Molecular Cancer Therapeutics

Ceritinib Enhances the Efficacy of Trametinib in *BRAF/NRAS*-Wild-Type Melanoma Cell Lines

Daniel Verduzco[1], Brent M. Kuenzi[2], Fumi Kinose[2], Vernon K. Sondak[3], Zeynep Eroglu[3], Uwe Rix[2], and Keiran S.M. Smalley[1,3]

Abstract

Targeted therapy options are currently lacking for the heterogeneous population of patients whose melanomas lack *BRAF* or *NRAS* mutations (~35% of cases). We undertook a chemical biology screen to identify potential novel drug targets for this understudied group of tumors. Screening a panel of 8 *BRAF/NRAS*-WT melanoma cell lines against 240 targeted drugs identified ceritinib and trametinib as potential hits with single-agent activity. Ceritinib enhanced the efficacy of trametinib across the majority of the *BRAF/NRAS*-WT cell lines, and the combination showed increased cytotoxicity in both three-dimensional spheroid culture and long-term colony formation experiments. Coadministration of ceritinib and trametinib led to robust inhibition of tumor growth in an *in vivo* xenograft *BRAF/NRAS*-WT melanoma model; this was not due to ALK inhibition by ceritinib. Mechanistic studies showed the ceritinib–trametinib combination to increase suppression of MAPK and TORC1 signaling. Similar results were seen when *BRAF/NRAS*-WT melanoma cells were treated with a combination of trametinib and the TORC1/2 inhibitor INK128. We next used mass spectrometry–based chemical proteomics and identified known and new ceritinib targets, such as IGF1R and ACK1, respectively. Validation studies suggested that ceritinib could suppress mTORC1 signaling in the presence of trametinib through inhibition of IGF1R and/or ACK1 in a cell line–dependent manner. Together, our studies demonstrated that combining a specific inhibitor (trametinib) with a more broadly targeted agent (ceritinib) has efficacy against tumors with heterogeneous mutational profiles. *Mol Cancer Ther; 17(1); 73–83. ©2017 AACR.*

Introduction

Major breakthroughs have been made in the development of therapeutic strategies to treat advanced melanoma. At this time, the choice of frontline therapy is determined both by the *BRAF* mutational status of the tumor and by the kinetics of disease progression (1, 2). For the approximately 45% to 50% of patients whose melanomas harbor activating position 600 mutations in the serine–threonine kinase BRAF, the combination of a BRAF inhibitor (e.g., vemurafenib or dabrafenib) with an MEK inhibitor (e.g., cobimetinib or trametinib) leads to high response rates, with progression-free survival rates of close to a year and survival durations approaching 2 years (3). The BRAF–MEK inhibitor combination has the advantage of working relatively rapidly with mild off-target effects (3, 4). This combination is thought to be ineffective, however, and may even be deleterious in individuals whose melanomas are wild-type (WT) for *BRAF* (5). The current frontline treatment for patients with *BRAF*-WT melanoma is immunotherapy, with the combination of ipilimumab plus nivolumab being associated with response rates similar to that achieved with targeted therapy in *BRAF*-mutant melanoma (6, 7). Although highly effective, the immune-related adverse effects associated with this combination are frequently severe with nearly 40% of those treated having to discontinue therapy. Other therapeutic options, and in particular second-line therapies, are urgently needed for patients with *BRAF*-WT melanoma.

Melanoma is one of the most genetically diverse tumors and carries a high mutational burden (8). Approximately 15% to 20% of melanoma patients have tumors that are *NRAS*-mutant. Another recently identified group of melanomas are those with mutation/inactivation of the tumor suppressor NF1 (9, 10). These mutations, which can also co-occur with *BRAF* and *NRAS*, are found in approximately 13% of melanomas (9–11).

The goal of precision medicine is to identify genetic predictors of response that will help guide therapy selection. Although there are numerous instances in which strong predictions can be made (e.g., EML4–ALK fusions and sensitivity to ALK inhibitors, TSC2 mutations, and sensitivity to rapalogs), tumor genetic profiles are complex and sensitivity predictions are often confounded by the presence of bypass pathways (e.g., EGFR signaling in *BRAF*-mutant colorectal carcinoma; refs. 12–14). With this in mind, we have undertaken the first comprehensive screen of curated (FDA-approved or in clinical development) anticancer drugs to identify potential therapeutic strategies for *BRAF/NRAS*-WT melanoma.

Materials and Methods

Cell lines

M257 and M285 were a gift from Antoni Ribas (UCLA Medical Center). 1205Lu, WM1366, WM209, WM1963, WM3438,

[1]The Department of Tumor Biology, The Moffitt Cancer Center and Research Institute, Tampa, Florida. [2]The Department of Drug Discovery, The Moffitt Cancer Center and Research Institute, Tampa, Florida. [3]The Department of Cutaneous Oncology, The Moffitt Cancer Center and Research Institute, Tampa, Florida.

Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

Corresponding Author: Keiran S.M. Smalley, H. Lee Moffitt Cancer Center and Research Institute, 12902 Magnolia Drive, Tampa, FL 33612. Phone: 813-745-8725; Fax: 813-449-8260; E-mail: keiran.smalley@moffitt.org

doi: 10.1158/1535-7163.MCT-17-0196

WM3681, and WM3918 were acquired from the Wistar Institute. A375 and SK-MEL-23 were acquired from the ATCC. The identities of all cell lines were confirmed by STR fingerprinting performed by Bio-synthesis Inc. All cell lines were grown in RPMI + 5% FBS. Mycoplasma testing was performed using Plasmotest every 3 months (Invivogen). Fresh cells were thawed every 3 months.

Western blotting

Western blotting was performed as previously described in ref. 15. See Supplementary Materials for antibody details.

Cell proliferation assay

Cell proliferation was measured as previously described (15).

Compounds

Compounds used included trametinib (Chemitek #CT-GSK212), ceritinib (Selleck #S7083), INK128 (Cayman #11811; ref. 16), and AIM-100 (Bio-Techne #4946; ref. 17).

RNAi knockdown

RNAi was performed using Dharmacon Smartpools: IGF1R (Dharmacon #L-003012-00-0005), ALK (#L-003103-00-0005), and ACK (#L-003102-01-0005).

Three-dimensional spheroid assay

Spheroids were grown as previously described (15). Spheroids were treated for 24 hours prior to 2X washes with PBS and staining with calcein-AM and propidium iodide (20 ng/mL). Images were acquired with an AMG Evos FL system.

Colony formation assay

Colony formation assays were performed as previously described (18).

Drug screening

Cells were seeded in 384-well plates at 1,000 cells/well. All compounds were diluted to 0.5 or 2.5 μmol/L, and all cell lines were treated in duplicate. A total of 240 compounds from an in-house library were tested. Compounds were aliquoted by a Biotek Precision Pipetting robot. Cell viability was measured by CellTiter-Glo (Promega G7572) at 72 hours after treatment.

Xenograft studies

WM209 were virally transduced with eGFP-NanoLuc plasmid (19) and tumor xenografts generated following injection of 2.5×10^6 cells in Matrigel into each flank of NSG mice. Mice were randomized at day 10 ($n = 5$), and treatment was started. Each mouse was dosed daily with vehicle, 25 mg/kg ceritinib, 1 mg/kg trametinib, or the combination of 25 mg/kg ceritinib and 1 mg/kg trametinib. Drugs were formulated in 0.5% methycellulose + 0.5% Tween-80. Luciferase expression was visualized on an IVIS-100. The work was performed under the approval of the Institutional Animal Care and Use Committee of the University of South Florida (IS00000324).

