Antiproliferative Effects of Continued Mitogen-Activated Protein Kinase Pathway Inhibition following Acquired Resistance to BRAF and/or MEK Inhibition in Melanoma

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Abstract

Inhibitors of the mitogen-activated protein kinases (MAPK), BRAF, and MAP–ERK kinase (MEK) induce tumor regression in the majority of patients with BRAF-mutant metastatic melanoma. The clinical benefit of MAPK inhibitors is restricted by the development of acquired resistance with half of those who benefit having progressed by 6 to 7 months and long-term responders uncommon. There remains no agreed treatment strategy on disease progression in these patients. Without published evidence, fears of accelerated disease progression on inhibitor withdrawal have led to the continuation of drugs beyond formal disease progression. We now show that treatment with MAPK inhibitors beyond disease progression can provide significant clinical benefit, and the withdrawal of these inhibitors led to a marked increase in the rate of disease progression in two patients. We also show that MAPK inhibitors retain partial activity in acquired resistant melanoma by examining drug-resistant clones generated to dabrafenib, trametinib, or the combination of these drugs. All resistant sublines displayed a markedly slower rate of proliferation when exposed to MAPK inhibitors, and this coincided with a reduction in MAPK signaling, decrease in bromodeoxyuridine incorporation, and S-phase inhibition. This cytostatic effect was also associated with diminished levels of cyclin D1 and p-pRb. Two short-term melanoma cultures generated from resistant tumor biopsies also responded to MAPK inhibition, with comparable inhibitory changes in proliferation and MAPK signaling. These data provide a rationale for the continuation of BRAF and MEK inhibitors after disease progression and support the development of clinical trials to examine this strategy. Mol Cancer Ther; 12(7); 1332–42. ©2013 AACR.

Introduction

Treatment of BRAF-mutant metastatic melanoma with the potent BRAF inhibitors, vemurafenib and dabrafenib, and the MAP–ERK kinase (MEK) inhibitor, trametinib, yields response rates of 25% to 60% and prolongs progression-free and overall survival (1–7). The combination of dabrafenib and trametinib prolongs progression-free survival compared with dabrafenib alone, and the addition of trametinib reduces the development of cutaneous squamous cell carcinomas, a common side effect of BRAF inhibitors (8, 9).

The clinical benefit associated with inhibition of the mitogen-activated protein kinase (MAPK) cascade is restricted by the development of acquired drug resistance, usually within 2 to 18 months of initial treatment. Multiple mechanisms of resistance have been described including elevated expression of the kinases CRAF, COT1, or mutant BRAF (10–13), activating mutations in N-RAS, MEK1, or AKT1 (14–16), aberrant splicing of BRAF (17), or persistent activation of receptor tyrosine kinases, including platelet-derived growth factor receptor ß (PDGFRß) and insulin-like growth factor-I receptor IGF-1R (14, 18).

In vitro data indicate that PTEN loss (19), cyclin D1 overexpression (20), hepatocyte growth factor expression (21), fibroblast growth factor receptor 3 activation (22), and EGF receptor (EGFR) amplification (23) may also contribute to MAPK inhibitor resistance. There is currently no standard approach for the management of resistance to targeted drug therapy of melanoma. Clinical benefit beyond progression has been shown for the continuation of trastuzumab in HER-2–positive breast cancer (24, 25) and the dose escalation of imatinib in resistant gastrointestinal stromal tumors (GIST; refs. 26–29).

In this report, we examined the concept that targeted RAF and MEK inhibitors retain antitumor activity in...
resistant melanoma tumor cells. This is particularly relevant as RAF inhibitors are used after disease progression in selected patients in some centers, particularly in patients with localized or asymptomatic disease progression (30). We illustrate the clinical rational for treatment beyond progression with 2 patients showing a marked increase in the rate of progression (“flare”) after discontinuation of MAPK inhibitor treatment. Furthermore, we confirm that RAF and MEK inhibitors retain MAPK inhibitory and antiproliferative activity in melanoma tumor cells with acquired resistance to MAPK inhibition and in 2 short-term melanoma cell cultures derived from biopsies of patients who have progressed on treatment with dabrafenib or trametinib. These data confirm that MAPK inhibitors retain tumor suppressor activity in resistant melanoma cells and highlight the importance of investigating this activity in vivo.

