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Ceritinib enhances the efficacy of trametinib in *BRAF/NRAS*-wild type melanoma cell lines

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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 3D spheroid culture and long-term colony formation experiments. Co-administration 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.

Keywords

melanoma; ceritinib; BRAF; NRAS; chemical biology; combination therapy

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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 ~45–50% 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 a 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 15–20% 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 ~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) (12–14). With this in mind, we have undertaken the first comprehensive screen of curated (FDA-approved or in clinical development) anti-cancer 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, 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. (Lewisville, TX). All cell lines were grown in RPMI + 5% FBS. Mycoplasma testing was performed using Plasmotest every three months (Invivogen, San Diego, CA). Fresh cells were thawed every 3 months.

Western blotting

Western blotting was performed as previously described in (15). See Supplemental Materials for antibody details.

Cell proliferation assay

Cells proliferation was measured as previously described (15).

Compounds

Compounds used included trametinib (Chemitec #CT-GSK212), ceritinib (Selleck #S7083), INK128 (Cayman #11811) (16) and AIM-100 (Bio-Techne #4946) (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 24h prior to 2X washes with PBS and staining with calcein-AM and propidium iodide (20ng/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 1000 cells/well. All compounds were diluted to 0.5 or 2.5 μ M, 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 Cell-Titer-Glo (Promega G7572) at 72h post 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 day (n=5), and treatment was started. Each mouse was dosed daily with vehicle, 25mg/kg ceritinib, 1mg/kg trametinib, or the combination of 25mg/kg ceritinib and 1mg/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 IACUC 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 NHS-activated Sepharose for Fast Flow resin (GE Healthcare) and blocked with ethanolamine overnight. SK-MEL-23 cells were lysed and total cell lysate containing 5mg of protein was

added to the affinity matrix for 2hr. Competition experiments were conducted by incubating total cell lysates with 20 μ M ceritinib or 20 μ M GSK1838705A for 30 min prior to affinity chromatography. Following affinity chromatography, SDS-PAGE and in-gel digestion with trypsin was performed.

Mass Spectrometry and Bioinformatic Analysis

A nanoflow ultra-high performance liquid chromatograph (RSLC, Dionex, Sunnyvale, CA) coupled to an electrospray bench top Orbitrap mass spectrometer (Q-Exactive plus, Thermo, San Jose, CA) 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 60sec exclusion for previously sampled peptide peaks. Data was searched by Mascot (v2.4.1) using the Swiss-Prot human database (downloaded 9/2015). Following protein ID, the data was 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 was allowed. A minimum of two unique spectrum counts was 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 (Malvern, PA) using the “HotSpot” assay platform (22).

Statistics

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* wild-type 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 72h (Figure 1A) (raw data and drug list in Supplemental Table 1). 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 (Figure 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 trametinib and the glycogen synthase kinase (GSK3)- β inhibitor LY2090314 that all showed activity against >50% of the cell lines, suggesting some selectivity (Figures 1C–D).

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

Cutaneous melanoma is uniquely addicted to signals through the mitogen-activated protein kinase (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*-wild-type 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 *NFI*. Western blot analysis revealed NF1 expression to be either reduced or absent in 3/7 cell lines tested including M285, WM3681 and WM3918 (Figure 2A), with other cell lines harboring one or more non-synonymous *NFI* mutations. Only the WM209, WM3438, and SK-Mel-23 melanoma cell lines had no identifiable mutations in *NFI*. All of the *BRAF/NRAS*-WT cell lines tested showed constitutive phosphorylation of phospho-ERK (Figure 2B) and treatment with the MEK inhibitor trametinib (10nM, 24h) 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 (Figure 2C). Across the panel, the growth inhibitory effect of trametinib was highly variable and although there was some trend towards increased trametinib sensitivity in NF1 intact cell lines, this was not true in all cases (Figure 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 agents. In the majority of the cell lines tested, concentrations of ceritinib that are clinically achievable (2.5 μ M) reduced cell growth to <50% when combined with trametinib (Figure 3A). The combination also had activity in melanoma cell lines with *BRAF*-V600E mutations (1205Lu and 1205LuR), and 3 *NRAS*-mutant (M245, IPC-298, WM1366) melanoma cell lines (Figure 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, SK-MEL-23) compared to either agent alone (Figure 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 (Figure 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 1mm³ in size before being treated with vehicle, ceritinib (25mg/kg), trametinib (1mg/kg) or the combination of ceritinib (25mg/kg) and trametinib (1mg/kg) for 24 days. At experiment