Drug affinity chromatography

Drug affinity chromatography experiments were conducted as described previously (20). Briefly, the immobilizable analog c-ceritinib (21) and ampicillin were tethered to N-Hydroxysuccinimidyl-activated Sepharose for Fast Flow resin (GE Healthcare) and blocked with ethanolamine overnight. SK-MEL-23 cells were lysed and total cell lysate containing 5 mg of protein was added to the affinity matrix for 2 hours. Competition experiments were conducted by incubating total cell lysates with 20 μmol/L ceritinib or 20 μmol/L GSK1838705A for 30 minutes prior to affinity chromatography. Following affinity chromatography, SDS-PAGE and in-gel digestion with trypsin were performed.

Mass spectrometry and bioinformatic analysis

A nanoflow ultra–high-performance liquid chromatograph (RSLC; Dionex) coupled to an electrospray bench top Orbitrap mass spectrometer (Q-Exactive plus, Thermo) was used for tandem mass spectrometry (MS) peptide-sequencing experiments. Sixteen tandem mass spectra were collected in a data-dependent manner following each survey scan. Both MS and MS/MS scans were performed in the Orbitrap to obtain accurate mass measurement using 60-second exclusion for previously sampled peptide peaks. Data were searched by Mascot (v2.4.1) using the Swiss-Prot human database (downloaded 9/2015). Following protein ID, the data were filtered (95% minimum peptide threshold, 95% protein threshold; 0.3% peptide FDR, 0.0% protein FDR) using Scaffold 4.6.1 (Proteome Software). A maximum of two missed cleavages were allowed. A minimum of two unique spectrum counts were required for protein identification.

In vitro kinase assays

In vitro profiling of a panel of kinases selected from the chemical proteomic experiments was performed at Reaction Biology Corporation using the "HotSpot" assay platform (22).

Statistical analysis

For all experiments in which *P* values are shown, the unpaired Student *t* test was used. A *P* value of ≤ 0.05 was considered statistically significant.

Results

Screening for small-molecule inhibitors with activity against *BRAF*/*NRAS*-WT melanoma

We began by screening a panel of 8 *BRAF*/*NRAS*-WT melanoma cell lines against a customized library of 240 drugs. The screen involved treating the cells with two concentrations of each drug, with cell viability measured after 72 hours (Fig. 1A; raw data and drug list in Supplementary Table S1). The library covered all major target classes, such as kinases, receptor tyrosine kinases, phosphatases, receptor agonists, proteases/proteasome, PARP1, epigenetic enzymes, Hedgehog, HSP90, and Notch, and was chosen to reflect the current landscape of targeted agents approved for use or have been considered for clinical development (Fig. 1B). Our analysis identified several classes of compounds with activity across all of the cell lines. These included the proteasome inhibitor bortezomib, pan-histone deacetylase inhibitors, HSP90 inhibitors, and histone methyltransferase inhibitors. These compounds were not selected for further analysis based upon prior poor performance in melanoma clinical trials. The selected compounds included the ALK inhibitor ceritinib, the MET/VEGFR2 inhibitor foretinib, the cyclin-dependent kinase (CDK) inhibitor SNS-032, the NEDD8-activating enzyme (NAE) inhibitor MLN-4924, the CDK1/2/9 inhibitor SCH727965, the polo-like kinase (PLK1) inhibitor ON-01910, the mTORC1/2 inhibitor INK128, the MEK inhibitor

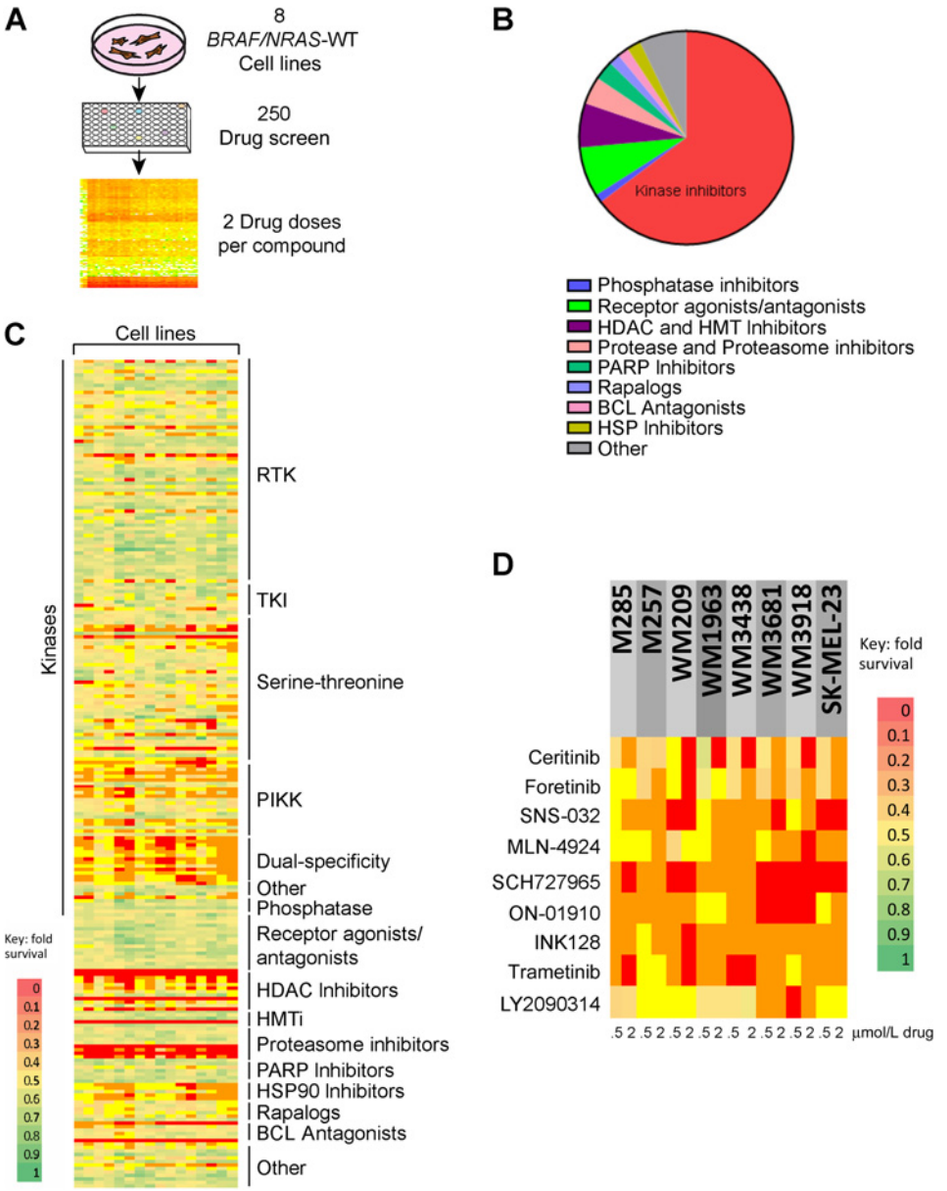

Figure 1.
Identification of drugs with single-agent activity against *BRAF/NRAS*-WT melanoma. **A,** Overview of the workflow of the drug screen. Eight *BRAF/NRAS*-WT melanoma cell lines were treated with two concentrations of each drug (0.5 and 2.5 µmol/L) for 72 hours before cell viability was assessed using the Cell-Titer-Glo assay. **B,** Overview of the drugs in the panel by type. **C,** Response of individual cell lines to each drug in the panel (RTK, receptor tyrosine kinase; TKI, tyrosine kinase inhibitor; PIKK, Phosphatidylinositol 3-kinase–related kinase; Dual-Specificity, dual specificity phosphatases; HDACi, histone deacetylase inhibitors; HMTi, histone methyl transferase inhibitors). Scale indicates the percentage growth inhibition at 0.5 and 2.5 µmol/L of drug relative to vehicle. **D,** Detailed view of the responses of the drugs selected for follow-up in the cell line panel. Data show the inhibition of growth per cell line at 0.5 and 2.5 µmol/L of drug relative to vehicle.