Materials and Methods

Patients and clinical assessment

Patients participated in the phase I study of dabrafenib (NCT00888321; ref. 3) or the phase I trial of dabrafenib in combination with trametinib (NCT01072175; ref. 31). Date of disease progression was determined by Response Evaluation Criteria in Solid Tumors (RECIST) 1.0 in the case of dabrafenib monotherapy and RECIST 1.1 in the case of dabrafenib combined with trametinib. Combined tumor diameter was measured as the sum of the maximal diameter of all lesions. Lactate dehydrogenase (LDH) was normalized as a ratio of the upper limit of normal (ULN) due to the variation in normal ranges between assays and laboratories used.

Both patients were imaged on dedicated positron emission tomography (PET)/computed tomography (CT) systems with 18-F-fluorodeoxyglucose (FDG) and fasted for at least 6 hours before radiopharmaceutical injection, and their blood glucose level did not exceed 7 mmol/L. Whole body PET imaging from vertex to toes started between 60 and 75 minutes after administration of FDG. A “low-dose” CT without intravenous contrast administration was acquired concurrently for attenuation correction and anatomical localization. Paired scans were conducted on the same camera under standard conditions and reconstruction parameters. Change in tumor metabolic activity was determined by total lesion glycolysis (TLG%) which was measured by multiplying the standard uptake value average by the region volume and for multiple regions summed together to measure the collective uptake for all lesions as described previously (32). The change in TLG%ID was calculated by dividing the difference in TLG%ID between scans by the TLG%ID of the initial scan TLG%ID.

Cell culture and compounds

SKMel28 and MelMS melanoma cells were obtained from Prof. P. Hersey (Immunology and Oncology Unit, Newcastle University, Newcastle, Australia). Cells were grown in Dulbecco’s Modified Eagle Medium (DMEM) with 10% FBS and glutamine (Gibco BRL) and cultured in a 37°C incubator with 5% CO₂. Stocks of dabrafenib (supplied by GlaxoSmithKline and Active Biochem) and trametinib (supplied by GlaxoSmithKline and Selleck chemicals) were made in dimethyl sulfoxide (DMSO). Cell authentication was confirmed using the StemElite ID system from Promega. To generate growth curves, cells were detached with trypsin and counted using an automated Beckman Coulter Z2 counter, a minimum of 2 independent counts, each conducted in triplicate, was carried out for each cell line and drug combination.

Patients consented to tumor biopsies and the creation of short-term cultures, which were derived according to a modified protocol (33, 34). Briefly, the tissue was collected in DMEM/10% fetal calf serum (FCS) containing 100 U/mL pencillin and 100 μg/mL streptomycin (GIBCO) and mechanically processed using scalpels and a 10 ml sterile syringe. The clarified cell suspension was maintained in DMEM/10% FCS containing pencillin and streptomycin (GIBCO). Contaminating fibroblasts were removed by treating with 100 μg/mL G418 (GIBCO) as required. Dual immunofluorescent staining with the pan-melanoma cocktail (HM454, MART1, tyrosinase; Applied Medical) and the fibroblast antibody TE7 (Millipore) was used to confirm that the derived cell lines were of melanocytic origin. BRAFV600E genotype of short-term melanoma cultures was also confirmed using PCR-based capillary sequencing (35).

Pharmacologic growth inhibition assay

Cultured cells were seeded into 96-well plates (1–2 × 10⁴ cells per well) and 24 hours after seeding, serial dilutions of each inhibitor prepared in media were added to cells. Cells were incubated for 72 hours following addition of drug. Cell viability was measured using the Cell proliferation Aqueous MTS assay (Promega) on a VICTOR² Multilabel counter (PerkinElmer). Viability was calculated as a percentage of control (untreated cells) after background (no cells) subtraction. A minimum of 2 independent viability assays, each conducted in triplicate, was carried out for each cell line and drug combination. GI5₀ values were generated from dose–response curves fitted using a regression fit in GraphPad PRISM 5 software (GraphPad) and are defined as the drug concentration required to reduce the growth of cells to half that of control DMSO-treated cells.

Cell-cycle and apoptosis analysis

Adherent and floating cells were combined and cell-cycle and apoptosis analyses were conducted as previously described (36).

RNA extraction and microarray gene expression analysis

Total RNA was extracted as described previously (37). Gene expression analysis was conducted using the Sentrix HumanRef-6 v4.0 Expression BeadChip (Illumina) and beadstation system from Illumina according to manufacturer’s instructions. The microarray platform and data
have been submitted to the Gene Expression Omnibus public database at the National Center for Biotechnology Information, following the minimum information about microarray gene experiment guidelines. The accession numbers is GSE44753.

