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Review Article

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Review on Development of BRAF Inhibitors as Promising Targets in Cancer Therapy

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Abstract The identification of mutationally activated BRAF in many cancers altered our conception of the part played by the RAF family of protein kinases in oncogenesis. In this Review, we describe the development of BRAF inhibitors and the results that have emerged from their analysis in both the laboratory and the clinic. We discuss the spectrum of RAF mutations in human cancer and the complex interplay between the tissue of origin and the response to RAF inhibition. Finally, we enumerate mechanisms of resistance to BRAF inhibition that have been characterized and postulate how strategies of RAF pathway inhibition may be extended in scope to benefit not only the thousands of patients who are diagnosed annually with *BRAF*-mutated metastatic melanoma but also the larger patient population with malignancies harbouring mutationally activated RAF genes that are ineffectively treated with the current generation of BRAF kinase inhibitors.
Growth factors and mitogens use the Ras/Raf/MEK/ERK signaling cascade to transmit signals from their receptors

Keywords BRAF Inhibitors, Cancer Therapy, Ras/Raf/MEK/ERK

Introduction

Overview of the Ras/Raf/MEK/ERK signalling. The Ras/Raf/MEK/ERK cascade couples signals from cell surface receptors to transcription factors, which regulate gene expression. Furthermore, this cascade also regulates the activity of many proteins involved in apoptosis. This pathway is often activated in certain tumors by chromosomal translocations such as BCR-ABL, mutations in cytokine receptors such as Flt-3, Kit, Fms or overexpression of wild type or mutated receptors, e.g., EGFR. The Raf/MEK/ERK pathway also has profound effects on the regulation of apoptosis by the post-translational phosphorylation of apoptotic regulatory molecules including Bad, Bim, Mcl-1, caspase 9 and more controversially Bcl-2. This pathway has diverse effects which can regulate cell cycle progression, apoptosis or differentiation [1]. A survey of the literature documents the daily increase in the complexity of this pathway, as there are multiple members of the kinase, transcription factor, apoptotic regulator and caspase executioner families, which can be activated or inactivated by protein phosphorylation. Furthermore, this pathway can induce the transcription of certain genes. Raf, either through downstream MEK and ERK, or independently of MEK and ERK, can induce the phosphorylation of proteins, which control apoptosis. Additional signal transduction pathways interact with the Raf/MEK/ERK pathway to positively or negatively regulate its

activity. Abnormal activation of this pathway occurs in human cancer due to mutations at upstream membrane receptors, Ras and B-Raf as well as genes in other pathways (e.g., PI3K, PTEN, Akt), which serve to regulate Raf activity. The Raf/MEK/ERK pathway also influences chemotherapeutic drug resistance as ectopic activation of Raf induces resistance to doxorubicin and paclitaxel in breast cancer cells. Mutations at B-Raf have been frequently detected in some malignancies including melanoma and thyroid cancers [2]. For all the above reasons, the Raf/MEK/ERK pathway is an important pathway to target for therapeutic intervention. Inhibitors of Ras, Raf, and MEK and some downstream targets have been developed and many are currently in clinical trials. Naturally, some inhibitors are better than others and certain "specific" inhibitors may inhibit multiple kinases.

