inhibit growth of trametinib-treated cells, LY2875358 blocked HGF-mediated protection from trametinib (Fig. 3C). The viability of trametinib-treated UM001 and UM004 cells was increased to levels similar to the vehicle control when cells were treated with HGF; an effect that was decreased with LY2875358 by 44% in UM001 and 25% in UM004 compared with vehicle control (Fig. 3C). Together, these data demonstrate that targeting HGF signaling with clinical-grade cMET neutralizing antibody and inhibitor overcomes HGF-mediated resistance to trametinib in metastatic uveal melanoma cells.

**Primary hepatic stellate cell medium protects uveal melanoma cells against trametinib through HGF/cMET signaling**

Basal phosphorylation of cMET and downstream signaling is low in uveal melanoma lines, and understanding the communication between cancer cells and the stroma in the metastatic site is necessary for the development of optimal therapeutic regimens. Uveal melanoma frequently metastasizes to the liver. Hepatic stellate cells are intralobular connective tissue cells that are quiescent in a healthy liver but transition into myofibroblast-like cells and become activated during liver fibrosis and hepatocellular carcinomas (37). Current available stellate cell lines are either immortalized by hTERT or become activated because of long-time culture and therefore at least partially lose characteristics of their primary origins (38). Therefore, we utilized primary stellate cells that were isolated from human liver. These cells were cultured for up to 6 passages to minimize their activation, passages at which they did not express the fibroblast markers, α-SMA and FAP (Supplementary Fig. S3A).

To better understand the effects of hepatic stellate cells on uveal melanoma cells, we first performed high-throughput antibody-based RPPA analysis on UM001 and UM004 cells incubated for 1 or 48 hours with either unconditioned medium or stellate cell conditioned medium. Supervised clustering of proteins that were regulated by stellate cell conditioned medium and further significance analysis of microarrays (SAM) identified several proteins that were differentially regulated by addition of stellate cell conditioned medium (Fig. 4A; Supplementary Fig. S3B). In both UM001 and UM004 cells, PI3K/AKT and ERK/MAPK signaling were the most activated pathways by stellate cell medium (Fig. 4A). We performed Western blot analysis to validate the RPPA findings. In UM001 and UM004 cells, conditioned medium from stellate cells rapidly induced phosphorylation of ERK1/2, AKT, cMET, and Stat3 (Fig. 4B). We also demonstrated that HGF was present at ng/mL levels in the conditioned medium from early passages of primary hepatic stellate cells by ELISA (Supplementary Fig. S3C). In contrast, the conditioned medium from an immortalized human hepatic stellate cell line did not induce cMET activation (Supplementary Fig. S3D). Consistent with the known role of HGF (39), we showed that stellate cell conditioned medium promoted the migration and invasion of UM001 and UM004 cells (Supplementary Fig. S4).

To determine whether conditioned medium from stellate cells drives resistance to trametinib though HGF/cMET pathway activation, we cultured UM001 and UM004 cells in either unconditioned medium or stellate cell conditioned medium. Factors from stellate cells protected uveal melanoma cells from trametinib-induced growth inhibition, as the viability of uveal melanoma cells cultured in conditioned medium increased by 2- to 3-fold compared with trametinib treatment/nonconditioned medium conditions (Fig. 4C). Importantly, stellate cell conditioned medium protection to trametinib was restored by LY2875358 and LY2801653, with LY2801653 being more potent in sensitizing uveal melanoma cells (85%–90% reduction in cell viability compared with the vehicle control). This suggests that LY2801653 is a more effective cMET inhibitor, and/or signaling molecules other than cMET may play a role in response to trametinib in uveal melanoma cells (Fig. 4C). However, together these data indicate that factors from hepatic stellate cells elicit innate resistance to trametinib at least partially through HGF/cMET signaling.
HGF-mediated growth protection from MEK inhibitors is reversed by PI3K/δ-sparing inhibitors

