The potential for BRAF V600 inhibitors in advanced cutaneous melanoma: rationale and latest evidence

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Abstract: Historically, patients with advanced cutaneous melanoma have a poor prognosis and limited treatment options. The discovery of selective v-raf murine sarcoma viral oncogene homolog B1 (BRAF) V600 mutation as an oncogenic mutation in cutaneous melanoma and the importance of the mitogen-activated protein kinase (MAPK) pathway in its tumourigenesis have changed the treatment paradigm for melanoma. Selective BRAF inhibitors and now MEK inhibitors have demonstrated response rates far higher than standard chemotherapeutic options and we review the phase I–III results for these agents in this article. The understanding of mechanisms of resistance that may occur upstream, downstream, at the BRAF level or bypassing the MAPK pathway provides a platform for rational drug development and combination therapies.

Keywords: BRAF inhibitor, BRAF V600E, cutaneous melanoma, ipilimumab, MEK inhibitor, resistance, vemurafenib

Introduction

Malignant melanoma is the sixth most common type of new cancer in the UK and the fifth most common in the USA [National Cancer Institute, 2010]. Although it is the least common skin cancer, cutaneous melanoma is the most life threatening with metastases present in 10–15% of patients at diagnosis [National Cancer Institute - Surveillance Epidemiology and End Results, 2010]. The annual incidence of melanoma is escalating and in the UK the incidence rates have increased more rapidly than any of the top 10 cancers in men and women [Cancer Research UK, 2010]. Patients with advanced or metastatic disease confined to the skin, subcutis and lymph nodes have a median overall survival (OS) of 12 months compared with only 4–6 months for patients with visceral disease [Balch *et al.* 2001]. In this setting, the reported OS rates from studies with various chemotherapeutic agents, including dacarbazine and temozolamide, as well as immune modulators, is between 6 and 10 months [Chapman *et al.* 1999; Eigentler *et al.* 2003; Middleton *et al.* 2000]. The cytotoxic T-lymphocyte antigen 4 (CTLA-4) antibody, ipilimumab, was the first agent to demonstrate a benefit in OS in previously treated metastatic melanoma [Hodi *et al.*

2010] and more recently in the first-line setting in combination with dacarbazine [Robert *et al.* 2011]. In addition, the selective v-raf murine sarcoma viral oncogene homolog B1 (BRAF) inhibitor, vemurafenib (PLX4032), has also demonstrated an OS benefit compared with dacarbazine in the first-line setting [Chapman *et al.* 2011]. Thus, it is the advent of immunotherapy and agents targeting specific genetic aberrations that have significantly improved outcomes in malignant melanoma.

Melanoma: a heterogeneous disease

Although genetic aberrations and abnormal activity in the mitogen-activated protein kinase (MAPK) pathway drive tumourigenesis in the majority of cutaneous melanomas, there is an increasing body of evidence that other pathways and immunological mechanisms contribute to its complexity.

Aberrant activation of the MAPK pathway has been demonstrated in over 80% of cutaneous melanomas due to abnormalities at various levels along the RAS-RAF-MEK-ERK pathway [Platz *et al.* 2008].

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Activation of the Rat Sarcoma (RAS) family GTPases by growth factors or by RAS mutation is the first step driving this pathway. Activated RAS proteins can complex with and activate members of the RAF kinase family (ARAF, BRAF and CRAF), causing subsequent phosphorylation and activation of MEK 1 and MEK2, followed by extracellular signal-regulated kinases (ERK1 and ERK2) [Davies *et al.* 2002]. In turn, this leads to phosphorylation of the erythroblast transformation specific (ETS) protein family, nuclear transcription factor activation, expression of cell-cycle regulators such as cyclin D, finally leading to cell-cycle progression and regulation of cellular differentiation, senescence and apoptosis/survival [Platz *et al.* 2008]. Although activity along this pathway is essential for normal cell function, abnormal activation of the MAPK pathway due to mutations and aberrations at various levels has been implicated in a number of cancer types, not only malignant melanoma but also colorectal cancer and borderline ovarian cancer, among others [Davies *et al.* 2002].

Mutations along the MAPK pathway and other genetic alterations have been documented in varying frequencies in primary and metastatic melanomas based on site, previous sun exposure, skin damage and other factors [Curtin *et al.* 2005, Long *et al.* 2011a].

The BRAF mutation is among the most studied, occurring in 36–59% of primary melanomas and 42–66% of metastatic melanomas [Houben *et al.* 2004; Jakob *et al.* 2011; Long *et al.* 2011a] and has been characterized as an oncogenic mutation [Davies *et al.* 2002.; Karasarides *et al.* 2004]. The BRAF mutation has been demonstrated to occur more frequently in intermittently sun-exposed sites (i.e. trunk) and sites without chronic sun-induced damage and are also present in 10–15% of primary cases of mucosal and acral melanomas [Curtin *et al.* 2005; Long *et al.* 2011a].