Immunoblotting
Total cellular proteins were extracted at 4°C using radioimmunoprecipitation assay lysis buffer containing protease inhibitors and phosphatase inhibitors (Roche). Proteins (40 μg) were resolved on 12% SDS-polyacrylamide gels and transferred to Immobilon-P membranes (Millipore). Western blot analyses were probed with antibodies against phosphorylated extracellular signal-regulated kinase (p-ERK, E4), total ERK (137F5; Cell Signaling), p-PRbS807/811 (Cell Signaling), β-actin (AC-74; Sigma-Aldrich), p27Kip1 (Becton Dickinson), and cyclin D1 (G124-326 Becton Dickinson).

Indirect immunofluorescence for BrdUrd incorporation
Cells were seeded onto coverslips in 12-well plates at 2-4E4 cells per well and cultured in the presence of inhibitor or DMSO control for 72 hours. Cells were then pulsed with bromodeoxyuridine (BrdUrd) for 2 to 8 hours, dependant on cell line, then washed in PBS, and fixed with 4% formaldehyde/PBS for 15 minutes at room temperature. Cells were rinsed 3 times with PBS, permeabilized with 0.2% Triton-X100/PBS for 10 minutes, and subsequently treated for 30 minutes with approximately 7U RQ1 DNase (Promega). The cells were then immunostained for 50 minutes with an antibody to BrdUrd (BU-1; Amersham Biosciences) followed by a 50-minute exposure to Alexa Fluor 594-conjugated secondary immunoglobulin G (Molecular Probes). Nuclear DNA was stained with 1 mg/mL 4', 6-diamidino-2-phenylindole for 10 to 15 minutes. At least 400 cells were screened from at least 2 independent experiments.

Statistical analysis
Data are presented as the mean ± SD from at least 2 independent experiments. Paired t tests were used to compare mean values, results were considered statistically significant at P < 0.05.

Results
Cessation of MAPK inhibitors in patients with acquired resistance results in accelerated disease progression
A 50-year-old man with BRAFV600E-mutant metastatic melanoma was enrolled on part D of the phase I clinical trial of dabrafenib (75 mg twice daily) and trametinib (2 mg daily) in combination (31) and achieved RECIST 1.1 (38) partial response. He later progressed at the site of an intra-abdominal metastasis (Fig. 1A), which was resected because of the risk of small bowel obstruction, and combination dabrafenib/trametinib therapy was continued. His disease continued to progress slowly but he remained clinically stable with an Eastern Cooperative Oncology Group (ECOG) performance status of one. Dabrafenib and trametinib were discontinued 154 days after documented disease progression and 94 days after surgery for a 30-day “washout” period before enrolment in a clinical trial of the anti-PD-1 antibody MK3475. During these 30 days off dabrafenib and trametinib, disease progression accelerated rapidly (Fig. 1A). TLG increased by 38% on serial PET scans with FDG taken 7 days before and 22 days after cessation of therapy (Fig. 1B). In keeping with this radiological and metabolic progression, the patient deteriorated rapidly with the development of abdominal pain and a fall in his ECOG performance status to 2.

Accelerated disease metabolic activity, 6 days after cessation of dabrafenib, was also shown in a 53-year-old male with BRAFV600E-mutant metastatic melanoma who was treated on the phase I trial of dabrafenib (3) at a total daily dose of 150 mg twice daily. After an initial RECIST partial response, his disease progressed 28.3 months after commencing treatment, but he was continued on dabrafenib because he was asymptomatic and the pace of progression was slow. Dabrafenib was ceased 34.6 months after commencing treatment and 6.3 months after disease progression in preparation for entry into another clinical trial. His ECOG performance status remained 0 throughout dabrafenib treatment. During a 7-day washout period, the patient developed pain at the sites of subcutaneous and intramuscular metastases requiring opioid analgesia, and his ECOG performance status fell to one. 18-F-FDG PET/CT scanning was conducted 5 days before and 6 days after ceasing dabrafenib. During this time, TLG increased by 34%, new lesions appeared, and rapidly growing lesions developed necrosis (Fig. 2A). Furthermore, serum LDH increased markedly (Fig. 2B).