termination, it was noted that the growth of the tumors treated with the combination was significantly reduced compared to those receiving trametinib or ceritinib monotherapy (Figure 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 (Supplemental Figure 1). 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 (Supplemental Figure 2). 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, WM1936) while some (WM3681, WM3918) showed the opposite effect (Supplemental Figure 3). We next focused upon MAPK and TORC1, as dual inhibition of these pathways are 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 (Figure 4A). Ceritinib had little effect upon pS6 levels as a single agent, indicating the importance of 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 (Figure 4A). The effects of ceritinib-trametinib on signaling were highly durable, with no recovery of pERK or pS6 signaling seen up to 72h of treatment (Supplemental Figure 4). No alterations in the MAPK signaling regulator Sprouty-2 were noted (Supplemental Figure 5). Further studies showed that the combination of trametinib and the mTOR inhibitor INK128 inhibited both pS6 and MEK signaling (Figure 4B) and that co-treatment of SK-MEL-23 cells with trametinib and INK128 led to enhanced cytotoxic effects in 3D collagen-implanted spheroid assays, in a manner that was equivalent to the ceritinib-trametinib combination (Figure 4C and Figure 3B).

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 analogue 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 (Figure 5A: Structure shown in Supplemental Figure 6 (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 (Figure 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 (IC₅₀s 15.2nM and 33.6nM, respectively) (Figure 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 (Figure 6A). We next

turned our attention to other potential targets of ceritinib. Seven of the cell lines (aside from M285) expressed IGF1R (Supplemental Figure 7), 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 (Supplemental Figure 8). The WM1963 and WM209 cells showed a requirement for IGF1R signaling with its siRNA knockdown being associated with reduced viability (Supplemental Figure 9). 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 (Figure 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 (Supplemental Figures 10–11). All of the *BRAF/NRAS*-WT melanoma cell lines expressed similar levels of ACK1 expression (Figure 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 (Figure 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 (Supplemental Figure 12). Combined knockdown of ACK1 and IGF1R in combination with trametinib blocked pS6 signaling in WM3681 and M257 cells (Figure 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).