Because PI3K/AKT is a major pathway activated by HGF, we examined the dependency of PI3K isoforms on HGF-mediated AKT phosphorylation and HGF-mediated resistance to trametinib in uveal melanoma cells. We utilized PI3K isoform–specific inhibitors: GDC0032 is a PI3K/δ-sparing isoform inhibitor targeting PI3Kδ/γ; TGX221 is a PI110δ-specific inhibitor; BYL719 is a selective PI3Kε inhibitor; and IPI145 is a selective PI3Kδ/γ inhibitor. We first pretreated UM001 (Supplementary Fig. S5A) and UM004 cells (Fig. 5) with increasing doses of individual inhibitors followed by HGF stimulation. All these 4 p110 isoforms were expressed in both UM001 and UM004 cells, and expression was unchanged by HGF/ inhibitor treatments (Fig. 5A; Supplementary Fig. S5A). The PI3Kδ-sparing isoform inhibitor GDC0032 effectively blocked HGF-mediated AKT phosphorylation at 250 nmol/L in UM001 cells and 50 nmol/L in UM004 cells. PI3Kε inhibitor BYL719 and PI3Kδ/γ inhibitor IPI145 significantly inhibited HGF-mediated AKT phosphorylation at 500 nmol/L, whereas the PI3Kδ-specific inhibitor TGX221 did not block AKT phosphorylation even at 2.5 μmol/L (Fig. 5A; Supplementary Fig. S5A). We evaluated the ability of PI3K isoform–specific inhibitors to overcome HGF-mediated resistance to trametinib in these cells. Individual inhibitors at 500 nmol/L elicited no effect or minimal effect on cell growth (Fig. 5B; Supplementary Fig. S5B). HGF-mediated growth protection in trametinib-treated UM001 cells (Supplementary Fig. S5B) and UM004 cells (Fig. 5B) was reverted by GDC0032 (37% reduction in UM004 cell growth compared with vehicle control) and partially reverted by BYL719 and IPI145 (12%–17% inhibition of UM004 cell growth compared with vehicle control). In addition, PI3Kδ-specific inhibitor TGX221 failed to markedly induce growth inhibition in HGF- and trametinib-treated cells. These data suggest PI3Kδ/γ, but not PI3Kε, account for HGF-mediated AKT phosphorylation and resistance to MEK inhibition in uveal melanoma cells.

LY2875358 combines with MEK1/2 targeting to promote apoptotic index in metastatic uveal melanoma explants

To test whether combined therapies targeting MEK1/2 and HGF/cMET signaling improve the response in metastatic uveal melanoma, we next extended our study to analyze a mutant GNAQ harboring uveal melanoma patient sample using an ex vivo treatment approach (Fig. 6A; Supplementary Table S1). Tumor tissue pieces were treated with DMSO, trametinib, LY2875358, or a combination of trametinib and LY2875358. As expected, treatment with trametinib inhibited the phosphorylation of ERK1/2 (Fig. 6B), suggesting that ex vivo treatment of patient-derived explants is a feasible strategy for testing drug response in uveal melanoma. Ex vivo treatment with trametinib also promoted apoptosis as evidenced by an increased expression of cleaved PARP. Interestingly, combination of trametinib with LY2875358 further upregulated the expression of cleaved PARP. These data are supportive that a combined therapy with MEK and cMET inhibition may represent a novel and effective strategy in treating patients with metastatic uveal melanoma.

Discussion

The majority of uveal melanoma metastases show a tropism for the liver and are highly resistant to targeted therapies such as MEK inhibitors. How the tumor microenvironment regulates the response in uveal melanoma to targeted inhibitors is poorly understood. Here, we utilized cell lines derived from metastatic uveal melanoma and conditioned medium derived from stromal cells in the liver microenvironment. We provide evidence that the use of cMET targeting agents as a part of combinational approach may counteract tumor microenvironment-mediated primary resistance to MEK inhibitors in mutant GNAQ/11 metastatic uveal melanoma.

Recent results from the phase III, randomized trial (NCT01974752) of the MEK inhibitor, selumetinib, in combination with dacarbazine in patients with metastatic uveal melanoma were disappointing with only 3 of 97 patients treated with the combination eliciting a partial response on the basis of a central review. These results are in contrast to findings in cutaneous melanoma, which led to the FDA approval of trametinib for the treatment of BRAF V600E/K unresectable or metastatic cutaneous melanoma (40). HGF is abundant in the liver microenvironment and, when supplied exogenously, rescues the growth of MEK-inhibited mutant GNAQ human metastatic uveal melanoma cell lines (20). Our data herein indicate that HGF-mediated resistance to MEK inhibitors in uveal melanoma cells involves silencing of the proapoptotic Bim-EL and Bmf. These data are similar to the role of Bim-EL and Bmf in resistance to the BRAF inhibitor, PLX4720, in cutaneous melanoma cells (41).