RAF and MEK inhibitors inhibit the proliferation of acquired resistant melanomas
To explore the activity of MAPK inhibitors in resistant melanoma, we generated drug-resistant clones from the human-derived melanoma cell line, SK-Mel28 (39) or its single-cell-derived subclone, SK-Mel28#8. Both SKMel28 lines carry activating mutations in BRAF (V600E), EGFR (P753S), and CDK4 (R24C; refs. 20, 40) and are highly sensitive to dabrafenib (GI50 < 8 nmol/L) and trametinib (GI50 < 1 nmol/L; Fig. 3A). We derived BRAFi-resistant sublines after prolonged exposure of SKMel28 cells to dabrafenib, as described previously (drug-resistant clones in prior publication now referred to as BR in figures; ref. 41). The trametinib-resistant and dabrafenib + trametinib (combination resistant) were derived from the SK-Mel28#8 cell line after chronic drug exposure. Two single-cell–derived drug-resistant clones from each drug treatment were expanded and characterized. All resistant sublines were screened for FGFR, IGF-1R, and MET activation, BRAF-splicing defects, MEK1 exon 3 and 6 mutations, N-RAS mutations, loss of PTEN, and over-expression of BRAF, CRAF, and CCND1 (data not
shown). Only the dabrafenib-resistant BR6 clone had an established driver of resistance, an NRASQ61H oncogenic mutation (41).

All drug-resistant sublines retained the activating BRAF, EGFR, and CDK4 mutations found in the parental cell line (data not shown) and were continuously cultured in the inhibitor used to acquire resistance (Fig. 3A). As expected, MAPK reactivation was detected in all resistant sublines, and p-ERK expression was maintained in the presence and absence of drug (Fig. 3B). Nevertheless, gene expression analyses indicated that the MAPK signaling output was diminished in resistant melanoma cells in the presence of drug, although the degree of suppression varied and was consistently less than that in the sensitive parental lines (Fig. 3C). The gene set used to measure MAPK signaling is indicative of persistent MEK–ERK activation and included many known transcription targets of ERK signaling, including MYC, ETV5, and the negative feedback regulators, DUSP4/6 and SPRY1/2 (14). Furthermore, there was also little evidence of inhibitor-induced cell death in the resistant sublines (see Supplementary Fig. S1) even though both parental and resistant sublines showed reduced S-phase entry (Fig. 4A) that was associated with p27kip1 accumulation, and diminished levels of cyclin D1 and p-pRb (Fig. 3B). Similarly the resistant sublines showed a proliferative transcriptome signature that was reduced in the presence of drug, and again the degree of suppression was less than that in the drug-treated parental line (see Supplementary Fig. S2).

To explore the impact of drug-induced S-phase inhibition in resistant melanoma cells, the proliferative rate of the resistant sublines was examined. As shown in Fig. 4B, all resistant melanoma cells displayed a markedly slower rate of proliferation when exposed to MAPK inhibitors, with significantly lower doubling times ($P < 0.001$ for all clones), and this coincided with a significant decrease in BrdUrd-positive cells ($P < 0.05$ for all clones; Fig. 4C).

Activity of MAPK inhibitors is not due to off-target effects or the effect of cell culture

To exclude the possibility that dabrafenib and trametinib impede cell proliferation via nonspecific effects, we examined the impact of these MAPK inhibitors on 2 BRAF wild-type melanoma cell lines. The WMM1175 cells are heterozygous for the N-RASQ13R mutation and the MelMS cells depend on oncogenic c-Kit receptor for survival (42). Dabrafenib is a highly selective RAF kinase inhibitor, and as expected, had no effect on the activation of ERK, proliferation, or survival of the BRAF wild-type WMM1175 and MelMS melanoma cells (see Supplementary Fig. S3). In contrast, treatment with trametinib, an inhibitor of wild-type MEK kinases, diminished cell proliferation and ERK phosphorylation, without inducing
cell death in the WMM1175 and MelMS cells (see Supplementary Fig. S3).

**MAPK inhibition also suppresses the proliferation of short-term tumor cultures acquired from patients with dabrafenib or trametinib resistance**

To validate the activity of MAPK inhibitors on resistance acquired in vivo, 2 short-term cultures generated from tumor biopsies derived from patients who had progressed on treatment with dabrafenib or trametinib were analyzed. Biopsies were conducted on lesions, which had progressed on drug treatment (see Supplementary Fig. S4).

Patient WMD013 was a 71-year-old male with BRAFV600E-mutant metastatic melanoma who, following progression on vemurafenib, was treated with trametinib at a dose of 2 mg daily. The WMD013 short-term culture was established after progression on trametinib from a subcutaneous lesion that increased in size as shown on CT (see Supplementary Fig. S4). Although this short-term culture was resistant to and continued to proliferate in the presence of 10 nmol/L trametinib (Fig. 5 and Supplementary Fig. S5; GI50 > 40 nmol/L), cells displayed a significant decrease in DNA replication and proliferation in the presence of drug. Proliferative inhibition was associated with reduced ERK phosphorylation, accumulation of p27kip1, and decreased levels of cyclin D1 and p-pRb (Fig. 5).