trametinib, and the glycogen synthase kinase (GSK3)-β inhibitor LY2090314 that all showed activity against >50% of the cell lines, suggesting some selectivity (Fig. 1C and D).

BRAF/*NRAS*-WT melanomas show frequent aberrations in the MAPK pathway and show sensitivity to MEK inhibition

Cutaneous melanoma is uniquely addicted to signals through the MAPK pathway. Despite this, and the fact that melanomas frequently harbor mutations in MAPK pathway drivers such as *BRAF* or *RAS*, the sensitivity of *BRAF*/*NRAS*-WT melanoma cell lines to MAPK inhibitors has been little studied. One gene that was frequently mutated in the *BRAF*/*NRAS*-WT melanoma cell lines was the tumor suppressor *NF1*. Western blot analysis revealed NF1 expression to be either reduced or absent in 3 of 7 cell lines tested including M285, WM3681, and WM3918 (Fig. 2A), with other cell lines harboring one or more nonsynonymous *NF1* mutations. Only the WM209, WM3438, and SK-Mel-23 melanoma cell lines had no identifiable mutations in *NF1*. All of the *BRAF*/*NRAS*-WT cell lines tested showed constitutive phosphorylation of phospho-ERK (Fig. 2B), and treatment with the MEK inhibitor trametinib (10 nmol/L, 24 hours) was associated with phospho-ERK inhibition. Treatment of the *BRAF*/*NRAS*-WT melanoma cell lines with increasing concentrations of trametinib led to decreased cell growth (Fig. 2C). Across the panel, the growth-inhibitory effect of trametinib was highly variable, and although there was some trend toward increased trametinib sensitivity in NF1-intact cell lines, this was not true in all cases (Fig. 2C).

Ceritinib enhances the activity of the MEK inhibitor trametinib in *BRAF*/*NRAS*-WT melanoma

Drug combinations that limit the adaptive signaling are typically associated with more durable responses than the single

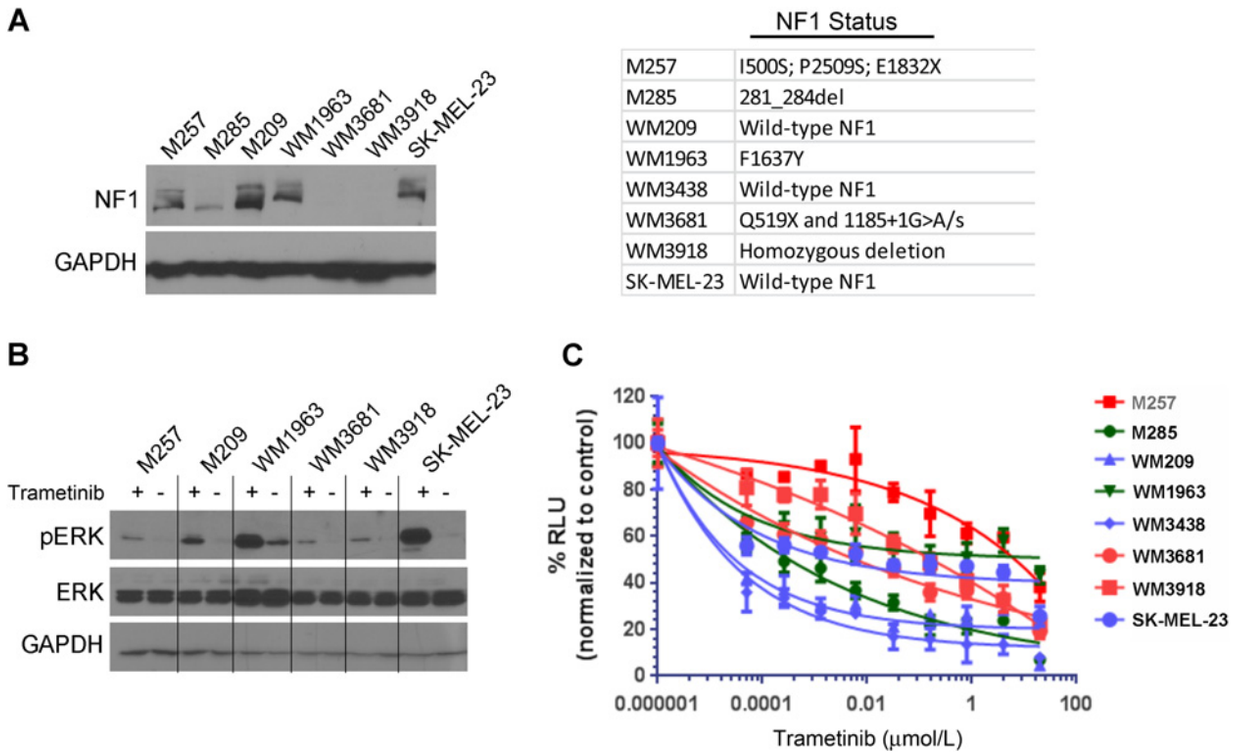

Figure 2.

BRAF/NRAS-WT melanoma cell lines have constitutive ERK activity and show a range of MEK inhibitor sensitivities. **A,** Western blot showing the expression of NF1 in *BRAF/NRAS*-WT melanoma cells. Panel, *NF1* mutations in the *BRAF/NRAS*-WT cell lines. **B,** Trametinib inhibits pERK in the majority of *BRAF/NRAS*-WT melanoma cells. Cells were treated with trametinib (10 nmol/L, 24 hours) before being subject to Western blot for pERK. **C,** Trametinib inhibits the growth of *BRAF/NRAS*-WT cell lines. Cells were treated with increasing concentrations of trametinib for 72 hours before being analyzed by Cell Titer Glo assay. Data show the mean of three experiments ± SEM. Blue indicates cell lines with wild-type *NF1*; green indicates cell lines with heterozygous *NF1* mutations; and red indicates cell lines with no NF1 expression/*NF1* mutation.

agents. In the majority of the cell lines tested, concentrations of ceritinib that are clinically achievable (2.5 μmol/L) reduced cell growth to <50% when combined with trametinib (Fig. 3A). The combination also had activity in melanoma cell lines with *BRAF*-V600E mutations (1205Lu and 1205LuR), and 3 *NRAS*-mutant (M245, IPC-298, and WM1366) melanoma cell lines (Fig. 3A). Under more physiologically relevant collagen-implanted spheroid culture conditions, the combination of ceritinib and trametinib enhanced cell death in a representative panel of 3 *BRAF/NRAS*-WT melanoma cell lines (WM209, WM3681, and SK-MEL-23) compared with either agent alone (Fig. 3B). The effects of the trametinib–ceritinib combination were also durable, suppressing the outgrowth of resistant clones in long-term colony formation assays in *BRAF/NRAS*-WT (WM3681), *BRAF*-mutant (1205Lu, A375), and *NRAS*-mutant (WM1366) melanoma cell lines (Fig. 3C).