To investigate the effect of liver microenvironment on response to MEK inhibitors in uveal melanoma cells, we examined factors derived from human hepatic stellate cells. Early-passage human stellate cells do not display activation markers and do secrete HGF, indicating that they may be an appropriate model for studying stromal contributions from the metastatic uveal melanoma tumor microenvironment. Pro-HGF is subsequently cleaved to form HGF, which acts as a growth factor for hepatocytes (42). cMET is expressed in both primary and metastatic uveal melanoma.

Figure 3.

Clinical-grade cMET targeting agents, LY2801653 and LY2875358, overcome HGF-mediated resistance to trametinib in uveal melanoma cells. A, Dual cMET/RON small-molecule inhibitor LY2801653 and a bivalent cMET monoclonal antibody LY2875358 block HGF/cMET signaling. UM001 cells and UM004 cells were pretreated with increasing doses of LY2801653 (left) and LY2875358 (right) overnight. The next day, cells were cultured with 10 ng/mL HGF for 30 minutes. Phosphorylation of cMET, AKT, and ERK1/2 was assessed by Western blotting. B, HGF-induced resistance to trametinib is reversed by LY2801653. UM001 cells (top) and UM004 cells (bottom) were treated with DMSO or 50 nmol/L trametinib, in combination with 10 ng/mL HGF and/or 100 nmol/L of LY2801643 as indicated for 4 hours (UM004) or 72 hours (UM001). Cells were washed and stained with crystal violet. Images were taken (100× magnification). Scale bar is equal to 100 μm. The mean of percentage crystal violet from triplicate experiments following normalization to vehicle control is shown. *, P < 0.05; **, P < 0.01; *** P < 0.001. C, HGF-induced resistance to trametinib is reversed by LY2875358. UM001 cells (top) and UM004 cells (bottom) were first treated with 10 μg/mL LY2875358 for 45 minutes, followed by 10 ng/mL HGF and 50 nmol/L trametinib for 48 hours (UM004) or 72 hours (UM001). Cells were stained with crystal violet. Representative microscopic images are shown (100× magnification). Scale bar is equal to 100 μm. The percentage of crystal violet is normalized to vehicle control, and the mean of percentage crystal violet from triplicate experiments is shown. *, P < 0.05; **, P < 0.01; *** P < 0.001.
Figure 4. Human hepatic stellate cells promote resistance to trametinib through HGF/cMET signaling in uveal melanoma cells. 

A, UM001 and UM004 cells were incubated with unconditioned medium or passage 2 HHSteC conditioned medium for 1 hour or a total of 48 hours. Cell lysates were subjected to RPPA against 295 validated antibodies. SAM for RPPA of UM001 cells (left) and UM004 cells (right) growing in unconditioned medium or stellate cells conditioned medium was shown.

B, Stellate cells conditioned medium stimulates cMET signaling cascades. UM001 and UM004 cells were incubated with unconditioned medium and stellate cells conditioned (passages 2, 5, and 6) for 1 hour. Levels of pcMET, pStat3, pAKT, and pERK1/2 were assessed by Western blotting.

C, HHSteC conditioned medium renders resistance to trametinib through cMET in uveal melanoma cells. UM001 cells (left) and UM004 cells (right) were cultured in unconditioned medium or passage 5 (p5) HHSteC conditioned medium and treated with 50 nmol/L trametinib, with or without 25 ng/mL LY2875358 and 100 nmol/L LY2801653 for 48 hours (UM004) or 72 hours (UM001). Cell growth was determined by crystal violet staining. Representative microscopic images are shown (100x magnification). Scale bar is equal to 100 μm. The percentage of crystal violet is normalized to non-CM control, and the mean of percentage crystal violet from triplicate experiments is shown. *; P < 0.05; **; P < 0.01; ***; P < 0.001.
Figure 5. HGF-mediated resistance to trametinib is dependent on PI3Kβ-sparing isoforms in uveal melanoma cells. A, Isoform-specific PI3K inhibitors differentially block HGF-mediated activation of AKT in uveal melanoma cells. UM004 cells were pretreated with increasing doses of GDC0032, TGX221, BYL719, and IPI145 for 6 hours. Cells were then stimulated with 10 ng/mL HGF for 30 minutes. Cell lysates were probed with pAKT, AKT, PI3Kα, PI3Kβ, PI3Kγ, PI3Kδ, and actin antibodies.

