Published in final edited form as:

Pigment Cell Melanoma Res. 2014 November ; 27(6): 1154–1158. doi:10.1111/pcmr.12303.

Vertical inhibition of the MAPK pathway enhances therapeutic responses in *NRAS*-mutant melanoma

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Summary

The MEK inhibitor MEK162 is the first targeted therapy agent with clinical activity in patients whose melanomas harbor *NRAS* mutations; however, median PFS is 3.7 months, suggesting the rapid onset of resistance in the majority of patients. Here we show that treatment of *NRAS*-mutant melanoma cell lines with the MEK inhibitors AZD6244 or trametinib resulted in a rebound activation of phospho-ERK (pERK). Functionally, the recovery of signaling was associated with the maintenance of cyclin-D1 expression and therapeutic escape. The combination of a MEK inhibitor with an ERK inhibitor suppressed the recovery of cyclin-D1 expression and was associated with a significant enhancement of apoptosis and the abrogation of clonal outgrowth. The MEK/ERK combination strategy induced greater levels of apoptosis compared to dual MEK/CDK4 or MEK/PI3K inhibition across a panel of cell lines. These data provide the rationale for further investigation of vertically co-targeting the MAPK pathway as a potential treatment option for *NRAS*-mutant melanoma patients.

Keywords

melanoma; NRAS; trametinib; ERK; AZD6244

Introduction

AZD6244, MEK162 and trametinib are allosteric, second generation, ATP non-competitive inhibitors with nanomolar activity against MEK1/2. MEK162 is the first targeted therapy agent to demonstrate clinical activity in patients whose melanomas harbor activating mutations in *NRAS* (Ascierto et al., 2013). In *BRAF*-mutant melanoma, treatment with BRAF inhibitors such as vemurafenib and dabrafenib is nearly always associated with the acquisition of resistance (Chapman et al., 2011; Hauschild et al., 2012). The importance of

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MAPK signaling recovery in therapeutic escape is demonstrated by the fact that the majority of patients (<70%) failing BRAF inhibitor therapy show constitutive pERK in their tumors post-relapse (Shi et al., 2014). Strategies to vertically target MAPK signaling in *BRAF*^T mutant melanoma have been preclinically and clinically successful with the combination of trametinib and dabrafenib being FDA-approved in early 2014 (Flaherty et al., 2012; Paraiso et al., 2010). In the current study we have addressed the role of adaptive MAPK signaling in the escape of *NRAS*-mutant melanoma cell lines from MEK inhibitor therapy.

Treatment of a panel of *NRAS*-mutant melanoma cell lines with either AZD6244 or trametinib inhibited pERK signaling (Figure 1A). Long-term colony formation assays revealed that MEK inhibition reduced the growth of WM1361A, WM1366 and WM1346 cells, however a significant number of resistant clones remained after 4 weeks of chronic treatment (Figure 1B). We next studied the time course of pERK signaling recovery. Treatment of drug-naïve *NRAS* mutant melanoma cells with AZD6244 (1 μ M) or trametinib (30 nM) showed the pathway to be rapidly inhibited, with robust recovery of pERK signaling within 24-48 hours (Figure 1C and Supplemental Figures 1A,B). The recovery of signaling still occurred when drug was replenished and remained stable over the course of a 10-day treatment schedule (Figures 1D). Reactivation of MAPK signaling was also noted following washout and re-treatment with AZD6244 and trametinib after 24 hrs (Supplemental Figures 1BD).