We next determined the efficacy of the trametinib–ceritinib combination in a *BRAF/NRAS*-WT melanoma cell mouse xenograft model. Here, luciferase-tagged WM209 cells were injected subcutaneously into the flanks of NSG mice and allowed to grow to 1 mm^3 in size before being treated with vehicle, ceritinib (25 mg/kg), trametinib (1 mg/kg), or the combination of ceritinib (25 mg/kg) and trametinib (1 mg/kg) for 24 days. At experiment termination, it was noted that the growth of the tumors treated with the combination was significantly reduced compared with those receiving trametinib or ceritinib monotherapy (Fig. 3D).

Ceritinib–trametinib inhibits MAPK and TORC1 signaling

To determine the mechanism of action of trametinib–ceritinib, we began by performing kinome arrays on *BRAF/NRAS*-WT melanoma cells (Supplementary Fig. S1). The combination had marked effects upon signaling through the MAPK, AKT/mTOR, RSK1/2, and JNK/c-JUN pathways. As RSK1/2 are downstream targets of both PI3K/AKT and MEK/ERK, we decided to focus on the MAPK, AKT/mTOR, and JNK/c-JUN pathways (Supplementary Fig. S2). Analysis of c-JUN phosphorylation under drug treatment showed variable results across the cell line panel, with some cell lines showing the expected inhibition of c-JUN phosphorylation and loss of c-JUN expression (WM209, SK-MEL-23, and WM1936) while some (WM3681 and WM3918) showed the opposite effect (Supplementary Fig. S3). We next focused upon MAPK and TORC1, as dual inhibition of these pathways is well correlated with BRAF inhibitor responses in *BRAF*-mutant melanoma (23). It was observed in the majority of the cell lines that maximal inhibition of pS6 occurred following treatment with both trametinib and ceritinib (Fig. 4A). Ceritinib had little effect upon pS6 levels as a single agent, indicating the importance of

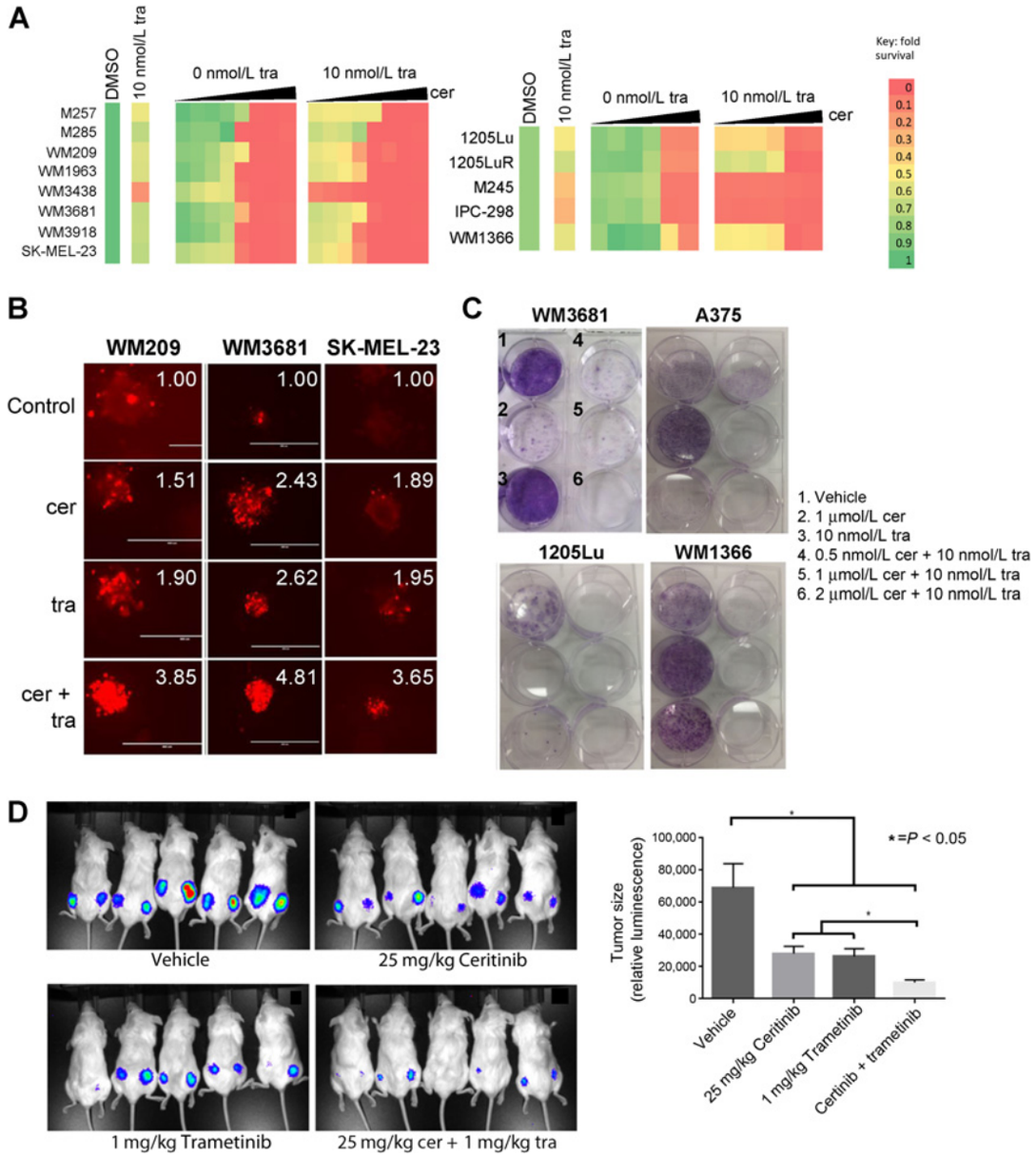

Figure 3.