B, PI3Kβ-sparing inhibitor GDC0032, but not PI3Kβ inhibitor TGX221, abrogates HGF-mediated resistance to trametinib in uveal melanoma cells. UM004 cells were treated with 50 nmol/L trametinib, 10 ng/mL HGF, 0.5 μmol/L of GDC0032, TGX221, BYL719, or IPI145, respectively, or in combination for 48 hours. Cells were stained with crystal violet. Representative microscopic images are shown (100× magnification). Scale bar is equal to 100 μm. The percentage of crystal violet is normalized to vehicle control, and the mean of percentage crystal violet from triplicate experiments is shown. *, P < 0.05; **, P < 0.01; ***, P < 0.001. ns, not significant.
melanomas, but metastatic lesions tend to have higher cMET expression levels (43), which is activated in the majority of uveal melanoma liver metastases (20). Indeed, cMET signaling is constitutively activated in uveal melanoma cells when cultured in conditioned medium from stellate cells. These data support a role for tumor microenvironment in regulating HGF/cMET signaling in metastatic uveal melanoma, which is mediated by stellate cell–cancer cell communication in the liver.

While uveal melanoma cells are sensitive to trametinib in regular growth medium, they are resistant when grown in conditioned medium derived from stellate cells. Importantly, resistance is overcome by cMET targeting agents. These data suggest that innate/intrinsic resistance of uveal melanoma to MEK inhibitors is driven, at least in part, by HGF from stellate cells in the liver microenvironment. We demonstrate that cMET targeting agents such as LY2801653 and LY2875358 may improve the response to MEK inhibitors in metastatic patients with uveal melanoma. We extended our studies to analyze a uveal melanoma surgical specimen in an ex vivo treatment approach, which maintains the tumor microenvironment. Interestingly, we observed that LY2801653 treatment promoted the expression of cleaved PARP, an indicator of apoptosis. We acknowledge that further studies using preclinical models are important to address the effect of combination MEK1/2 and cMET–based target therapy in metastatic uveal melanoma.

The main activated downstream pathway of HGF/cMET is PI3K/AKT signaling. In the presence of trametinib, HGF promotes the activation of PI3K/AKT, which compensates for loss of MEK–ERK1/2 activity in uveal melanoma cells (20). Despite the evidence highlighting the importance of the PI3K pathway activation in the development of resistance to targeted therapy in melanoma, initial testing of class I PI3K inhibitors in patients has not produced dramatic results mainly due to the overlapping toxicities with MEK inhibitors that limits their effective dosing (44). One possible way to overcome this limitation is to utilize PI3K isoform–specific inhibitors. We identified that PI3Kγ/δ isoforms, but not PI3Kβ, are responsible for HGF–mediated AKT activation and HGF–mediated resistance to MEK inhibitors. These data suggest that the use of PI3Kβ-sparing inhibitors may represent a useful strategy to overcome HGF–mediated resistance and subsequently improve responses to MEK inhibitors in metastatic uveal melanoma. Of note, in cutaneous BRAF-mutated GEM melanoma models, the combination of MEK inhibitor plus the PI3Kβ-sparing inhibitor enhanced initial tumor regression and forestalled the onset of tumor resistance (45).

In summary, the data presented here show for the first time that stellate cells from the liver provide innate resistance to MEK inhibitors in metastatic uveal melanoma through HGF/cMET signaling. We have provided evidence that downregulation of the BH3-only proteins, Bim-EL and Bmf, contributes to HGF–mediated resistance. Blocking HGF signaling with either clinical-grade cMET targeting agents or PI3Kγ/δ inhibitors in uveal melanoma cells overcome resistance to MEK inhibitors mediated by stellate cells or exogenous HGF. Ongoing efforts include testing anti-cMET monoclonal antibodies in combination with MEK inhibitors in preclinical uveal melanoma studies. In addition, profiling other factors within the hepatic cellular architecture that regulate response to targeted therapy may identify novel targets for more effective therapeutic strategies.

Disclosure of Potential Conflicts of Interest

M.A. Davies reports receiving commercial research grants from GlaxoSmithKline, AstraZeneca, Roche/Genentech, and Sanofi-Aventis and is a consultant/advisory board member for Novartis, Roche/Genentech, GlaxoSmithKline, Sanofi-Aventis, and Vaccinex. T. Sato reports receiving commercial research grant from Eli Lilly and Company and other commercial research support from Gaerbett LLC and is a consultant/advisory board member for Immunocore. A.E. Aplin reports receiving commercial research grant from Pfizer. No potential conflicts of interest were disclosed by the other authors.

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