Patient WMD009 was a 58-year-old female with BRAFV600E-mutant metastatic melanoma treated with dabrafenib as part of a phase I trial (3), and had an initial partial response. The cell line analyzed was derived from a subcutaneous lesion, which initially responded and then progressed on treatment as shown on CT (see Supplementary Fig. S4). The resected lesion expressed the exon 2 to 10 BRAFV600E-splicing variant, an established mechanism of resistance to BRAF inhibitors (17), which was not present in the pretreatment biopsy (see Supplementary Fig. S6). The derived WMD009 short-term culture also expressed this BRAF splice variant and survived in the presence of dabrafenib (Fig. 5A and Supplementary Figs. S5 and S6; GI50 > 1000 nmol/L). Nevertheless, dabrafenib promoted a slight S-phase inhibition that was associated with a reduced rate of proliferation and diminished BrdUrd incorporation (Fig. 5), without significant changes in p27kip1, cyclin D1, and p-pRb (Fig. 5).

**Discussion**

The phase III clinical trial of vemurafenib confirmed that BRAF inhibitors are the standard of care in BRAF-mutant metastatic melanoma (5). Despite the shown clinical benefits, responses to MAPK inhibitors are short lived and improved durability of response is a critical goal. We suggest that in selected patients, including those with slow and/or asymptomatic progression, continuation of the MAPK inhibitor(s) may be a viable alternative to
Figure 3. Acquired resistance to MAPK inhibition is associated with reactivation of MAPK signaling. Dabrafenib (BRAFi)-resistant clones referred to as BR, trametinib (MEKi)-resistant clones as MR, and combination (dabrafenib and trametinib) resistant as CR. A, viability curves of the parental SK-Mel28/SK-Mel28#8 and derived BR, MR, and CR sublines. The data are shown relative to DMSO-treated controls (mean ± SD; n = 6). B, parental cells and MAPK inhibitor-resistant sublines cells were treated with DMSO (−) or 100 nmol/L dabrafenib (BR clones), 10 nmol/L trametinib (MR clones) or 100 nmol/L dabrafenib, and 5 nmol/L trametinib (CR clones; +) for 24 hours and effects on ERK activation and cell-cycle regulators were determined by immunoblotting. C, heatmap for MAPK activation signature in the indicated cell lines treated with DMSO (−) or 100 nmol/L dabrafenib (BR clones), 10 nmol/L trametinib (MR clones) or 100 nmol/L dabrafenib, and 5 nmol/L trametinib (CR clones; +) for 24 hours. Color scale, log2-transformed expression (red, high; green, low) for each gene (row) normalized by the mean of all samples (left). The mean log2-transformed expression of all transcripts included in the MAPK activation signature (see left) was determined. The histogram shows mean values of the treated samples relative to their corresponding untreated cells (right).
Figure 4. Melanoma cells with acquired resistance to MAPK inhibitors display reduced proliferation in the presence of these inhibitors. Dabrafenib (BRAFi)-resistant clones referred to as BR, trametinib (MEKi)-resistant clones as MR, and combination (dabrafenib and trametinib)-resistant as CR. A, cell-cycle distribution of parental and resistant subclones treated with indicated MAPK inhibitor(s) or DMSO control for 72 hours (mean ± SD; n = 4). B, cell proliferation analyses of DMSO and MAPK inhibitor–treated sublines (mean ± SD; n = 6). C, analyses of BrdUrd incorporation in cells exposed to DMSO or MAPK inhibitors for 72 hours (mean ± SD; n = 4).
changing treatment, particularly given the lack of evidence-based treatment strategies after failure of MAPK inhibition. It is important to note, however, that melanoma progression on MAPK inhibitor therapy is clinically and biologically heterogeneous, and patients who progress rapidly during treatment are unlikely to benefit from continuation of MAPK inhibitor therapy. The proposed benefit of continuing therapy contrasts with a recent report showing that withdrawal of vemurafenib promoted melanoma regression of established vemurafenib-resistant melanomas in mice. In this instance, vemurafenib resistance was driven by overexpression of oncogenic BRAF, and resistant sublines became dependent on drug for proliferation (43). Vemurafenib withdrawal also diminished the clonogenicity of a resistant melanoma cell line, expressing a 61 kDa splice variant of BRAFV600E.
although the impact of vemurafenib on the proliferation and tumorigenicity of this cell line was not reported (43). It remains to be determined whether mechanisms of BRAF inhibitor resistance, other than BRAF overexpression, confer a fitness deficit to the tumor in the absence of drug, and certainly our data indicate that melanoma cells with resistance driven by BRAF splice variants or oncogenic NRAS continue to respond partially to drug. Furthermore, in 2 patients with melanoma, that remained clinically stable with slowly progressing disease while on MAPK inhibitor therapy, the cessation of MAPK inhibitor therapy was associated with accelerated disease activity and rapid deterioration. Indeed, tumor biopsies taken on progression from these patients did not show evidence of BRAF copy number gains (data not shown). Taken together, these data suggest that treatment strategies after failure of MAPK inhibitors will need to be individualized and account for the mechanisms of tumor resistance.