In contrast, mutational analyses of melanoma in patients with high sun exposure and chronic sun-induced skin damage more often have high cyclin D1 (CCND1) and cyclin-dependent kinase 4 (CDK4) gene copy numbers. Increased CCND1 copy number has also been demonstrated in acral melanoma (44%), lentigo maligna melanoma (10%) and superficial spreading melanoma (6%) [Sauter *et al.* 2002].

Another frequently occurring mutation is the CKIT mutation, specifically in melanomas of both ultraviolet (UV)-protected sites, acral and mucosal melanomas (in 36% and 39%, respectively), as well as melanoma on chronically sun-damaged skin (28%) [Curtin *et al.* 2006].

Increased copy number of GAB2, a scaffolding protein that mediates interactions with various signalling pathways, including RAS-RAF-MEK-ERK and phosphoinositide 3-kinase (PI3K)-AKT signalling, has also been demonstrated in up to 26% of acral and mucosal melanomas and is mutually exclusive of BRAF, NRAS and KIT mutations [Chernoff *et al.* 2009]. NRAS mutations have been demonstrated in 20–29% of melanomas of all subtypes and are associated with a higher Clark level of invasion and older age compared with BRAF mutation tumours [Curtin *et al.* 2006; Edlundh-Rose *et al.* 2006].

Improved understanding of the genetic heterogeneity in melanoma, the detection of oncogenic mutations and the ability to target these mutations has dramatically expanded the treatment options available for this disease.

Types of BRAF mutations

BRAF is a serine/threonine protein kinase, encoded on chromosome 7q34, that activates the MAPK/ERK signalling pathway. There are now over 100 somatic mutations identified in BRAF [Wellcome Trust Sanger Institute, 2011].

The most common somatic mutation, found in 66–90% of BRAF-mutant melanomas [Cheng *et al.* 2011; Wellcome Trust Sanger Institute, 2011; Long *et al.* 2011a], occurs in the activating segment in exon 15 and involves the substitution of glutamic acid for valine at codon 600 (GTG to GAG, known as V600E) [Davies *et al.* 2002; Platz *et al.* 2008]. This leads to elevated kinase activity compared with BRAF wild type (wt) disease, stimulated phosphorylation of downstream endogenous ERK and subsequent cellular proliferation and survival [Davies *et al.* 2002; Dhomen and Marais, 2009].

A number of other clinically relevant, but less common mutations have also been described, including V600K and V600G/R. The V600K mutation has been reported in 16–30% of patients with BRAF-mutant metastatic melanoma [Cheng

et al. 2011; Long *et al.* 2011; Rubinstein *et al.* 2010; Thomas *et al.* 2007]. It involves two point mutations (GTG to AAG) resulting in a substitution of lysine for valine. The frequency of non-V600E mutations is particularly important when interpreting the clinical trials of the BRAF inhibitors, which vary in the mutation subtypes that are included.

In primary melanomas, BRAF-mutant status has been associated with younger age (age ≤ 40 years), histopathologic subtype (superficial spreading and nodular melanoma), presence of mitoses, single or occult primary melanoma and truncal location [Long *et al.* 2011b]. Recent data in patients with advanced disease demonstrate BRAF mutation rates greater than 80% in those less than 40 years, with V600E more common at younger ages and V600K more common at older ages [Menzies *et al.* 2011]. Other reported associated factors include fewer markers of chronic sun damage in surrounding skin, higher total body nevus counts, early life UV exposure and histopathologic findings, including heavy melanization and prominent upward scatter of melanocytes [Liu *et al.* 2006; Thomas *et al.* 2007; Viros *et al.* 2008].

BRAF mutations are a negative marker for survival with a strong association with inferior OS demonstrated in the metastatic setting [Houben *et al.* 2004; Long *et al.* 2011b; Flaherty, 2011]. It has also been demonstrated that patients with BRAF mutation treated with a BRAF inhibitor have an improved OS compared with those with BRAF wt and BRAF-mutant status not receiving a BRAF inhibitor [Long *et al.* 2011a], findings which have been confirmed in later phase trials.

Early preclinical studies of RAF inhibition demonstrated that specific inhibitory nucleic acids or chemical RAF inhibition in cell lines and xenograft models caused growth arrest and induction of apoptosis [Calipel *et al.* 2003; Hoeflich *et al.* 2006].