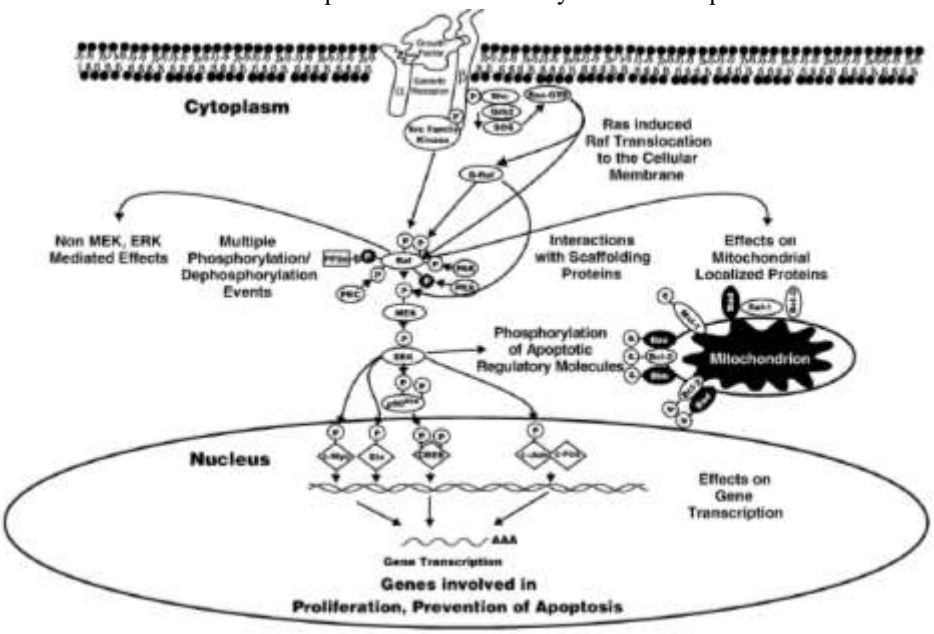

Figure 1: Overview of Raf/MEK/ERK Pathway. The Raf/MEK/ERK pathway is regulated by Ras as well as various kinases, which serve to phosphorylate S/T and Y residues on Raf. Some of these phosphorylation events serve to enhance Raf activity (shown by a black P in a white circle) whereas others serve to inhibit Raf activity (shown by a white P in a black circle. Moreover, there are phosphatases such as PP2A, which remove phosphates on certain regulatory residues. The downstream transcription factors regulated by this pathway are indicated in diamond shaped outlines.

RAF kinases have been associated with cancer since their discovery in 1983, when Ulf Rapp and colleagues first described *v-Raf*, a murine retroviral oncogene with a mammalian cell homologue, termed *CRAF* (also known as *RAF1*). Contemporaneously, Bister and colleagues characterized *v-mil*, an avian retroviral oncogene that is orthologous to *v-Raf*. In 1984, both v-RAF and v-MIL oncoproteins were shown to have serine/threonine kinase activity — they were the first oncoproteins identified to have such activity [3]. Two genes that are related to *CRAF* were subsequently found in mice and humans: *ARAF* and *BRAF* [4,5]. Furthermore, homologues of *BRAF* were identified in *Drosophila melanogaster* (*D-Raf*) and in *Caenorhabditis elegans* (*lin-45*), which function during development downstream of receptor tyrosine kinase (RTK) signalling [6,7]. Ten years after the identification of *CRAF* [8–10], the dual-specificity protein kinase MEK1 was identified as a physiological substrate of CRAF [11]. Concurrently, several groups identified a direct interaction between RAF proteins and GTP-bound RAS proteins, implicating RAF proteins as direct effectors of activated RAS [12,13]. Interaction with RAS–GTP at membranes promotes RAF kinase activation that, in turn, leads to direct RAF-mediated activating phosphorylation of MEK1 and MEK2. In turn, MEK1 and MEK2 activate the ERK1 and ERK2 MAP kinases via phosphorylation. Thus, RAF proteins are crucial regulators of the ERK MAP kinase signalling cascade, relaying signalling cues from the extracellular environment throughout the cell, thereby directing cell proliferation, differentiation, migration and survival.

In 2002, sequencing efforts identified a high frequency of *BRAF* point mutations in melanoma and in other human cancers [14]. The ensuing decade witnessed myriad publications that further characterized the roles of mutant BRAF in numerous solid tumours and haematological malignancies. Furthermore, it has become evident that mutations in *CRAF* and *ARAF* also occur in cancer, thereby implicating the RAF family protein kinases both as drivers of oncogenesis and as direct targets for therapeutic intervention. Discovery of the *BRAF* oncogenes prompted several structure-based drug design campaigns that have yielded several highly potent and selective ATP-competitive small molecule BRAF inhibitors. Two compounds (vemurafenib and dabrafenib) have achieved approval by the US Food and Drug Administration (FDA) for the treatment of metastatic and unresectable *BRAF*-mutated melanomas. Initially, the success of BRAF inhibitors seemed to unequivocally reinforce the paradigm of using predictive markers to molecularly stratify patients in clinical trials testing pathway-targeted therapeutics. However, it has since become apparent that *BRAF* mutational status alone does not predict therapeutic response in all cancers.

Key points

• Mutationally activated BRAF is expressed in melanoma, glioblastoma, thyroid, lung and colon cancers and in a subset of haematological malignancies.

• The most common *BRAF* mutation leads to the substitution of a glutamic acid for valine at amino acid 600 (V600E) in the kinase domain of the protein. This substitution mimics phosphorylation of the activation loop, thereby inducing constitutive BRAF protein kinase activity.

• Point mutations in the related *ARAF* and *CRAF* protein kinases, although very rare, have been reported as oncogenic drivers in some human cancers. In addition to point mutation, gene fusion events are reported to activate BRAF and CRAF.

• Numerous non-V600E alterations in BRAF have been reported in cancer and in a rare developmental disorder. Many of these promote kinase activity by relieving autoinhibitory mechanisms or promote activation of other RAF isoforms in a RAS-dependent manner.

• ATP-competitive BRAF kinase inhibitors are currently under investigation for the treatment of BRAF-mutated cancers. However, to date, efficacy is limited to a subset of melanomas owing to primary or adaptive resistance mechanisms in colorectal and thyroid cancers that reactivate signalling downstream of receptor tyrosine kinases.