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), NEDD8 activation

enzyme (NAE) (MLN-4924), mTOR (INK128) and MEK (trametinib). Many of these classes of drugs, including CDK2, CDK1/2/9, GSK3 β , 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, ~13% of all *BRAF/NRAS*-WT melanomas harbor inactivating mutations in the RAS-GAP *NFI*, with a further 15–36% 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, *NFI* mutations frequently co-occur with lesions in other “Ras-opathy” genes including *SOS1*, *RASA2* and *PTPN1* (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 *NFI*. 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, *NFI* 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 one 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 non-receptor 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 non-receptor 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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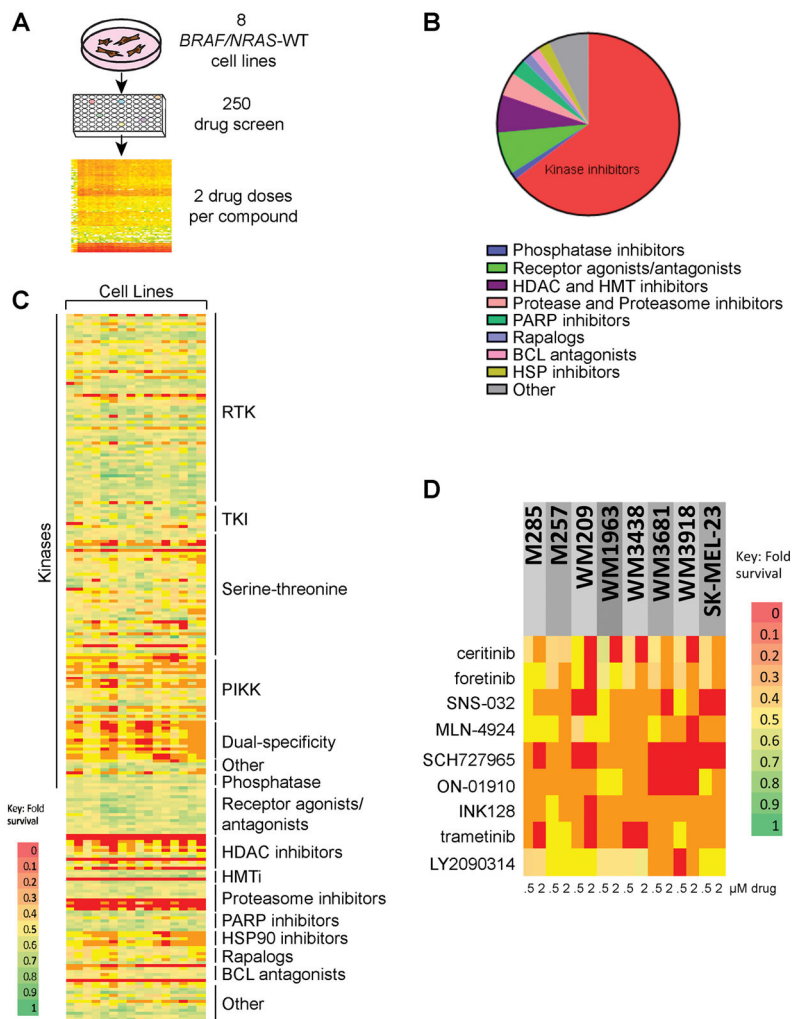


Figure 1. Identification of drugs with single agent activity against *BRAF/NRAS* wild-type (WT) melanoma

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 μ M) for 72h 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 μ M of drug relative to vehicle. **D:** Detailed view of the responses of the drugs selected for follow-up in the cell line panel. Data shows the inhibition of growth per cell line at 0.5 and 2.5 μ M of drug relative to vehicle.

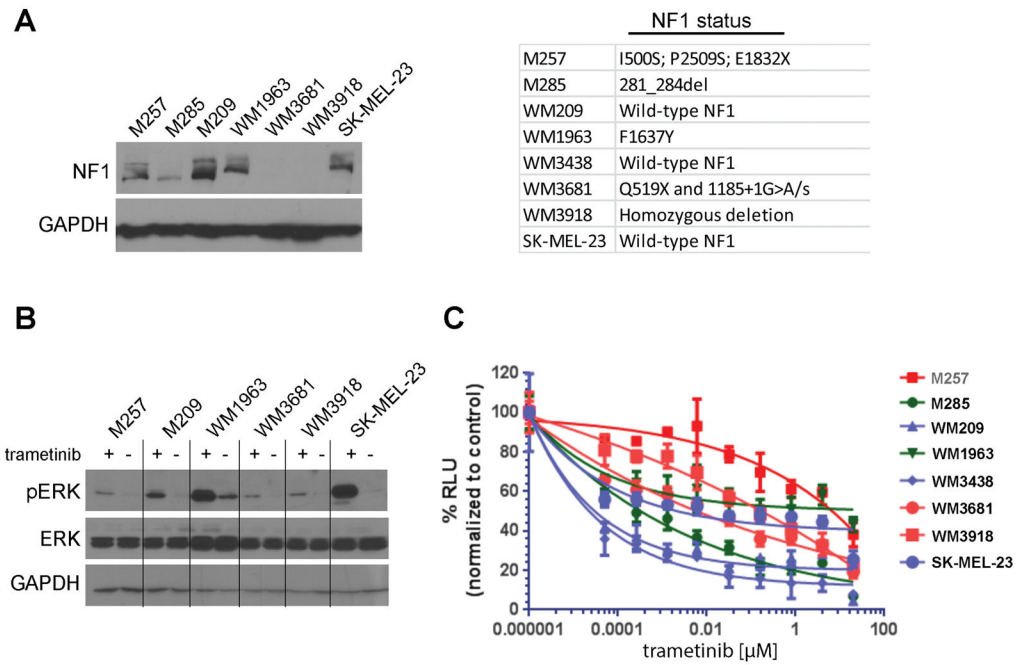


Figure 2. *BRAF/NRAS*-WT melanoma cell lines have constitutive ERK activity and show a range of MEK inhibitor sensitivities

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 (10nM, 24h) 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 72h before being analyzed by Cell Titer Glo assay. Data shows the mean of three experiments \pm 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.