The earliest clinical trials of RAF inhibition with metastatic melanoma involved the multiple tyrosine kinase inhibitor sorafenib. Although it was initially developed as a RAF inhibitor, sorafenib has inhibitory effects on vascular endothelial growth factor receptor 2 (VEGFR2), VEGFR3, platelet-derived growth factor receptor β (PDGFR β), cKIT and fms-like tyrosine kinase receptor 3 (FLT3). A randomized phase II study of sorafenib

showed disease stabilization in a few unselected patients with advanced melanoma, but without any impact on survival [Eisen *et al.* 2006]. Another phase II study of sorafenib in combination with dacarbazine in patients with untreated melanoma showed an improvement in median progression-free survival (PFS), but this did not translate into an OS benefit [McDermott *et al.* 2008]. In addition, there was no activity demonstrated in a phase III trial in the second-line setting combining sorafenib with carboplatin and paclitaxel [Hauschild *et al.* 2009].

BRAF inhibitors

The two most advanced agents currently in clinical development are the selective BRAF inhibitors, vemurafenib (PLX4032, RG 7204) and GSK2118436 (GSK436).

Vemurafenib is an orally available, highly potent, ATP-competitive inhibitor of mutant BRAF. The phase I (dose extension phase), II and III trials for this agent included patients with BRAF V600E-mutant metastatic melanoma, confirmed by means of a polymerase chain reaction (PCR) assay (cobas 4800 BRAF V600 Mutation Test, Roche Molecular Systems Inc., Pleasanton, CA, USA). This assay involves hybridizing a probe, specific to the 1799T $\rightarrow$ A substitution that results in the V600E BRAF mutation, with DNA isolated from formalin-fixed, paraffin-embedded tumour tissue and determining the presence or absence of amplification after repeated chain-reaction cycles. Patients with other DNA alterations giving rise to V600E and non-V600E mutations were thus excluded.

The phase I study included a dose-escalation phase (from 160 mg twice daily to 1120 mg twice daily) and dose-extension phase (recommended phase II dose of 960 mg twice daily) and demonstrated a response rate (RR) of 69% (11 of 16 patients) in the dose-escalation phase and 81% (26 of 32 patients) in the dose-extension phase. At the time of publication, the estimated median PFS was more than 7 months with duration of response ranging from 2 months to over 18 months [Flaherty *et al.* 2010].

The subsequent BRAF in Melanoma 2 (BRIM-2) phase II study included 132 patients and showed RR of 53%, stable disease (SD) in a further 29%, median PFS of 6.7 months and OS at 6 and 12 months of 77% and 58%, respectively.

At the time of the report, the median OS had not been reached [Ribas *et al.* 2011].

The recent phase III BRAF Inhibitor in Melanoma 3 trial (BRIM-3) compared vemurafenib (960 mg twice daily) with dacarbazine (1000 mg/m²) as first-line treatment in patients with BRAFV600E-mutant metastatic melanoma [Chapman *et al.* 2011]. The RR was 48% for vemurafenib and 5% for dacarbazine with a significant prolonged median PFS of 5.3 months in the vemurafenib arm compared with 1.6 months on dacarbazine [hazard ratio (HR) 0.26; 95% confidence interval (CI) 0.20–0.33, $p < 0.0001$]. Treatment with vemurafenib resulted in a 63% relative risk reduction for death and a 74% risk reduction for either death or disease progression. At 6 months, the OS was 84% for patients who received vemurafenib compared with 64% for patients who received dacarbazine. The adverse events (AEs) were consistent with those previously described in earlier trials and included grade 2 (G2) and G3 arthralgias (18% and 3%), rash (10% and 8%), photosensitivity (12% G2 or G3), fatigue (11% and 2%), cutaneous squamous-cell carcinoma (SCC, 12%), keratoacanthoma (2% and 6%), nausea (7% and 1%) and diarrhoea (5% and <1%). Dose interruption and modification were required in 38% of patients.

Interestingly, BRIM-3 also included 10 patients with a V600K mutation, 4 of whom demonstrated a good clinical response. Comparison of the PCR assay (cobas 4800 BRAF V600 Mutation Test) and Sanger sequencing has demonstrated higher sensitivity in the detection of V600E mutations with the PCR test; however, 6.8% of samples identified by the PCR assay were shown to have a V600K rather than V600E mutation, confirmed on Sanger sequencing [Bloom *et al.* 2011].

GSK436 is an ATP-competitive, reversible inhibitor of mutant BRAF V600E, as well as V600K and V600G kinases. A phase I–II trial enrolled 61 patients including 52 with BRAF-mutant melanoma [Kefford *et al.* 2010]. There was a high RR with 18 of 30 patients (60%) demonstrating partial response (PR) at first restaging during weeks 8–9. The PFS at the expanded dose of 150 mg twice daily was 8.3 months.