• In clinical trials of BRAF-mutated melanoma, BRAF inhibitors tend to induce high rates of response that show transient durability due to the onset of drug-resistant disease.

• Acquired resistance to BRAF-V600E inhibitors is strikingly complex but frequently involves reactivation of MEK–ERK MAP kinase signalling. Drug resistance due to overexpression of oncogenic BRAF-V600E leads to ‘oncogene overdose’ following cessation of drug administration — a phenomenon that could be clinically exploitable to forestall the onset of drug resistance.

• The efficacy of RAF inhibitors in tumours with other RAF mutations is mostly unknown, although preclinical studies indicate varied responses that are inhibitor-specific and depend on the biochemical mechanism of oncogene activation.

Efficacy of BRAF inhibitors is limited to a subset of cancer patients with *BRAF*-mutated metastatic melanoma, despite the abundance of *BRAF*-mutated tumours identified in colorectal cancers, thyroid cancers, glioblastoma and non-small-cell lung cancers (NSCLCs), as well as the minority of *ARAF* and *CRAF* mutations that have been found in lung adenocarcinoma. Furthermore, the durability of responses in *BRAF*-mutated melanoma is restricted by the onset of drug resistance. Although the era of RAF-targeted therapeutics remains in its infancy, the challenge in the coming years lies in determining how to use RAF inhibitors across multiple tumour types to achieve the greatest immediate clinical benefit, while simultaneously forestalling the emergence of drug-resistant disease.

RAF mutations in cancer

The spectrum of BRAF mutations

The identification of *BRAF* mutations in cancer ushered in a new era in the treatment of advanced melanomas. *BRAF* is mutated in ~8% of all cancers, and approximately one-half of all melanomas harbour a *BRAFT1799A*

transversion, which encodes the constitutively active BRAF-V600E oncoprotein. In the original description of *BRAF* mutations in cancer, *BRAFT1799A* was only one of 14 *BRAF* alterations identified in cell lines and primary tumour samples14. Since then, nearly 300 [15] distinct missense mutations have been found in tumour samples and cancer cell lines. These missense mutations encompass 115 of the 766 BRAF amino acids, but most of the mutations occur in the activation loop (A-loop) near V600, or in the GSGSFG phosphate-binding loop (P-loop) at residues 464–469 [16]. Crystallographic analysis revealed that the inactive conformation of BRAF is stabilized by interactions between the A-loop and P-loop of the BRAF kinase domain, specifically involving V600 interacting with F468 [17]. Under normal circumstances, reversible phosphorylation of T599 and S602 in the A-loop regulates the A-loop–P-loop interaction, thereby allowing BRAF to convert back and forth from its kinase-active state to its kinase-inactive state. Consequently, *BRAF* mutations that lead to amino acid substitutions in either the A-loop or the P-loop mimic T599 and S602 phosphorylation and, by disrupting the A-loop–P-loop interaction, irreversibly shift the equilibrium of BRAF to the kinase-active conformation.

BRAFT1799 point mutations are clearly the most common oncogenic driver in melanoma, but BRAF-V600E melanoma represents only a subset of tumours with BRAF alterations. *BRAF* point mutations also occur in 60% of thyroid cancers, 10% of colorectal carcinomas and in 6% of lung cancers, as well as in nearly all cases of papillary craniopharyngioma [18], classical hairy-cell leukaemia (HCL-C) [19,20] and metanephric kidney adenoma [21]. Unlike other indications where V600 mutations predominate, BRAF alterations in lung cancer often occur in the P-loop at G466 and G469. Although the frequencies of *BRAF* mutation in colon cancer and lung cancer are considerably lower than in the other types of cancer in which BRAF mutations have been found, the relative morbidity for these indications (50,000 and 158,000 deaths per year, respectively, in the United States) [22] suggest that an even larger population of patients with BRAF-mutated cancers are currently ineffectively treated. If 10% of patients with colorectal cancer and 6% of patients with lung cancer carry *BRAF* mutations, that amounts to nearly 16,000 deaths annually owing to *BRAF*-mutated cancers.

The biochemistry of the various altered BRAF proteins varies substantially. Although the V600E alteration markedly increases kinase activity, several of the less common alterations decrease BRAF kinase activity, but they promote MEK phosphorylation in a CRAF-dependent manner [23]. In one genetically engineered mouse model (GEMM), conditional melanocyte-specific expression of either KRAS-G12D or BRAF-D594A (a kinase-impaired A-loop mutation) was insufficient to induce nevi or melanomas, but co-expression of both mutant proteins promoted cellular dimerization of the catalytically inert BRAF-D594A with catalytically competent CRAF and elicited rapidly growing, pigmented tumours [24]. These data strongly indicate that kinase-impaired BRAF mutations are oncogenic drivers but require RAS and a catalytically competent RAF isoform to activate downstream signalling. Additionally, the P-loop contains an autophosphorylation site that markedly reduces the activity of the wild-type enzyme [25]. At least some of the BRAF-mutant proteins seem to bypass this effect, which may explain why even mutations that impair intrinsic kinase activity can constitutively activate the enzyme by preventing autoinhibition. Therefore, the various BRAF P-loop and A-loop mutations may have different mechanisms of activation, relying partly on constitutive kinase activity or functioning entirely as a scaffold for RAS signal transduction through CRAF.