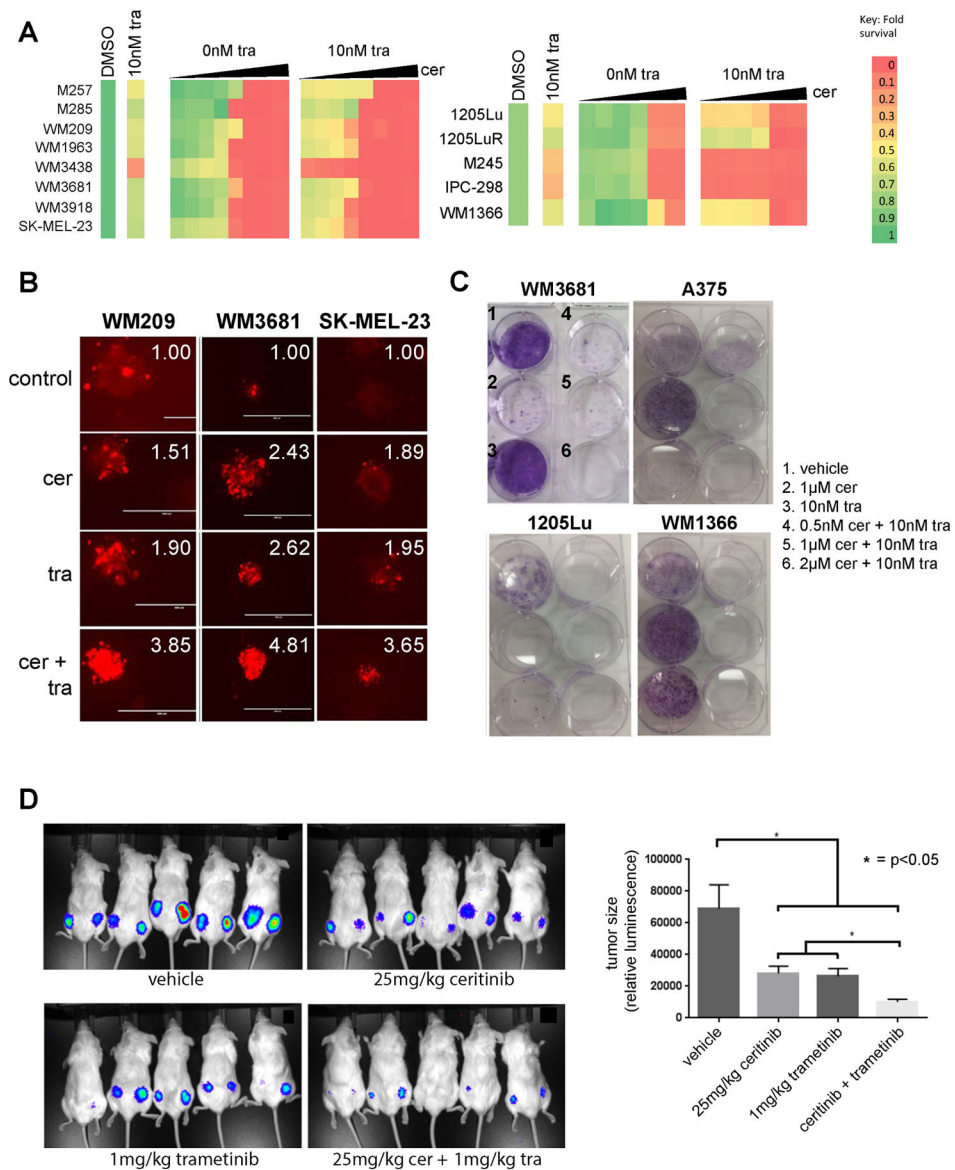


Figure 3. Ceritinib enhances the efficacy of trametinib

A: Heatmap showing the inhibition of growth to a selection of drugs identified from the screen in Figure 1 \pm 10nM trametinib. Cells were treated with each drug alone and in the presence of trametinib for 72h 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 μ M), trametinib (10 nM) or ceritinib + trametinib (2 μ M/10nM). 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 (25mg/kg), trametinib (1mg/kg) or the ceritinib-trametinib combination (25mg/kg