Ceritinib enhances the efficacy of trametinib. **A,** Heatmap showing the inhibition of growth to a selection of drugs identified from the screen in Fig. 1 ± 10 nmol/L trametinib. Cells were treated with each drug alone and in the presence of trametinib for 72 hours before being analyzed by MTT assay. **B,** WM209, SK-MEL-23, and WM3681 spheroids were implanted into a gel of collagen before being treated with vehicle, ceritinib (2 μmol/L), trametinib (10 nmol/L), or ceritinib + trametinib (2 μmol/L/10 nmol/L). Spheroids were stained with propidium iodide to indicate fold-increase in dead cells. Panels show fold increase in dead cells. **C,** The combination of trametinib and ceritinib prevents outgrowth of WM3681, A375, 1205Lu, and WM1366 cells in long-term colony formation assays. Cells were seeded out and treated with vehicle, ceritinib, trametinib, or ceritinib plus trametinib for 2 weeks before being stained with crystal violet. **D,** Luciferase-expressing WM209 cells were grown as xenografts in NSG mice before being dosed daily with vehicle, ceritinib (25 mg/kg), trametinib (1 mg/kg), or the ceritinib–trametinib combination (25 and 1 mg/kg) daily by oral gavage. Left plot shows luminescence following 24 days of treatment. Right plot shows quantification of mean fluorescence following treatment with the drugs (day 24).

ceritinib target kinases in the escape from trametinib. Ceritinib had some minor inhibitory activity against pERK in some cell lines, including WM3918 and SK-MEL-23 (Fig. 4A). The effects of ceritinib–trametinib on signaling were highly durable, with no recovery of pERK or pS6 signaling seen up to 72 hours of treatment (Supplementary Fig. S4). No alterations in the MAPK signaling regulator Sprouty-2 were noted (Supplementary Fig. S5). Further studies showed that the combination of trametinib and the mTOR inhibitor INK128 inhibited both pS6 and MEK signaling (Fig. 4B) and that cotreatment of SK-MEL-23 cells with trametinib and

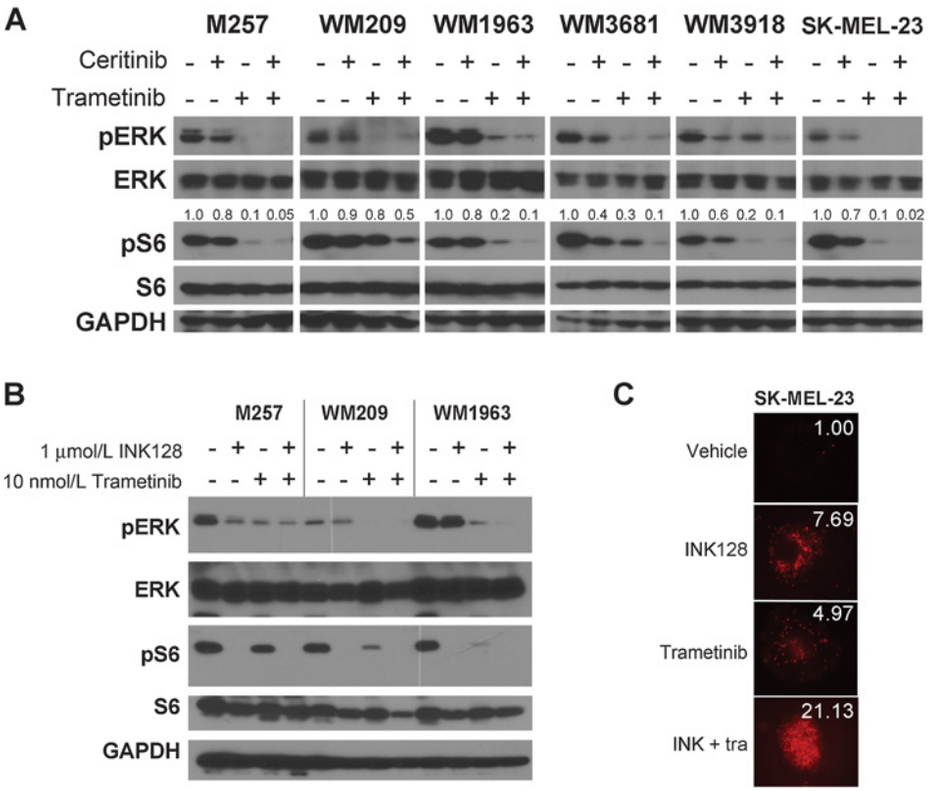

Figure 4.
The combination of trametinib and ceritinib leads to enhanced suppression of TORC1 signaling. **A,** *BRAF/NRAS*-WT cell lines were treated with vehicle, ceritinib, trametinib, or ceritinib–trametinib for 24 hours before extraction of protein and Western blot for pERK or pS6. Numbers above pS6 indicate the densitometry measurements expressed as a proportion of total S6. **B,** The combination of trametinib and the TORC inhibitor INK128 causes the dual suppression of MAPK and S6. *BRAF/NRAS* WT cell lines were treated with vehicle, INK128 (1 μmol/L), trametinib (10 nmol/L), or INK128–trametinib (1 μmol/L/10 nmol/L) for 24 hours before the extraction of protein and Western blot for pERK and pS6. **C,** The ceritinib–trametinib combination shows equivalent effects to trametinib–INK128 in a 3D collagen–implanted spheroid assay. SK-MEL-23 spheroids were implanted into a collagen gel before being treated with vehicle, INK128 (1 μmol/L), trametinib (10 nmol/L), or INK128–trametinib (1 μmol/L/10 nmol/L). Spheroids were stained with propidium iodide to indicate dead cells. Plots show foldincrease in dead cells relative to controls.

INK128 led to enhanced cytotoxic effects in three-dimensional (3D) collagen–implanted spheroid assays, in a manner that was equivalent to the ceritinib–trametinib combination (Figs. 3B and 4C).

Ceritinib inhibits multiple targets in *BRAF*/*NRAS*-WT melanoma

Ceritinib was developed as an inhibitor of the ALK fusion protein. As ALK fusions are rare in melanoma, we performed a chemical proteomic screen to identify potential interactors/binding partners of ceritinib. In these studies, an immobilized ceritinib analog was used for drug affinity chromatography with total cell lysates from SK-MEL-23 melanoma cells, and the resulting drug pull downs were analyzed by LC-MS/MS (Fig. 5A: Structure shown in Supplementary Fig. S6; ref. 21). Using ampicillin beads and ceritinib competition as independent controls, these studies identified the known ceritinib targets ALK, IGF1R, and InsR, as well as several new ceritinib target candidates, such as ACK1, FER, FAK, and CAMKK2 (Fig. 5B). *In vitro* kinase assays were then performed to validate the chemical proteomics studies (Table 1). Dose-response analysis showed ceritinib to potently inhibit IGF1R and ACK1 (IC_{50}s 15.2 nmol/L and 33.6 nmol/L, respectively; Fig. 5C).

Ceritinib limits TORC1 signaling via inhibition of ACK1 and IGF1R

Among all of the cell lines, only SK-MEL-23 expressed any ALK, with siRNA studies showing ALK knockdown to have little impact upon cell survival (Fig. 6A). We next turned our attention to other potential targets of ceritinib. Seven of the cell lines (aside from M285) expressed IGF1R (Supplementary Fig. S7), with IGF1R being the only receptor tyrosine kinase to be inhibited following the treatment of WM3681 and SK-MEL-23 *BRAF*/*NRAS*-WT melanoma cells with ceritinib (Supplementary Fig. S8). The WM1963 and WM209 cells showed a requirement for IGF1R signaling with its siRNA knockdown being associated with reduced viability (Supplementary Fig. S9). A role for IGF1R in the maintenance of pS6 signaling in WM1963 cells was suggested by the observation that knockdown of IGF1R in combination with trametinib led to a suppression of both pS6 and pERK signaling (Fig. 6B). None of the other cell lines showed any decrease in pS6 following knockdown of IGF1R (alone and in combination with trametinib). Systematic siRNA knockdown of ceritinib targets showed that only ACK1 appeared to be a regulator of TORC1 signaling following MEK inhibition. In particular, no effects of ceritinib upon pFAK were noted, nor were dramatic effects seen upon siRNA knockdown of FER (Supplementary Figs. S10 and S11). All of the *BRAF*/*NRAS*-WT melanoma cell lines expressed similar levels of ACK1 expression (Fig. 6C). siRNA knockdown of ACK1 in combination with trametinib led to the near-complete suppression of pS6 in both the WM209 and SK-MEL-23 cell lines (Fig. 6C). The ACK1 inhibitor AIM-100 was noted to suppress long-term colony formation growth in WM209 and SK-MEL-23 cells when combined with trametinib (Supplementary Fig. S12). Combined knockdown of ACK1 and IGF1R in combination with trametinib blocked pS6 signaling in WM3681 and M257 cells (Fig. 6D). The observation that ACK1 and IGF1R inhibition had distinct, cell line–specific effects upon pS6 indicated the value of combining a broad specificity drug (ceritinib) with a highly specific drug (trametinib).