We also confirm that MAPK inhibitors retain antiproliferative activity in resistant melanoma tumor cells. Specifically, single-cell–derived melanoma sublines with acquired resistance to BRAF and/or MEK inhibitors responded to these inhibitors with reduced MAPK signaling and diminished proliferation, but showed no evidence of apoptosis. Similarly, short-term melanoma cultures derived from patients who had progressed on treatment with dabrafenib or trametinib also responded to these MAPK inhibitors with reduced proliferation and no cell death. These data suggest that these resistant tumor cells retain some dependency on MAPK signaling, and that any disruption in MAPK activity will be reflected in slower proliferation. Thus, we suggest that examples of disease flare upon cessation of MAPK inhibitors can be explained, at least in part, by partial drug activity on resistant cells.

The discontinuation of tyrosine kinase inhibitors has been shown to result in disease flare and a significant risk of accelerated disease progression in EGFR-mutant non–small cell lung cancer (44, 45). Indeed, reinitiating kinase inhibition leads to disease control in a majority of patients with EGFR-mutant cancer (45). Similarly, cessation of imatinib treatment in patients with imatinib-resistant GISTs can result in disease flare as visualized on FDG-PET scanning (46).

The continuation of targeted therapy beyond disease progression may produce clinical benefits because of tumor heterogeneity where growth-inhibited sensitive and proliferating resistant tumor cells coexist. In this instance, the cessation of targeted therapy would enable the proliferation of sensitive tumor clones resulting in rapid progression (44–46). This has been shown radiologically in imatinib-treated GIST where resistant tumor clones were visualized as new nodules within stable metastases of presumably sensitive cells (47). Data from the phase I trial of vemurafenib support this model in BRAF-mutant metastatic melanoma; a subset of patients continued responding to vemurafenib for over 3 months after local treatment of focal progression (48). Heterogeneity may explain the rapid increase in disease activity after drug discontinuation in the cases reported here. Alternatively, our data on single-cell–derived resistant melanoma cell lines suggest that MAPK inhibitors also retain some antiproliferative activity, which seems sufficient for clinical benefit.

Determination of disease progression by RECIST criteria is frequently used by clinicians to determine change in systemic treatment. Our data support anecdotal reports of tumor flare upon discontinuation of MAPK inhibition and provide experimental evidence for the use of continued MAPK inhibition beyond disease progression. These observations also contain important implications for treatment of and clinical trial design for metastatic melanoma. First, the unregulated and unmeasured continuation of MAPK inhibitors beyond disease progression may have a major impact on measurement of overall survival in certain trials where cessation of drug was not mandated, but permitted for extended periods of perceived clinical benefit. Second, the risk of disease flare on cessation of MAPK inhibitors needs to be considered when deciding on how best to sequence therapies. This is particularly relevant when considering treatment with ipilimumab where almost 50% of the patients treated with ipilimumab after BRAF inhibitor failure had rapid disease progression resulting in death and were unable to complete ipilimumab treatment as per protocol (49). Finally, given the biologic activity of continued MAPK inhibitors, placebo-controlled clinical trials, or trials where the control arm is a relatively ineffective agent such as chemotherapy, may overestimate the benefit of second line therapies. A randomized discontinuation trial comparing continued treatment at the time of progression to discontinuation would test and quantify any clinical benefit of MAPK inhibitor treatment beyond disease progression.

Disclosure of Potential Conflicts of Interest
G.V. Long is a consultant/advisory board member of GSK and Roche. R.F. Kefford is a consultant/advisory board member of GSK, Roche, and Novartis. No potential conflicts of interest were disclosed by the other authors.

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