and 1mg/kg) daily by oral gavage. Left panel shows luminescence following 24 days of treatment. Right panel shows quantification of mean fluorescence following treatment with the drugs (day 24).

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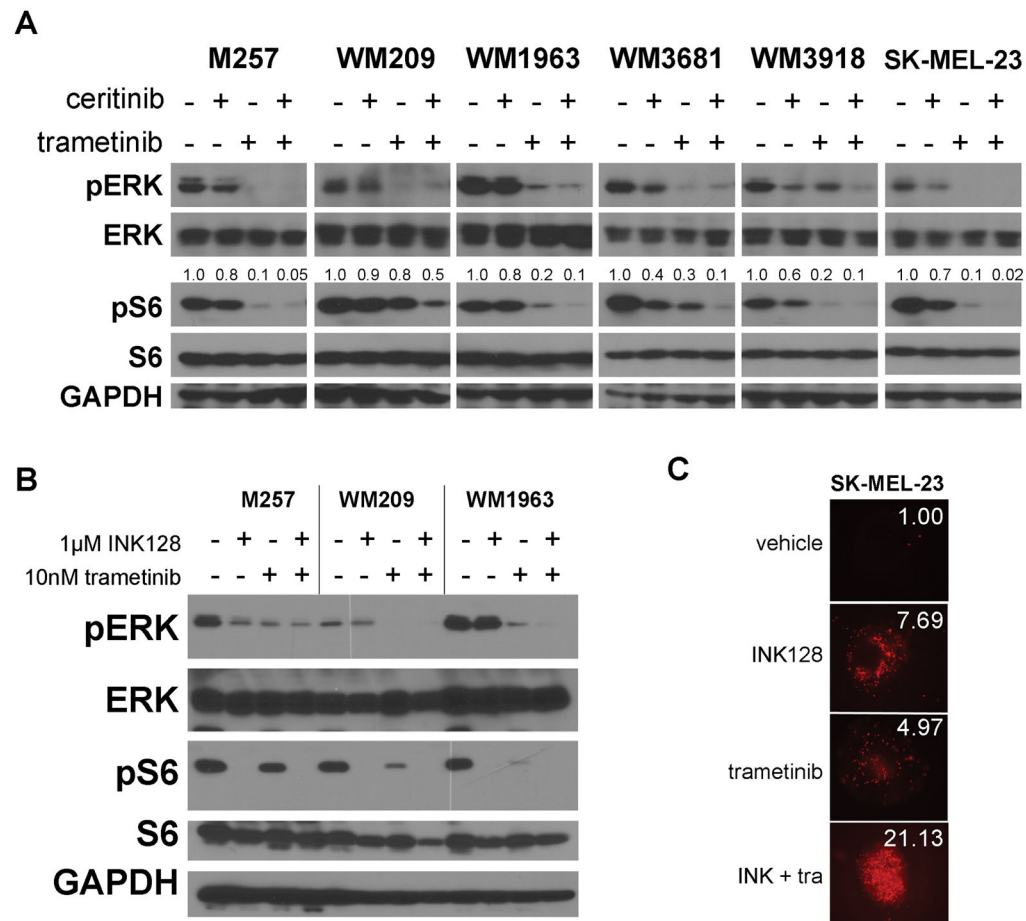


Figure 4. The combination of trametinib and ceritinib leads to enhanced suppression of TORC1 signaling

A: *BRAF/NRAS*-WT cell lines were treated with vehicle, ceritinib, trametinib or ceritinib-trametinib for 24h 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 μ M), trametinib (10nM) or INK128-trametinib (1 μ M/10nM) for 24h 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 μ M), trametinib (10nM) or INK128-trametinib (1 μ M/10nM). Spheroids were stained with propidium iodide to indicate dead cells. Panels show fold-increase in dead cells relative to controls.