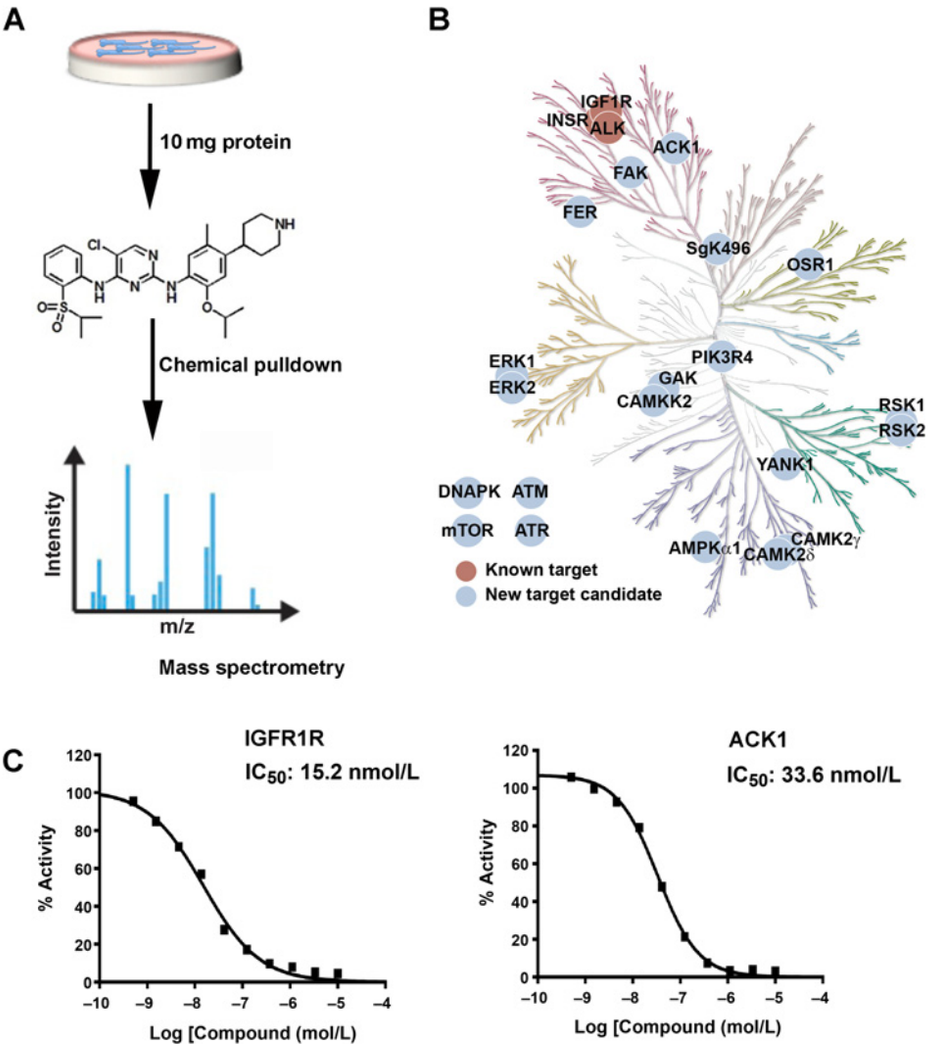

Figure 5.
Chemical proteomics identifies IGF1R and ACK1 as potential targets of ceritinib in *BRAF/NRAS*-WT melanoma. **A,** Workflow of the chemical proteomic experiment. **B,** Kinome tree of potential kinase targets of ceritinib in SK-MEL-23 melanoma cells. **C,** Ceritinib inhibits IGF1R and ACK1 in *in vitro* kinase assays.

Discussion

Although targeted therapies have proven effective for *BRAF*-mutant melanoma and immune therapies are showing great promise across all genotypes (both *BRAF*-mutant and WT), new treatment strategies are still urgently needed. As a subgroup, *BRAF/NRAS*-WT melanomas have been little studied and attempts to date have not uncovered consistent, therapeutically tractable, oncogenic drivers (8, 24). This analysis has been complicated by the extremely high mutational load of melanomas, a likely result of the role of UV-radiation exposure in the etiology of the disease (25). It therefore seems likely that the transformation of *BRAF/NRAS*-WT melanomas may depend upon the complex interplay of multiple genetic hits that work together to drive the pathways required for maintenance of the oncogenic state. At this time, immunotherapy approaches such as nivolumab and the ipilimumab–nivolumab combination are routinely used to treat *BRAF*-WT melanoma (6, 7). For those who fail checkpoint inhibitor therapy, the only second-line therapy options currently available are chemotherapy or a clinical trial. Against this backdrop, we sought to define novel targeted therapy strategies for *BRAF/NRAS*-WT melanoma. As our goal was to define drug combinations with potential for rapid clinical translation, we focused upon drugs and targeted inhibitors that were either (1) FDA-approved or (2) currently in clinical development.

Our drug screen identified multiple hits. The compounds identified were diverse and included pan-RTK inhibitors (ceritinib, foretinib), inhibitors of CDK2 (SNS-032), CDK1/2/9 (SCH727965), GSK3-β (LY2090314), PLK1 (ON-01910),

Table 1. Inhibitory potency of ceritinib and staurosporine against the kinases identified in the chemical proteomic screen

Kinase	Ceritinib	Staurosporine
ACK1	33.7 nmol/L	255 nmol/L
AMPK	2360 nmol/L	0.00813 nmol/L
CAMKK2	75.3 nmol/L	455 nmol/L
FAK	16.3 nmol/L	173 nmol/L
FER	11.7 nmol/L	0.303 nmol/L
IGF1R	15.2 nmol/L	52.7 nmol/L
IR	72.6 nmol/L	32.1 nmol/L
OSR1	881 nmol/L	58.4 nmol/L
RSK1	966 nmol/L	0.09 nmol/L
RSK2	418 nmol/L	0.11 nmol/L