In the dose expansion cohort of 20 patients, 77% had a BRAF V600E mutation and 19% had a V600K mutation [Kefford, 2010]. Patients with a BRAFV600E mutation demonstrated RR of 77%

and the subgroup of patients with a V600K mutation demonstrated a RR of 44% (four of nine).

Overall GSK436 was very well tolerated with side effects similar to those described with vemurafenib, including skin changes (37%, one G3), low-grade cutaneous SCC (two patients), headache (19%, one G3), nausea (18% G1), fatigue (15% G1) and vomiting (13%, four G2). Although the majority of side effects are similar between the two BRAF inhibitors, vemurafenib is associated with photosensitivity in up to 30% of patients (12% G2 or G3) while pyrexia is reported in 15% (2% G3) on GSK436.

Interestingly, in the dose-escalation phase with GSK436, a cohort of 10 patients with previously untreated brain metastases also demonstrated a significant RR to treatment. There was a reduction in size of the brain metastases in 9 of 10 patients, ranging from a 20% to a 100% reduction in brain metastases that were 3–15 mm in size prior to treatment. The reduction in brain metastases correlated with extra-cranial response [Long *et al.* 2010].

Further studies of GSK436 that have completed accrual and may be reported in 2012 include GSK436 *versus* dacarbazine in previously untreated patients with BRAF-mutant advanced or metastatic melanoma, as well as a study of GSK436 in BRAF-mutant metastatic melanoma to the brain [ClinicalTrials.gov Identifier NCT01227889].

Importantly, the response to BRAF inhibition with improvement in symptoms and performance status is usually rapid, occurring within the first 2 weeks, and has shown concordance with fluorodeoxyglucose positron emission tomography (FDG-PET) response [McArthur *et al.* 2010]. Interestingly, heterogeneous FDG-PET response at day 15 of treatment has been demonstrated in up to 26% of patients in a substudy of 23 patients, with significantly shorter time to progression compared with those with a homogeneous FDG-PET response [Carlini *et al.* 2011].

As evidenced with both selective BRAF inhibitors, though less commonly with GSK436 [Kefford *et al.* 2010], the increased incidence of cutaneous SCC generally develops between weeks 2 and 14 and is hypothesized to be due to upstream RAS mutations in pre-existing SCC skin lesions, occurring in approximately 15% of patients. Selective inhibition of downstream BRAF

can lead to CRAF signalling by mutant RAS with subsequent development of SCCs [Arkenau *et al.* 2010]. The majority of these SCCs are keratoacanthoma type, well differentiated with no metastatic potential and can be treated with surgical excision [Flaherty *et al.* 2010].

MEK inhibitors

In addition to inhibiting BRAF signalling with selective BRAF inhibitors, preclinical and clinical evidence also supports the antiproliferative activity of MEK inhibitors in melanoma [Zhang *et al.* 2003].

The earliest MEK inhibitors in preclinical and clinical development were PD98059, UO126 and CI-1040 [Messersmith *et al.* 2006]; however limited clinical activity did not warrant further investigation of these agents.

PD0325901 was a second-generation MEK inhibitor also evaluated in phase I and II trials with some preliminary evidence of response and disease stabilization in patients with metastatic melanoma [Lorusso *et al.* 2005]. Dose-limiting diarrhoea and rash prevented further dose escalation and phase II trials were suspended due to the occurrence of retinal vein thrombosis in several patients.

AZD6244 was another MEK inhibitor assessed in early phase trials. The phase I trial enrolled 57 patients, 20 of whom (35%) had melanoma [Adjei *et al.* 2008]. One patient had an objective response and seven patients achieved disease stabilization after two cycles. A further randomized phase II study comparing AZD6244 and temozolamide demonstrated an RR of 12% (5 of 42 patients) in BRAF-mutant metastatic melanoma [Dummer *et al.* 2008] but no significant difference in the primary endpoint of PFS. Further single-agent clinical trials have not been pursued but phase II combination trials with dacarbazine, docetaxel and temsirolimus are currently underway in BRAF-mutant metastatic melanoma in the first-line setting [ClinicalTrials.gov Identifier NCT00936221, ClinicalTrials.gov Identifier NCT01256359, ClinicalTrials.gov Identifier NCT01166126 respectively].

The phase I–II study of the MEK inhibitor GSK1120212 in patients with advanced BRAF-mutant melanoma showed good tolerability and encouraging RRs. At the recommended phase II dose of 2 mg once daily, RRs were 40% (8 of 20

patients) and a further 18% had SD [Falchook *et al.* 2010].