We next determined the utility of dual MEK/ERK inhibition in preventing therapeutic escape. Treatment of WM1361A and WM1366 cells with AZD6244 led to the initial inhibition of pRSK-1 (an ERK substrate) below detectable levels, followed by its recovery - an effect that mirrored the reactivation in pERK (Figure 2A). The addition of the ERK inhibitor VTX-11e induced high levels of pERK, a likely consequence of feedback inhibition being relieved upstream (Lito et al., 2012). The addition of VTX-11e to AZD6244 prevented the reactivation of pRSK1 (Thr359/Ser353), abrogated cyclin-D1 expression, retinoblastoma protein (pRB) phosphorylation and induced a greater expression of p27^{KIP1} compared to either single agent (Figures 2A,B). In apoptosis studies the combination of AZD6244 and VTX-11e was associated with an increase in cell death, as seen by increased Annexin-V binding, compared to either AZD6244 or VTX-11e (Figure 2C). It was further found that the combination of VTX-11e with AZD6244 and the combination of VTX-11e with trametinib was more effective at preventing the onset of resistant clones in colony formation assays (Figure 2D and Supplemental Figure 2A). The combination of VTX-11e (30nM) and AZD6244 (10nM) was found to be synergistic with a CI of 0.07 (Supplemental Figure 2B). A good correlation was not always observed between the extent of apoptosis induction and the inhibition of long-term colony formation; a likely consequence of MAPK inhibition also leading to the induction of senescence, autophagy and ER stress (Ma et al., 2014).

In *BRAF*-mutant melanoma, reactivation of the MAPK pathway typically occurs through increased signaling via Ras and CRAF (Lito et al., 2012). We next determined whether upstream targeting of CRAF enhanced the effects of MEK inhibition in *NRAS*-mutant melanoma. SiRNA knockdown of CRAF partly reversed the AZD6244-mediated recovery of pMEK, and abrogated the recovery of pRSK1 and pERK (Figure 3A). CRAF knockdown

also more fully suppressed the expression of cyclin-D1 and enhanced the expression of p27 (Figure 3A). MEK/CDK4 and MEK/PI3K are two combinations currently under investigation for *NRAS* mutant melanoma (Kwong et al., 2012; Posch et al., 2013). Treatment of melanoma cells with the MEK/ERK inhibitor combination led to higher levels of apoptosis and better long-term growth suppression than either the MEK/CDK4 or the MEK/PI3K inhibitor combination (Figures 3B,C). There was no evidence that the combination of two MEK inhibitors (trametinib and AZD6244) significantly enhanced the inhibition of pERK, pRSK1 or cyclin D1 protein expression or increase apoptosis beyond additive levels compared to either drug alone (Supplemental Figure 2C). Even though MAPK signaling is a prerequisite for Ras-mediated tumor initiation and maintenance, MEK inhibitors have shown limited efficacy in *Ras*-mutant cancers (Ishii et al., 2013; Lito et al., 2014). Recent work in *KRAS* mutant cancer cell lines supports our findings and shows that MEK inhibition is frequently associated with rebound MAPK signaling and can be prevented through CRAF knockdown (Ishii et al., 2013; Lito et al., 2014). Newer generation MEK inhibitors such as CH5126766, which prevent CRAF/MEK binding, give more durable therapeutic responses than allosteric MEK inhibitors (Ishii et al., 2013). In summary we have shown for the first time that MAPK signaling recovers rapidly following MEK inhibition in *NRAS*-mutant melanoma and that vertical targeting of MEK and ERK enhances therapeutic efficacy in this underserved melanoma subtype.

METHODS

Melanoma Cell Lines and drugs

The melanoma cell lines WM1361A, WM1366, M202, M318 and WM1346 were described previously (Rebecca et al., 2014). PD0332991, AZD6224 and GDC-0941 were purchased from Selleck (Houston, TX). Trametinib was from Chemietek (Indianapolis, IN).

ERK inhibitor synthesis

The ERK inhibitor VTX-11e was synthesized (0.5-1g scale) using the protocols reported in (Aronov et al., 2009) with > 99% purity. VTX-11e was characterized using ¹H NMR, LCMS, HRMS, HPLC-MS and HPLC to confirm its structure and determine its purity. The ¹H-NMR of the in-house synthesized compound was consistent with the ¹H-NMR reported by (Aronov et al., 2009) (Supplemental Figure 3).

Western blotting

The primary antibodies to phospho-ERK, total ERK, pRSK-1, total RSK1, Cyclin D1, BIM, pRB, Rb, p27 and cleaved-PARP were from Cell Signaling Technology (Danvers, MA). GAPDH was from Sigma.