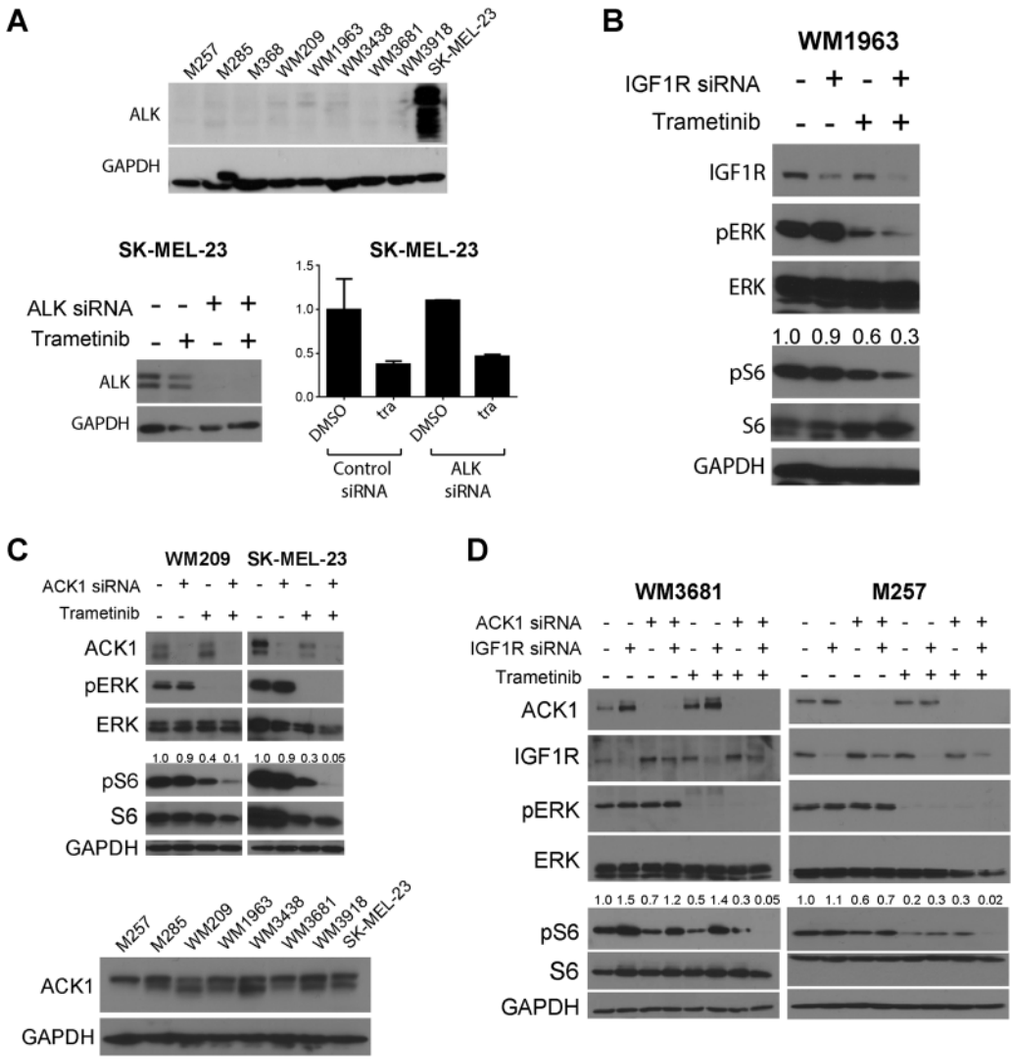

Figure 6.

Silencing of IGF1R or ACK1 enhances suppression of pS6 when combined with trametinib. **A,** (top plot) Expression of ALK in the eight *BRAF/NRAS*-WT melanoma cell lines. Western blot shows expression of ALK and GAPDH. (Bottom plot) Silencing of ALK in SK-MEL-23 cells does not affect viability +/- trametinib in cell survival assays. Cells were treated with siRNA for 24 hours and then with ALK siRNA or nonsilencing control alone and in the presence of trametinib (10 nmol/L) for 72 hours. Cell numbers were measured by MTT assay. **B,** Silencing of IGF1R in WM1963 leads to enhanced pS6 suppression when combined with trametinib. Cells were treated with siRNA for 24 hours and then with vehicle or drug for 24 hours. Numbers are the densitometry values corresponding to pS6/tS6. **C,** (Top) Silencing of ACK1 enhances the suppression of pS6 in WM209 and SK-MEL-23 cells when combined with trametinib. Cells were treated with siRNA for 24 hours and then with vehicle or drug for 24 hours before Western blot analysis for pERK and pS6. (Bottom) Western blot shows the expression of ACK1 across the cell line panel. **D,** Combined silencing of ACK1 and IGF1R suppresses pS6 signaling in combination with trametinib (10 nmol/L). WM3681 and M257 cells were treated with siRNA as above and then with trametinib for 24 hours before Western blot analysis for pERK and pS6.

NEDD8 activation enzyme (NAE; MLN-4924), mTOR (INK128), and MEK (trametinib). Many of these classes of drugs, including CDK2, CDK1/2/9, GSK3β, NAE, mTOR, and MEK inhibitors, have been previously shown to have efficacy against melanoma cell lines in *in vitro* and *in vivo* studies (26–31). We next determined the sensitivity of our *BRAF*/*NRAS*-WT melanoma cell line panel to the MEK inhibitor trametinib. Our focus upon MEK inhibition was based upon previous work demonstrating the reliance of nearly all melanomas upon the MAPK pathway (32). Although *BRAF*/*NRAS*-WT melanomas lack more commonly known MAPK drivers such as mutant *BRAF* and *NRAS*, they do harbor other mutations that lead to MAPK pathway activation. In particular, approximately 13% of all *BRAF*/*NRAS*-WT melanomas harbor inactivating mutations in the RAS-GAP *NF1*, with a further 15% to 36% of melanoma specimens showing loss of or reduced NF1 expression (10, 11). Loss of NF1 function typically leads to increased MAPK signaling that is secondary to loss of negative regulation of Ras signaling (11). In *BRAF*/*NRAS*-WT melanomas, *NF1* mutations frequently co-occur with lesions in other "Rasopathy" genes including *SOS1*, *RASA2*, and *PTPN*1 (11), which often lead to increased MAPK signaling. In line with published work, our *BRAF*/*NRAS*-WT cell line panel showed recurrent

mutations in and loss of expression of the tumor suppressor *NF1*. All of the cell lines in the *BRAF*/*NRAS*-WT panel exhibited high levels of pERK activation, with some cell lines showing good sensitivity to trametinib monotherapy (30). In agreement with previously published work, *NF1* status was not highly predictive of MEK inhibitor sensitivity (11, 30).

Single-agent trametinib has been evaluated in *BRAF*-WT melanoma patients with some partial responses noted (33, 34). In light of this, and the general observation that kinase inhibitor monotherapy typically leads to signaling adaptation and resistance (5, 35), we reasoned that trametinib would make a good backbone therapy for *BRAF*/*NRAS*-WT melanoma. We then asked which of the candidates from our screen would constitute a suitable combination partner and identified ceritinib on the basis of its positive interactions with trametinib and its clinical availability (36).