The most common AEs were an acneiform rash (all grade 85%; $\geq$ G3 2%) usually on the face, torso and arms, diarrhoea (all grade 48%; $\geq$ G3 2%), fatigue (all grade 37%; $\geq$ G3 7%) and nausea (all grade 20%; $\geq$ G3 0). Less common events requiring monitoring in future studies included left ventricular systolic dysfunction (9 of 162 patients), central serous retinopathy (3 of 162 patients, at dose levels higher than 2 mg daily) and retinal vein occlusion (1 of 162 patients at 2 mg daily). Importantly, central serous retinopathy is reversible on drug cessation [Infante *et al.* 2010]. Retinal vein occlusion is not reversible; however, the one patient affected had a significant improvement in vision with intraocular anti-VEGF therapy [Infante *et al.* 2010].

Further single-agent activity is being assessed in an ongoing phase III trial, randomizing patients to GSK1120212 *versus* first or second-line chemotherapy.

Combination therapy with MEK and BRAF inhibitors

There is early clinical evidence that the combination of BRAF and MEK inhibitors shows clinical activity in BRAF V600-mutant melanoma with a lower incidence of rash and BRAF-induced hyperproliferative skin lesions [Infante *et al.* 2011]. The rationale for combining both agents is based on preclinical studies that demonstrate potential reduction in drug resistance as well as decreased incidence of BRAF inhibitor induced hyperproliferative skin changes and SCCs. Preliminary results at doses of GSK436 150 mg twice daily and GSK1120212 2 mg daily (19 patients) showed a complete response (CR) rate of 11%, a total RR (CR + PR) of 74% and clinical benefit rate (CR + PR + SD) of 100%. Compared with single-agent toxicities there was also a lower incidence of rash (all grade 25%; $\geq$ G3 2%) and hyperproliferative skin lesions. Other common G2 toxicities were pyrexia (11%), vomiting (4%) and fatigue (4%). Significantly, only 1 of 109 patients developed cutaneous SCC, supporting the preclinical evidence that the combination leads to reduction of SCCs, potentially by switching off the CRAF-activated pathway through downstream MEK inhibition.

A number of clinical trials assessing the combination of BRAF and MEK inhibitors or the

combination of inhibitors of the MAPK pathway with other agents are underway. The combination of vemurafenib with the MEK inhibitor GDC0973 is being assessed in patients with BRAF V600E-mutant metastatic melanoma whose disease has progressed after treatment with vemurafenib. Vemurafenib is also being studied in combination with ipilimumab.

More general studies that may show relevance in the setting of melanoma include studies of MEK inhibitors (e.g. GSK1120212 or GDC0973) and PI3K inhibitors (e.g. BKM120 or GDC0941) in advanced solid tumours. Other novel agents in development have dual BRAF and CRAF activity and include PLX4720 and RAF265.

Resistance to BRAF inhibitors

Recent studies investigating primary and secondary resistance to BRAF inhibition have identified both upstream, downstream and bypass mechanisms that act in an ERK-dependent or ERK-independent manner [Corcoran *et al.* 2011], as illustrated in Table 1. Primary resistance, when patients have progressive disease as their best response to treatment, is present in less than 15% of patients treated with BRAF or MEK inhibitors for BRAF V600-mutant metastatic melanoma [Chapman *et al.* 2011; Flaherty *et al.* 2010; Kefford *et al.* 2010; Ribas *et al.* 2011]. However, secondary or acquired resistance develops in the majority of patients after an initial response, with the duration of this response ranging between 2 months and 1.8 years with a median of 8–9 months [Flaherty *et al.* 2010].

As part of the phase I vemurafenib trial, the effect on key signalling pathways and mechanisms of acquired resistance were investigated [McArthur *et al.* 2011]. Formalin-fixed paraffin-embedded samples were obtained from a total of 23 patients (5 patients from the dose-escalation cohort and 18 patients from the melanoma expansion cohort) at three different time points; at baseline, at day 15 of treatment and at disease progression while still receiving vemurafenib. Immunohistochemical (IHC) staining for a number of different molecular markers was performed as well as Sequenom MassARRAY of over 400 mutations in genes, including BRAF, RAS, PIK3CA, AKT1/2 and CDK4. At day 15, phosphorylated ERK (pERK) and phosphorylated MEK (pMEK) were markedly reduced, consistent with decreased signalling downstream and correlating with tumour

response. Cyclin D1 and Ki67 staining on IHC were also decreased, consistent with reduced proliferation. There were no significant effects on phosphorylation of AKT or on phosphatase and tensin homolog (PTEN) expression. Interestingly, on progression, some but not all tumours were found to have a return of ERK and MEK phosphorylation, indicating the presence of both ERK-dependent and ERK-independent mechanisms of resistance. Sequenom analysis demonstrated the presence of NRAS G12R mutation in 1 of 11 patients and MEK P124S mutation in 1 of 28 patients with resistant tumours. One patient was shown to have PTEN loss and an increase in phosphorylated AKT (pAKT) at progression, indicating the PI3K-AKT-mammalian target of rapamycin (mTOR) pathway may act as a bypass signalling pathway. Sequenom analysis also confirmed BRAF V600E mutation was present in all progression samples (10/10).