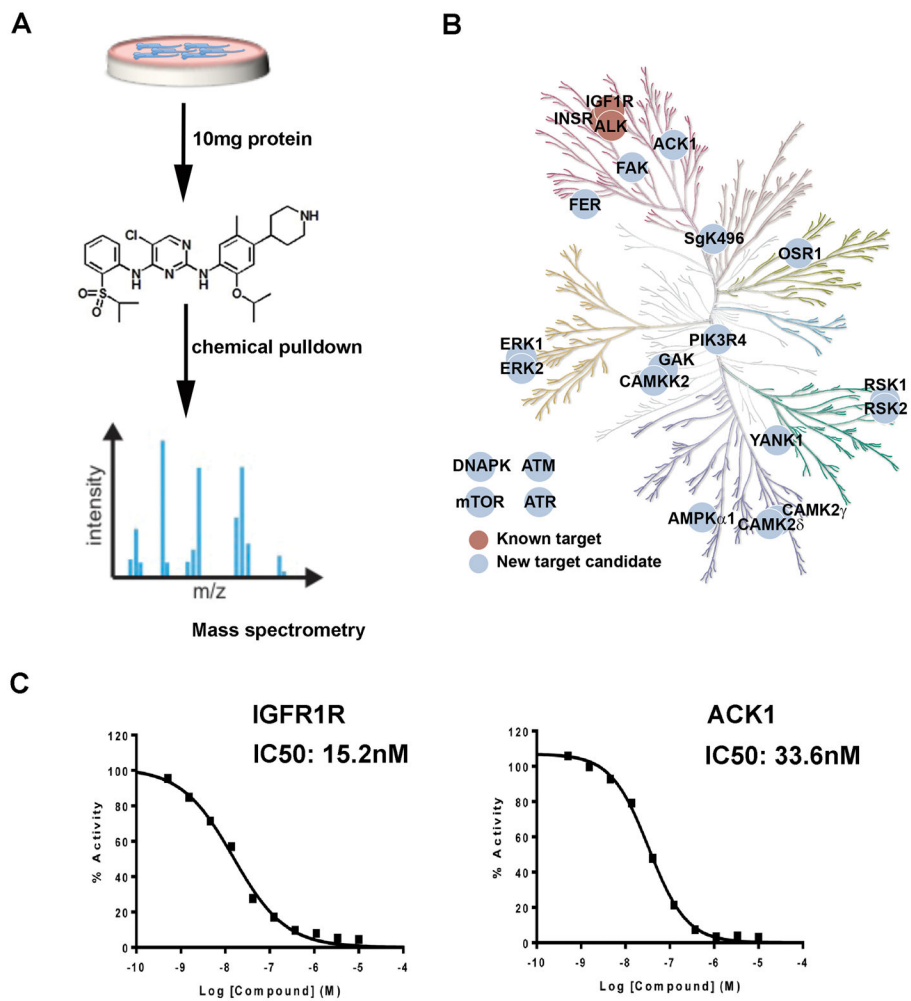


Figure 5. Chemical proteomics identifies IGF1R and ACK1 as potential targets of ceritinib in *BRAF/NRAS*-WT melanoma

A: Workflow of the chemical proteomic experiment. **B:** Kinome tree of potential kinase targets of ceritinib in SK-MEL-23 melanoma cells. **C:** Certinib inhibits IGF1R and ACK1 in *in vitro* kinase assays.