Although the tumour-initiating potential of many of the rare BRAF mutations has yet to be demonstrated *in vivo*, the oncogenicity of BRAF-V600E has been validated in numerous GEMMs. To study BRAF-V600E-driven tumorigenesis, a mouse carrying a Cre recombinase-activated allele of *Braf* (*BrafCA*) was developed such that normal BRAF is expressed prior to Cre-mediated recombination and mutant.

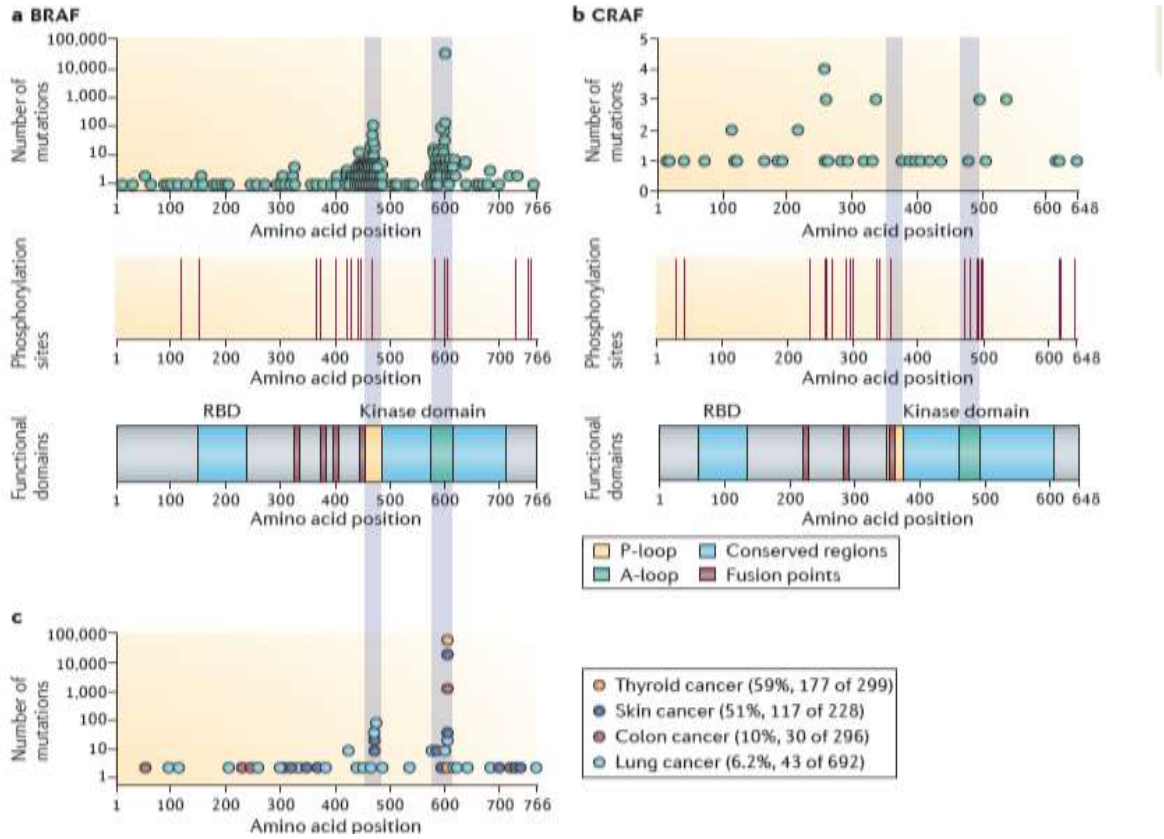

*Figure 2: BRAF and CRAF mutations in cancer. BRAF amino acid positions (1–766) (part **a**) and CRAF amino acid positions (1–648) (part **b**) are depicted on the x-axis. In both part **a** and part **b**, the top graphs show the number of mutations that are reported for each position; the middle panels show the position of putative phosphorylation sites that are reported to have a functional consequence on kinase activity, stability or localization; and the bottom panels show BRAF and CRAF functional domains: the RAS-binding domain (RBD) and the kinase domain are highlighted in blue, the phosphate-binding loop (P-loop) is highlighted in yellow, the activation loop (A-loop) is highlighted in green and fusion points are highlighted in magenta. Part **c** shows the spectrum of BRAF mutations compiled from multiple studies in thyroid, skin, colon and lung cancers (no equivalent graph is shown for CRAF mutations owing to their low frequency across all cancers). The sample size of the compiled sequencing data and the percentage of BRAF mutations for each indication are also shown.*