An exploratory tumour gene analysis of patients treated in the GSK436 phase I–II trial also demonstrated several potential mechanisms of resistance, including alterations in PTEN and mutations in CDK4 and β -catenin [Nathanson *et al.* 2011]. A MEK2 mutation was demonstrated in a tumour sample of one patient with SD. Pretreatment analysis demonstrated that abnormal PTEN (deleted or mutant) was associated with shorter PFS. Analysis of array-based comparative genomic hybridization also demonstrated that patients with CDKN2A or KIT deletion had a shorter PFS.

The results of the above studies suggest mechanisms of resistance that may occur upstream (NRAS mutation) or downstream of BRAF along the MAPK pathway (MEK mutation), as well as alternative bypass signalling pathways through the PI3K-AKT pathway and through abnormalities in CDK4/cyclin D1. Further resistance mechanisms have also been investigated in melanoma cell lines, tumour models and through individual case reports [Corcoran *et al.* 2010; Emery *et al.* 2009; Johannessen *et al.* 2010; Little *et al.* 2011; Montagut *et al.* 2008; Nazarian *et al.* 2010; Wagle *et al.* 2011]. The majority of these data focus on mechanisms of acquired resistance, though some overlap with primary resistance exists.

These substudies also demonstrate the necessity of longitudinal tumour biopsies to fully characterize the activity of targeted agents, investigate

Table 1. Possible mechanisms of resistance to selective BRAF inhibitors and potential therapeutic combinations.

Level of resistance	Genetic mutation/alteration	Status of ERK pathway	Potential combination therapy	Reference
Upstream in the MAPK pathway	NRAS mutation (e.g. G12R, Q61K)	Dependent	BRAF + MEKi	[Adjei <i>et al.</i> 2008; McArthur <i>et al.</i> 2011; Nazarian <i>et al.</i> 2010]
	PDGFR β upregulation	Independent	PDGFR β inhibitor $\pm$ BRAFi BRAF + IGF1Ri	[Corcoran <i>et al.</i> 2011; Nazarian <i>et al.</i> 2010]
At the RAF level	Other RTK upregulation (e.g. IGF1R signalling)	Independent	BRAF + PI3K/AKT/dual mTORi	[Corcoran <i>et al.</i> 2011; Villanueva <i>et al.</i> 2010]
	BRAF amplification	Dependent	BRAF + MEKi	[Corcoran <i>et al.</i> 2010; Little <i>et al.</i> 2011]
	Elevated CRAF activity	Dependent	BRAF + MEKi or CRAF/nonselective RAFi	[Johannessen <i>et al.</i> 2010]
	Increased COT/Tip2 levels (also acts downstream in the MAPK pathway)	Dependent	BRAF + MEKi	[Montagut <i>et al.</i> 2008; Johannessen <i>et al.</i> 2010]
Downstream in the MAPK pathway	MEK1 mutation (e.g. P124L, P124S, C121S)	Dependent	BRAF and MEKi for P124L mt (not C121S)	[Corcoran <i>et al.</i> 2011; Emery <i>et al.</i> 2009; McArthur <i>et al.</i> 2011; Wagle <i>et al.</i> 2011]
Bypass pathway signalling	PTEN loss	Independent	BRAF/MEKi + PI3K/mTORi	[Nathanson <i>et al.</i> 2011; Paraiso <i>et al.</i> 2011]
	PI3K-AKT pathway activation	Independent	BRAF/MEKi + PI3K/AKT/mTORi BRAF/MEKi + PI3K/AKTi	
	AKT3 mutation	Independent	BRAF + IGF1Ri or PI3K/AKTi	[Jiang <i>et al.</i> 2011]
	IGF1R signalling	Independent	BRAF + mTORi	[Shao and Aplin, 2010]
	CDK4 mutation/cyclin D1 amplification	Independent	BRAF + mTORi	[Villanueva <i>et al.</i> 2010]
	Co-overexpression of KIT/CDK4	Independent	BRAF/MEKi + imatinib	[Smalley <i>et al.</i> 2008b, 2008a]
BRAF ⁱ , selective v-raf murine sarcoma viral oncogene homolog B1 inhibitor; CDK4, cyclin-dependent kinase 4; ERK, extracellular signal-regulated kinase; IGF1R, insulin-like growth factor 1 receptor; MAPK, mitogen-activated protein kinase; MEKi, MEK inhibitor; mTORi, mammalian target of rapamycin inhibitor; PDGFR β , platelet-derived growth factor receptor β ; PI3K, phosphoinositide 3-kinase; PTEN, phosphatase and tensin homolog; RTK, receptor tyrosine kinase.				

resistance mechanisms and formulate rational drug combinations for future studies.