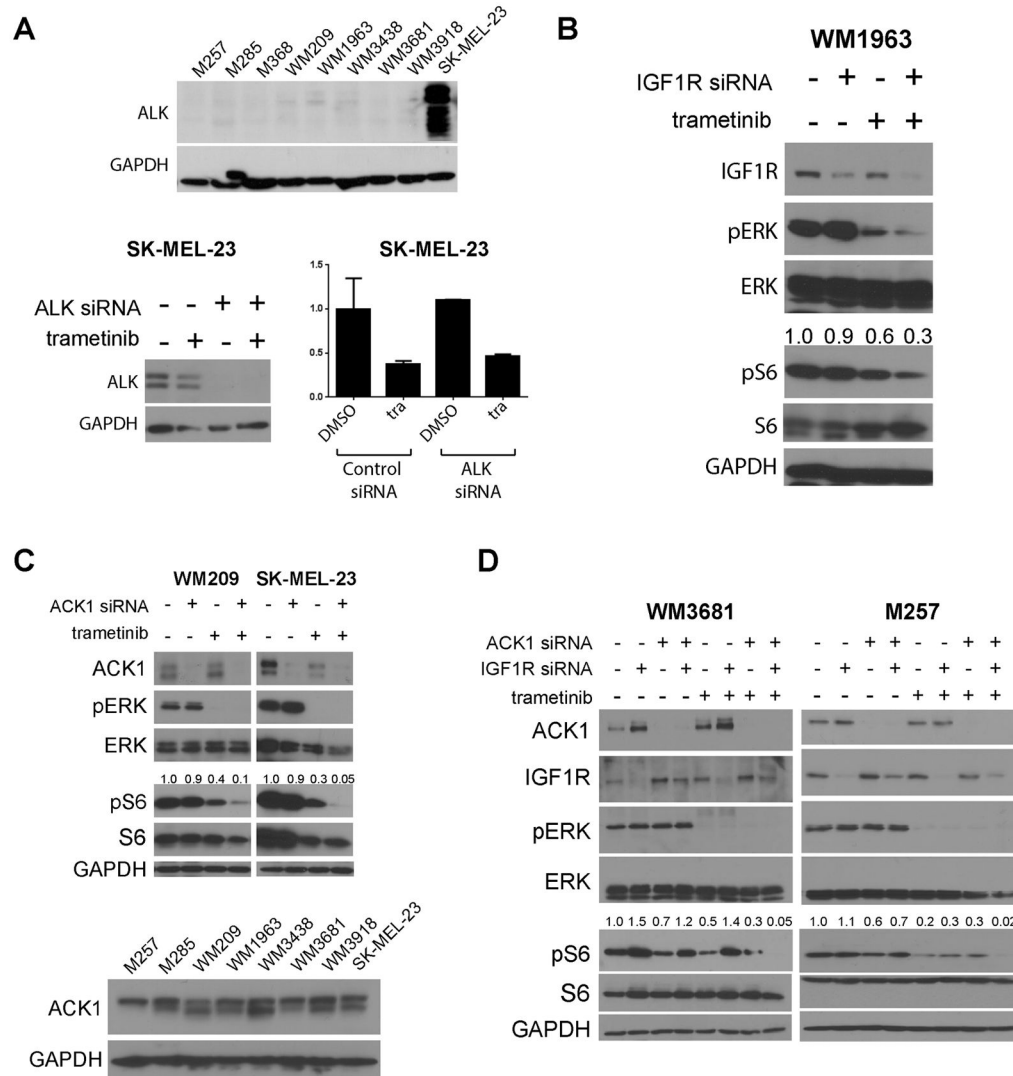


Figure 6. Silencing of IGF1R or ACK1 enhances suppression of pS6 when combined with trametinib

A: (Upper panel) Expression of ALK in the eight *BRAF/NRAS*-WT melanoma cell lines. Western Blot shows expression of ALK and GAPDH. (Lower panel) Silencing of ALK in SK-MEL-23 cells does not affect viability +/- trametinib in cell survival assays. Cells were treated with siRNA for 24h and then with ALK siRNA or non-silencing control alone and in the presence of trametinib (10nM) for 72h. 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 24h and then with vehicle or drug for 24h. Numbers are the densitometry values corresponding to pS6/tS6. **C:** (Upper) 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 24h and then with vehicle or drug for 24h before Western blot analysis for pERK and pS6. (Lower) 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 (10nM). WM3681 and M257 Cells were

treated with siRNA as above and then with trametinib for 24h before Western blot analysis for pERK and pS6.

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Table 1

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

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

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