Ceritinib is a multi-RTK inhibitor that is FDA-approved for the treatment of EML4–ALK fusion-positive non–small cell lung cancer (NSCLC). To our knowledge, ceritinib has never been evaluated in melanoma, either preclinically or clinically. The combination of ceritinib and trametinib was associated with increased cytotoxicity in long-term colony formation experiments and in 3D collagen–implanted spheroid assays that more closely mimic the *in vivo* tumor microenvironment (15). These responses were not limited to *BRAF*/*NRAS*-WT melanoma and were also observed in the *BRAF*- and *NRAS*-mutant melanoma cell lines we tested. Previous work has shown the progression of *BRAF*-mutant melanoma to be dependent upon signals through both the MAPK and PI3K/AKT/mTOR/S6 signaling pathways. A role for both of these pathways in melanoma initiation has been demonstrated by the observation that loss of PTEN is critical for both the escape of *BRAF*-mutant melanocytes from oncogene-induced senescence as well the initiation of *BRAF* mutant melanoma in GEM models (37, 38). Studies from our own group and others have shown adaptive AKT/mTOR signaling is a frequent event in the escape of *BRAF* mutant melanoma cells from BRAF and MEK inhibitor therapy (39–41). These findings are not restricted to *BRAF*-mutant melanoma, with synergy between inhibitors of MEK and TORC1/2 inhibitors also reported for *NRAS*-mutant melanoma (42). Mechanistically, it appears that MAPK and PI3K/AKT/mTOR converge on key cell survival hubs, with their combined inhibition leading to increased apoptosis (43).

We observed that the combination of trametinib and ceritinib increased the suppression of pS6. Ceritinib had little impact upon pS6 levels as a single agent, indicating its role in inhibiting the signaling adaptations that occurred secondary to MEK inhibition. The critical nature of dual MAPK and TORC1 signaling inhibition was demonstrated by the ability of the trametinib and the mTOR inhibitor INK128 combination to phenocopy the trametinib–ceritinib combination in 3D spheroid cytotoxicity assays.

The results that we describe here have parallels with studies in both *BRAF*-mutant melanoma and *PIK3CA*-mutant breast cancer, in which complete inhibition of TORC1 (indicated by suppression of pS6) is a biomarker of sensitivity to inhibitors of BRAF and PI3K, respectively (23, 44). In *BRAF*-mutant melanoma, complete suppression of TORC1 was required for apoptosis induction, with PUMA being identified as the key mediator of cell death (23). The importance of TORC1 inhibition as a biomarker of response to many different targeted therapies is reflective of the central role of pS6 as a signal activity readout from multiple upstream inputs. It is therefore likely that maximal pS6 inhibition reflects the near total shut down of adaptive signaling. Although the level of suppression effected in some cell lines by combined ceritinib–trametinib was sometimes only slightly more than single-agent trametinib, this is not say it is not significant. Even low levels of signaling are sufficient to allow survival following drug treatment. This is most clearly demonstrated in melanoma patients treated with BRAF inhibitors, in which >90% pERK inhibition is required for efficacy to be observed (45).

It is worth noting that the ceritinib–trametinib combination affected multiple signaling pathways in addition to MAPK and TORC1, including JNK and c-JUN. The JNK signaling pathway is known to be a critical mechanism of adaptation of BRAF-mutant melanoma cells following BRAF inhibitor treatment, where it drives RTK signaling that limits the effects of MEK/ERK inhibition (46). Although likely to be important in some of the *BRAF*/*NRAS*-WT cell lines evaluated here, the responses were variable across the cell line panel, indicating the complexity and cell line specificity of the drug combination.

Ceritinib is known primarily as an inhibitor of ALK. An examination of the *BRAF*/*NRAS*-WT melanoma cell lines demonstrated only 1 of the 8 to express any ALK protein by Western blot. In the one cell line that did express ALK (SK-MEL-23), its knockdown did not affect cell viability. It therefore seems unlikely that the effects of ceritinib are mediated through ALK inhibition. We next used chemical proteomics to define other potential targets of ceritinib. One of the top hits for ceritinib was IGF1R, a previously validated target of the drug in NSCLC (47). The importance of IGF1R as a ceritinib target in some melanoma cell lines was confirmed through RTK arrays, with no other RTK found to be significantly inhibited. The majority of melanomas express IGF1R, with its signaling being implicated in proliferation, cell invasion, and apoptosis resistance. In the monotherapy setting, IGF1R inhibition has proven relatively weak, a likely consequence of the high signaling redundancy in melanoma cells (48). In other contexts, IGF1R signaling may be important, with some studies implicating this RTK in BRAF inhibitor resistance (49). IGF1R signaling has also shown to be an important adaptive signaling mechanism in other tumor systems, such as following the knockdown of KRAS in colon cancer (50).

Although the majority of the *BRAF*/*NRAS* WT melanoma cell lines express IGF1R, its siRNA knockdown had minimal effects upon cell growth, both alone and in combination with trametinib. Despite this, one of the cell lines—WM1963—did show sensitivity to IGF1R knockdown and, more importantly, showed a decrease in pS6 when IGF1R knockdown was combined with trametinib. As melanoma cells are known to be genetically complex, we reasoned that multiple ceritinib targets were involved in the regulation of adaptive TORC1 signaling. One candidate was the nonreceptor tyrosine kinase ACK1/TNK2 (activated CDC42-associated kinase), whose knockdown suppressed pS6 signaling when combined with MEK inhibition in a further two *BRAF*/*NRAS*-WT cell lines. ACK1 is best characterized as an intermediary nonreceptor tyrosine kinase that links upstream receptor activation to downstream signaling pathways. Extensive work has already shown ACK1 to be an EGFR interactor, where EGF stimulation leads to the association of ACK1 and EGFR, resulting in EGFR degradation (51). ACK1 also interacts with many other RTKs, with its kinase activity being increased following treatment with GAS6, IGF-I, and heregulin (52).

Together, our data illustrate the utility of combining drugs that are highly specific (such as MEK inhibitors) with agents that are

broadly targeted (such as ceritinib). The utility of this approach is demonstrated in genetically complex cancers where the effects of potent inhibition of a critical growth pathway in combination with the simultaneous inhibition of multiple signaling adaptations can prove highly effective. This strategy can be particularly advantageous in instances where melanoma cell lines have multiple, simultaneous mechanisms of escape (e.g., ACK1, IGF1R) that converge upon single signaling nodes, such as S6. Our study also illustrates the strength of using unbiased drug screens combined with chemical proteomics to identify novel off-target effects of FDA-approved drugs that can be efficacious in cancers that lack effective targeted therapy options.

Disclosure of Potential Conflicts of Interest

V.K. Sondak is a consultant/advisory board member for Array, BMS, Genentech/Roche, Merck, and Novartis No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: D. Verduzco, U. Rix, K.S.M. Smalley
Development of methodology: D. Verduzco
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D. Verduzco, B.M. Kuenzi, F. Kinose
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D. Verduzco, B.M. Kuenzi, V.K. Sondak, Z. Eroglu, U. Rix
Writing, review, and/or revision of the manuscript: D. Verduzco, B.M. Kuenzi, V.K. Sondak, Z. Eroglu, U. Rix, K.S.M. Smalley
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): V.K. Sondak
Study supervision: V.K. Sondak, U. Rix, K.S.M. Smalley

Acknowledgments

The authors would like to thank Dr. Franz Schaub for technical assistance.

This work was supported by R01 CA161107-01 and P50 CA168536 (to K.S.M. Smalley), R01 CA181746 (to U. Rix), F99 CA212456 (to B.M. Kuenzi), and the Cancer Center Support Grant P30-CA076292.

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Received March 3, 2017; revised July 6, 2017; accepted October 13, 2017; published OnlineFirst November 13, 2017.

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