Mechanisms of resistance at the BRAF level

Secondary mutations in BRAF, as well as BRAF amplification have been surmised as possible primary or secondary resistance mechanisms, as has been demonstrated with secondary EGFR mutations in non-small cell lung cancer. There is evidence in mouse models regarding secondary BRAF mutations [Whittaker *et al.* 2010] and in BRAF-mutant colorectal cancer cell lines regarding BRAF amplification [Corcoran *et al.* 2010; Little *et al.* 2011] that suggests these mechanisms may play a role in resistance to BRAF or MEK inhibition. To date, however, there is limited clinical evidence from patients' tumour biopsies to support these preclinical findings.

Elevated activity of the alternative RAF isoform, CRAF, has also been identified in preclinical studies as a possible mechanism of both primary and secondary resistance through reactivation of the MAPK pathway [Johannessen *et al.* 2010; Montagut *et al.* 2008]. These findings require further validation in prospective analysis of tumour tissue and biopsies on progression but suggest that there may be therapeutic potential in combining a nonselective RAF inhibitor or selective CRAF inhibitor with a MEK or BRAF inhibitor.

Another resistance mechanism that acts both at the level of BRAF and downstream in the MAPK pathway is mediated by COT/Tpl2. COT/Tpl2, encoded by MAP3K8, activates ERK and the MAPK pathway primarily through MEK-dependent mechanisms but is RAF independent [Johannessen *et al.* 2010]. There is both preclinical and clinical evidence [Johannessen *et al.* 2010] demonstrating elevated COT/Tpl2 levels in resistant cell lines and biopsy specimens. It is hypothesized that high COT levels cause MEK hyperactivation, thus mediating resistance to both BRAF and MEK inhibition. Consistent with this hypothesis, combined BRAF and MEK inhibition has been demonstrated to overcome COT-induced resistance in experimental models [Johannessen *et al.* 2010].

Mechanisms of resistance downstream of BRAF in the MAPK pathway

Current preclinical and clinical evidence demonstrates that downstream resistance is primarily

mediated through mutations in MEK [Corcoran *et al.* 2011; Emery *et al.* 2009; Wagle *et al.* 2011]. Point mutations in MEK1 have been shown to cause secondary resistance to both BRAF inhibition and MEK inhibition in the clinical setting [Emery *et al.* 2009; Wagle *et al.* 2011]. A MEK point mutation has been implicated in acquired resistance to MEK inhibition [Emery *et al.* 2009] while another MEK1 mutation, P124L, led to both resistance to MEK inhibition and cross resistance to BRAF inhibition [Emery *et al.* 2009]. In addition, the point mutation C121S has been demonstrated in a post-relapse biopsy in a patient on the BRAF inhibitor vemurafenib [Wagle *et al.* 2011] and further analysis demonstrated it could confer resistance to both RAF and MEK inhibition. Interestingly, the combination of BRAF and MEK inhibition was able to overcome resistance conferred by the MEK1 P124L mutation in BRAF-mutant melanoma cells [Emery *et al.* 2009].

The significance of MEK2 mutations in mediating sensitivity or resistance is currently unknown.

Mechanisms of resistance upstream of BRAF in the MAPK pathway

NRAS mutations have been demonstrated *in vitro* and *in vivo* to cause resistance to BRAF inhibition [Nazarian *et al.* 2010]. NRAS Q61K mutation was demonstrated in preclinical cell line models to confer resistance to vemurafenib, supported by its isolation in a nodal biopsy from a patient whose disease had progressed after an initial response to treatment. The NRAS mutation is thought to signal through RAF isoforms other than BRAF, leading to persistently elevated pMEK and pERK levels despite BRAF inhibition [Nazarian *et al.* 2010]. Interestingly, these cell lines may retain sensitivity to MEK inhibition [Adjei *et al.* 2008].

PDGFR β upregulation, insulin-like growth factor 1 receptor (IGF1R) activation and signalling through other receptor tyrosine kinase (RTK) pathways have also been investigated as ERK-independent mechanisms of resistance, acting upstream of BRAF in the MAPK pathway and mediating alternative pathways that bypass MAPK [Corcoran *et al.* 2011; Nazarian *et al.* 2010].