BRAF-V600E is only expressed from the endogenous locus after exposure to Cre [26]. Using this model, lung-specific expression of BRAF-V600E caused lung adenomas, whereas concomitant BRAF-V600E expression and homozygous excision of floxed *Trp53* alleles caused progression to adenocarcinoma. Expression of BRAF-V600E in melanocyte lineage also cooperated with loss of tumour suppressors, *Pten* or *p16INK4A* (also known as *Cdkn2a*), to yield melanoma, with metastatic potential in *BrafT1799A*; *Pten*–/– melanocytes [27,28]. Targeted expression of *BrafT1799A* in thyrocytes produced papillary or anaplastic thyroid cancers [29–32]. Expression of BRAF-V600E in the proliferative cells of the mouse gastrointestinal tract has been shown to function as an early driver mutation in the pathogenesis of serrated colorectal cancer [33,34]. In addition, recent years have heralded the publication of additional BRAF-V600E-driven GEMMs of pancreatic ductal adenocarcinoma [35], prostate cancer [36], pediatric malignant astrocytoma [37], soft tissue sarcoma [38] and neoplasms of the monocyte–histiocyte lineage [39]. Together, these models of BRAF-V600E-driven malignancies highlight the oncogenicity of the mutant protein in a wide array of tissue types and emphasize the therapeutic potential of targeting BRAF oncoproteins.

CRAF and ARAF mutations

In contrast to *BRAF*, *ARAF* and *CRAF* mutations are exceptionally rare in cancer. Recent data indicate that a small subset (~1%) of patients with lung adenocarcinoma carry activating *ARAF* or *CRAF* mutations. It has not yet been determined whether all ARAF and CRAF mutations constitute oncogenic drivers in all cases, but initial cell culture studies confirmed the transforming potential of ARAF-S124C, CRAF-S257L and CRAF-S259A, as well as the sensitivity of these mutants to RAF inhibition [40].

Although somatic *CRAF* point mutations are rare in human cancers, several germline *CRAF* mutations are the cause of Noonan syndrome (germline mutations in seven other MAP kinase pathway genes are also reported to cause Noonan syndrome; thus, the disorder is described as a 'RASopathy' [41,42]. Noonan syndrome is an autosomal dominant genetic disorder that is characterized by craniofacial deformations, short stature, cardiac anomalies and a propensity for neurocognitive delay [43]. One-third of patients with Noonan syndrome who have *CRAF* mutations also display multiple nevi, lentigines or café au lait spots, suggesting that germline *CRAF* mutations may predispose patients to cutaneous hyperpigmented lesions, similar to the ability of *BRAFT1799A* to elicit benign nevi27, [44]. However, most of the *CRAF* point mutations identified in Noonan syndrome have not been found in human cancers, and fewer than 2% of human cancer-derived cell lines harbour mutated *CRAF* [45]. The oncogenic impotence of CRAF, compared to the potent oncogenicity of BRAF, is thought to arise because CRAF has low basal kinase activity compared with that of BRAF [46]. Importantly, one Noonan syndrome-associated mutation, CRAF-S259F, has also been identified in lung cancer. Additionally, CRAF-S259 mutations have been identified in patients with either colon (S259P) or ovarian (S259A) cancer. Phosphorylation of CRAF-S259 is associated with reduced activity and promotes direct binding to 14-3-3 proteins, which stabilize the inactive state [47].

Thus, dephosphorylation or mutation of S259 disrupts the stability of the inactive CRAF conformation and facilitates binding to RAS–GTP at the plasma membrane [48,49]. The Melan-a cell line — a non-transformed mouse melanocyte-derived cell line that is sensitive to BRAF-V600E-induced transformation — is also transformed by the expression of CRAF-S259A [50]. In contrast to the scarcity of *CRAF* point mutations identified in cancer, increased *CRAF* expression has been identified in several malignancies, the most notable being bladder cancer [51]. The oncogenic importance and therapeutic implications of *CRAF* amplifications remain unclear.

RAF fusion proteins as oncogenic drivers.