Flow cytometry

Cells were seeded in 6-well plates, as previously described (Paraiso et al., 2010). Cells were incubated with inhibitors for 120 hours, after which they were stained for annexin-V.

Colony Formation Assay

Plates were set up as described previously (Paraiso et al., 2010) and treated with vehicle (DMSO), 1 μ M AZD6244, 30nM Trametinib, 100-300 nM VTX, 1 μ M PD0332991, 1 μ M GDC-0941 or the labeled combination of these agents (4 weeks). All drugs were replaced twice per week. Relative colony density was determined by solubilizing the crystal violet dye in 10% acetic acid followed by measurement of absorbance at 450 nm.

Statistical analysis

Data show the mean of at least 3 independent experiments. GraphPad Prism 5 statistical software was used to perform the Student's t test where (*) indicates $P < 0.05$, (**) indicates $P < 0.05$, (***) indicates $P < 0.001$ and (****) indicates $P < 0.0001$. CompuSyn (CompuSyn, Inc.) synergy/antagonism analysis software was used to assess synergy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Grant support: Work in the Smalley lab is supported by 1P50CA168536-01A1 and R01 CA161107-01 from the NIH

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Significance

Although single agent MEK inhibition has some activity in *NRAS*-mutant melanoma patients, response times are limited. Here, we present new data demonstrating that MEK inhibition is associated with a rapid recovery of MAPK signaling in *NRAS*-mutant melanoma that can be overcome through the dual inhibition of MEK and ERK. We suggest that vertical MAPK inhibition may be of utility in *NRAS*-mutant melanoma.

Figure 1. MEK inhibition leads to MAPK reactivation

(A) A panel of *NRAS*-mutant cells were treated with AZD6244 (AZD, 1 μ M) or trametinib (TRA, 30 nM) for 1 hour before being blotted for pERK and total-ERK.

(B) Cells were treated chronically with AZD6244 or trametinib for 4 weeks (as above) and colonies stained with crystal violet.

(C) Cells were treated with AZD6244 for increasing periods of time before being probed for pERK and total-ERK

(D) Cells were treated chronically with AZD6244 (1 μ M) for up to 10 days with drug replaced according the schedule in the lower panel. Western blotting was performed for pERK, total-ERK, pRSK1, total-RSK, cyclin D1 and GAPDH.

Figure 2. Inhibition of MEK and ERK is more effective than either agent alone

(A) Cells were treated with AZD6244 (1 μ M) in the absence or presence of VTX-11e (300 nM, 72 hrs) before being analyzed by Western Blot.

(B) WM1361A cells were treated with AZD6244 (1 μ M) and/or VTX-11e (300 nM) for 72 hr and analyzed by Western blot.

(C) Cells were treated with VTX-11e (300 nM) in the presence or absence of AZD6244 (1 μ M) or with VTX-11e (300 nM) in the presence or absence of trametinib (30 nM) for 0-120 hours before being analyzed by flow cytometry.

(D) Cells were treated with VTX-11e (100 - 300 nM) in the presence or absence of AZD6244 or with VTX-11e in the presence or absence of trametinib for 4 weeks before being stained with crystal violet.

Figure 3. Dual MEK/ERK inhibition is more effective than MEK/CDK4/6 and MEK/PI3K inhibition

(A) WM1361A cells were treated with siRNA specific against CRAF or scrambled control, plus or minus AZD6244 (1 μ M, 72 hr). Cell lysates were analyzed by Western blotting, as indicated.

(B) Cells were treated with AZD6244 (1 μ M) in the presence or absence of VTX (300 nM), PD0332991 (1 μ M) or GDC-0941 (1 μ M) 0-120 hours. Identical combinations were utilized with trametinib (30 nM) as labeled. Apoptosis was assessed by annexin-V staining flow cytometry.

(C) Colony formation experiments in which cells were treated with drugs listed in (B) for 4 weeks. Lower panel shows quantification.