Nazarian and colleagues [Nazarian *et al.* 2010] demonstrated that overexpression of PDGFR β resulted in acquired resistance to vemurafenib in BRAF-mutant melanoma cell lines despite persistent pERK suppression, consistent with

ERK independence. Additionally, 4 of 11 clinical postrelapse biopsies from patients with melanoma treated with vemurafenib showed increased PDGFR β expression relative to pretreatment biopsies.

Mechanisms bypassing BRAF signalling

Signalling via the PI3K-AKT-mTOR pathway has been described as an escape pathway in various tumour models [Gopal *et al.* 2010; Shao and Aplin, 2010; Villanueva *et al.* 2010].

IGF1R signalling has been demonstrated as an ERK-independent mechanism of resistance and also plays a critical role in the PI3K-AKT-mTOR pathway.

In preclinical models, Villanueva and colleagues [Villanueva *et al.* 2010] identified IGF1R activation with enhanced IGF1R/PI3K signalling as a key RTK-driven acquired mechanism of resistance that could be overcome by combined IGF1R/PI3K and MEK inhibition. A number of other preclinical studies have also demonstrated aberrant activation in the PI3K-AKT pathway, evidenced by elevated pAkt, which contributes to both primary and secondary resistance and may be overcome through combining RAF and PI3K-AKT inhibition [Jiang *et al.* 2011; Shao and Aplin, 2010]. PTEN loss and subsequent lack of inhibition on the PI3K-AKT-mTOR pathway has also been demonstrated to confer acquired resistance to BRAF inhibition in preclinical models [Paraiso *et al.* 2011] and could be reversed with dual treatment with PLX4720 and a PI3K inhibitor [Paraiso *et al.* 2011].

Signalling via the PI3K-AKT-mTOR pathway mediates an important MAPK-independent mechanism of resistance and demonstrates a complex crosstalk between these pathways [Corcoran *et al.* 2011]. Measurement of pERK and pAKT to determine pathway activity may therefore help to guide therapeutic choices and combinations of selective BRAF, MEK or PI3K/AKT inhibitors, but this data require further clinical confirmation.

Finally, dysregulation of CDK4 or cyclin D1 has also been implicated in BRAF inhibitor resistance. In preclinical models, cyclin D1 overexpression demonstrated increased resistance, particularly in the presence of CDK4 mutation/overexpression [Smalley *et al.* 2008b]. Smalley and colleagues

[Smalley *et al.* 2008a] also demonstrated that co-overexpression of KIT/CDK4 also led to decreased sensitivity to BRAF inhibition in preclinical models but may increase sensitivity to imatinib. Although, the existence of this KIT/CDK4 subgroup has been confirmed in human melanoma samples, the clinical significance of these preclinical findings requires further investigation.

Future strategies

Increased understanding of the molecular pathways and aberrations in advanced and metastatic cutaneous melanoma has changed the treatment paradigm for a large subset of patients with melanoma. However, there remain several challenges. This review outlines a subset of possible strategies to overcome resistance mechanisms that inevitably develop to BRAF and MEK inhibition. Further challenges include combining immune therapy with inhibitors of the MAPK pathway, as well as treating those patients who do not have a BRAF mutation.

Intensive study continues into the complex resistance mechanisms of BRAF inhibition. Whether by inhibiting both the MAPK and bypass pathways, such as with BRAF-MEK and PI3K-AKT-mTOR inhibition, or by targeting IGF1R or other RTKs, a combination of these agents may be effective for overcoming secondary resistance to BRAF inhibition and may be utilized in an earlier setting to prevent or delay resistance [Corcoran *et al.* 2011]. Importantly, obtaining biopsies longitudinally during a patient's treatment course is key to this process. The combination of molecular characterization in the preclinical and clinical setting can further enhance identification of clinically relevant mutations and possible treatment options [Wagle *et al.* 2011].

Improved understanding of the effect of BRAF inhibition on immune response and T-cell function will also provide a rationale for the combination of these approaches. Treatment with selective BRAF inhibitors has been shown to preserve T-cell function whereas treatment with MEK inhibitors may impair T-cell function [Boni *et al.* 2010]. Improved recognition by antigen-specific T cells and increased intratumoural and peritumoural lymphocytes occurs early after exposure to BRAF inhibition. Conversely, patients who develop resistance to BRAF inhibition demonstrate a decrease in intratumoural and peritumoural lymphocytes [Boni *et al.* 2010; Long *et al.*

2011b]. Together, these early data provide a rationale for the combination of ipilimumab and BRAF inhibition.

Understanding the oncogenic drivers of melanoma, the complex mechanisms of resistance, the interaction between signalling pathways and the immune system and integration of new genomic technologies has increased exponentially over the last few years and provides a rationale for the current combination studies, with further clinical correlation required to strengthen the rationale for future studies.

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Conflict of interest statement

The authors declare that there is no conflict of interest.

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