Analyses of prostate cancer and pediatric astrocytoma or glioma have revealed that point mutation is not the only mechanism that can reveal the oncogenic potential of RAF protein kinases. The presence of the Philadelphia chromosome in chronic myelogenous leukemia is the archetypal example of an oncogenic protein kinase (BCR–ABL) that is generated via a chromosome translocation [52]. Similarly, RAF fusion transcripts have been identified in melanoma and gliomas, as well as in prostate, gastric, thyroid and breast cancers [53]. Early studies demonstrated the constitutive activity and transforming potential of RAF proteins with amino terminus truncations [54]. In every case in which chromosomal break-points have been identified within the *BRAF* locus in human cancer, the carboxy-terminal portion of the enzyme is fused in-frame using the start codon of another gene, resulting in a RAF kinase fusion protein that lacks the N-terminal domain. Several 5′ fusion partners have been identified for *BRAF*, including angiotensin II receptor-associated protein (*AGTRAP*) and solute carrier family 45, member 3 (*SLC45A3*) in prostate cancer; A-kinase anchor protein 9 (*AKAP9*) in thyroid cancer; and MARVEL domain containing 1 (*MARVELD1*), acylglycerol kinase (*AGK*) [55] and HSPB associated protein 1 (*HSPBAP1*; also known as *PASS1*) in melanoma. *CRAF* fusions with epithelial splicing regulatory protein 1 (*ESRP1*) have also been described in prostate cancers [56], although most RAF kinase fusion events seem to occur in pediatric gliomas. Despite the low frequency of *BRAF* point mutations in low-grade paediatric astrocytomas (~1%), *BRAF* gene fusions are observed in the majority (~70%) of such cases. To date, the *KIAA1549–BRAF* fusion is the most commonly observed, whereas *FAM131B–BRAF*55 and SLIT-ROBO RHO GTPase activating protein 3 (*SRGAP3*)–*CRAF* fusions seem to occur less frequently (~2%–8% of cases).

The biochemistry of RAF fusion proteins remains poorly characterized, but canonical RAF signalling is thought to heavily rely on RAS-mediated dimerization of BRAF and CRAF protomers. Deletion of the N-terminal RAS-

binding domain (RBD) has long been known to constitutively activate RAF kinases [57] and is thought to promote dimerization, suggesting that the N terminus of RAF contains an autoinhibitory domain that prevents dimerization. Dimerization is associated with increased MEK phosphorylation, and disruption of the dimerization interface identified by the crystal structures 17,23 nearly abolishes catalytic activity [58]. Indeed, disrupted dimerization also prevents KIAA1549–BRAF-induced oncogenic transformation *in vitro*, indicating that loss of the RAF RBD through fusion of the kinase domain to KIAA1549 may constitutively activate RAF proteins through a similar mechanism.

Development of ATP-competitive RAF inhibitors

In 2000, sorafenib was the first RAF inhibitor to enter clinical trials; this was prior to the discovery of BRAF, CRAF or ARAF mutations in cancer. Originally developed as a CRAF inhibitor that was intended to treat RAS-mutated cancers, sorafenib was discovered by screening a chemical library for inhibitors of recombinant, activated CRAF [59]. Sorafenib competes with ATP for binding directly to the active site of the CRAF kinase domain. Owing to the shared structural homology of the ATP-binding pocket, several additional kinase targets have been described, including FMS-like tyrosine kinase 3 (FLT3), platelet-derived growth factor receptor-β (PDGFRβ), KIT and vascular endothelial growth factor receptor 2 (VEGFR2) [60]. Although sorafenib is now approved for the treatment of renal cell, hepatocellular and thyroid cancers, developed specifically to target BRAF-V600E. Specificity for the mutated oncoprotein was first shown for the vemurafenib analogue PLX-4720, which has a 50% growth inhibition (GI_{50}) that is approximately 50-fold more potent for cells containing BRAF-V600E than for cells with wild-type BRAF [61]. Selectivity for the mutant protein was originally explained by the type I binding mode of vemurafenib (and dabrafenib), which favours the active enzyme conformation that is imparted by the oncogenic mutation. By contrast, sorafenib and other type II inhibitors preferentially bind to the inactive conformation and are thus relatively poor inhibitors of BRAF-V600E. Clinically, this oncoprotein binding selectivity has translated into an unusually high therapeutic index for BRAF-V600E inhibitors, which allows for high exposures of the drug while avoiding the acute toxicities that are associated with RAF inhibition [62]. The high therapeutic index of BRAF-V600E inhibitors has proven to be crucial for treatment of BRAF-mutated melanomas because >80% target inhibition is required for a clinical response [63]. Recent studies have shown that all ATP-competitive RAF inhibitors — including vemurafenib, dabrafenib and sorafenib — are not only poor inhibitors of wild-type BRAF but also lead to paradoxical activation of the MAP kinase pathway in BRAF wild-type cells. Thus, the high potency of BRAF-V600E inhibitors in the treatment of BRAF-V600E-mutated melanoma may be attributed to inhibitor binding mode, but the lack of potency in BRAF wild-type cells and the high therapeutic index probably stem from the unique mechanism of action for wild-type RAF kinase, as described below.

The RAF inhibitor paradox

The use of RAF inhibitors in RAS-mutated cancers has revealed the complexity of RAF signal transduction. In 2009, three groups showed that catalytic inhibition of RAF kinases resulted in 'paradoxical activation' of CRAF, increased proliferation of RAS-mutated cells *in vitro* and, in some cases, induction of tumorigenesis *in vivo* [64]. Although each group proposed a distinct mechanism to explain the effect, several themes overlapped. RAF inhibitors promoted co-immunoprecipitation of RAF isoforms, as well as association with RAS–GTP at cell membranes [65] suggesting that the inhibitors promote CRAF homodimerization and heterodimerization and RAS-mediated activation. Moreover, site-directed mutagenesis of the putative dimer interface rendered CRAF resistant to paradoxical activation. BRAF inhibitors also promoted binding to kinase suppressor of RAS 1 (KSR1), which functions as a scaffold to coordinate a RAF–KSR1–MEK complex but competes with CRAF for dimerization with BRAF. The drug-induced association of RAF with RAS–GTP, increased RAF dimerization and binding to KSR1 probably each contribute to the activation of ERK signalling, but it remains unclear whether each event alone is sufficient to induce paradoxical activation.[66]

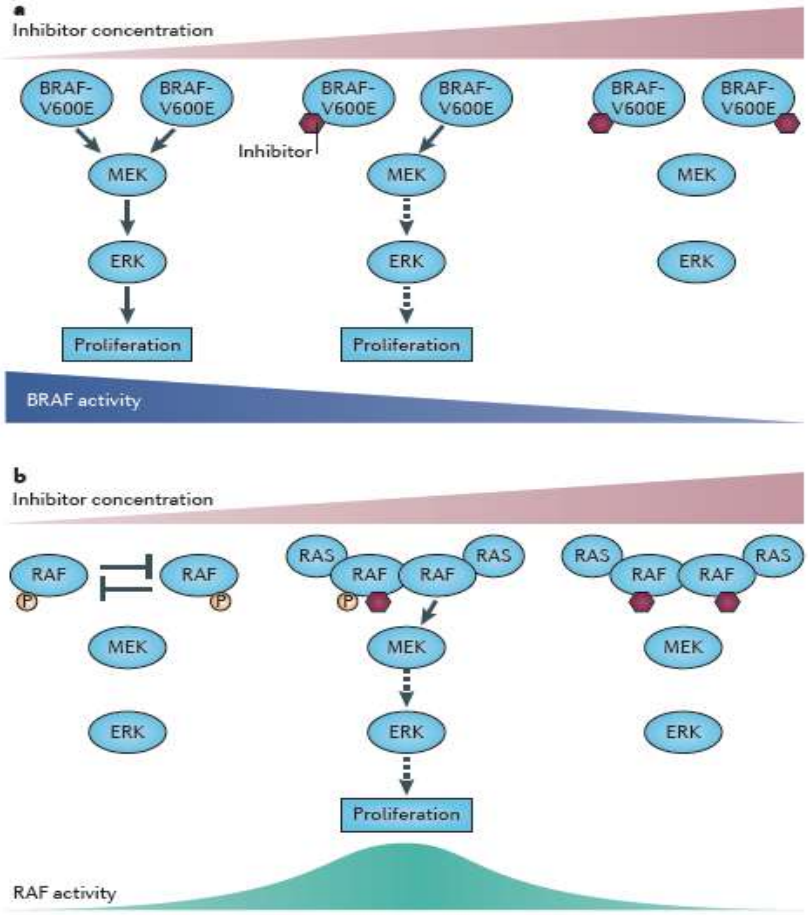

*Figure 3: Biochemistry of RAF inhibitors in BRAF-V600E and BRAF wild-type cells. BRAF oncoproteins promote proliferation by constitutively activating the MAP kinase pathway. **a** | ATP-competitive RAF inhibitors disrupt MEK activation and prevent proliferation of BRAF-V. 600E-mutated melanomas in a dose-dependent manner. **b** | Wild-type RAF proteins usually occur in an autoinhibited state. In the context of activated RAS, RAF inhibitors stimulate MEK activation at sub-saturating inhibitor concentrations by relieving autophosphorylation (P) of wild-type RAF, thereby promoting RAF dimerization and association with RAS. Saturating inhibitor concentrations prevent MEK phosphorylation by completely blocking RAF catalysis. Part **b** was adapted from REF. 148, Nature Publishing Group*

Interestingly, paradoxical activation by RAF inhibitors can be genetically recapitulated using catalytically impaired BRAF [67]. In this model, BRAF catalytically suppresses CRAF activity in RAS-mutated cells such that a disruption in BRAF function (either genetically or with a BRAF inhibitor) alleviates CRAF suppression and activates it in a RAS-dependent manner. This mechanism is further supported by the discovery of a RAF autoinhibitory phosphorylation site that is required for paradoxical activation, indicating that paradoxical activation may be intrinsic to all catalytic RAF inhibitors.

Clinical use of RAF inhibitors

Prior to the approval of BRAF inhibitors, patients with BRAF-mutated melanoma faced a worse prognosis than that for patients whose disease expressed wild-type BRAF. However, the recent approval of two BRAF inhibitors (vemurafenib and dabrafenib) and one MEK inhibitor (trametinib) for the treatment of metastatic BRAF-V600E-mutated melanoma has altered the situation; patients with BRAF-mutated melanoma who are treated with BRAF inhibitors now have a longer median survival than that of patients with wild-type BRAF melanoma [68]. In Phase I

clinical trials, treatment with vemurafenib induced disease stabilization, as well as some cases of marked regression of BRAF-V600E-mutated melanomas. The objective response rate (ORR) in Phase II increased from 19% in the dacarbazine control arm to 53% in the vemurafenib arm, and the average duration of response increased from 2.7 months to 6.7 months [69]. In 2011, the Phase III trial reported a 48% ORR when patients were treated with vemurafenib, compared with a 5% ORR when patients were treated with dacarbazine, as well as an estimated 5.3-month median progression-free survival (PFS) in the vemurafenib treatment group, compared with a 1.6-month median PFS in the dacarbazine treatment group [70]. Although the overall survival benefit was limited, approximately 14% of patients experienced durable responses and remained free from relapse after 18 months of treatment with vemurafenib [71]. Moreover, the full measure of the effect of vemurafenib on overall survival could not be calculated owing to the crossover design of the Phase III trial. A second BRAF inhibitor, dabrafenib, also gained FDA approval in 2013, for the treatment of not only BRAF-V600E-expressing melanomas but also BRAF-V600K-expressing melanomas [72]. Phase III results reported that treatment with dabrafenib resulted in a 50% ORR and a 5.1-month median PFS, compared with a 6% ORR and a 2.7-month median PFS for dacarbazine. FDA approval was also granted for trametinib — the first MEK inhibitor marketed for cancer treatment. The median PFS and the ORR increased from 1.5 months and 8%, respectively, with dacarbazine to 4.8 months and 22%, respectively, with trametinib.

The remarkable efficacy of BRAF inhibition in melanoma has been recapitulated in early data that are emerging from the treatment of patients with HCL-C. In 2011, sequencing of a cohort of samples from patients with HCL-C showed that the *BRAFT1799A* mutation occurred in virtually all patients with HCL-C. Anecdotes from the clinic suggest that patients with HCL-C whose disease is refractory to conventional chemotherapy respond well to vemurafenib. Thus, HCL-C may be a neoplasm in which mutated BRAF is a key oncogenic driver, and the paucity of BRAF-independent signalling pathways precludes the development of primary chemoresistance.

Cutaneous lesions induced by BRAF inhibitors

The ability of RAF inhibitors to paradoxically activate ERK MAP kinase signalling has crucial clinical implications. Results from the vemurafenib Phase III clinical trial indicate that 18% of patients experienced squamous cell carcinomas (SCCs) and/or keratoacanthomas. The list of secondary cutaneous lesions that are induced by BRAF inhibitors has since expanded to include papillomas, cystic lesions, milia, eruptive nevi and basal cell carcinomas. Although all drug-induced lesions are, so far, wild type for *BRAF*, multiple other genetic mutations have been identified. Importantly, samples of secondary keratoacanthomas show a high frequency of *HRAS* mutations [73]. This suggests that the ability of BRAF inhibitors to elicit paradoxical MAP kinase pathway activation induces a proliferative programme in dormant keratinocytes that harbour a latent RAS mutation. Indeed, studies using the keratin 5 (*K5*; also known as *Krt5*)–*SOS–F* transgenic mouse model, in which mice are predisposed to epidermal tumours through keratinocyte-specific expression of a dominant active, farnesylated son of sevenless (SOS-F), have shown that treatment with a RAF inhibitor promotes RAF dimerization, [74] activates ERK signalling and suppresses RHO-associated, coiled-coil containing protein kinase 2 (ROCK2) to potentiate RAS-driven epidermal tumorigenesis. In addition to RAS mutations, oncoviruses have been implicated as initiating factors in a subset of cutaneous lesions. Human papilloma virus (HPV) and Merkel cell polyomavirus DNA were identified in samples from patients who had been treated with BRAF inhibitors, and vemurafenib promoted tumorigenesis in an HPV-driven mouse model of SCC94. So far, the secondary cutaneous malignancies arising in patients receiving BRAF inhibitor therapy have been well-differentiated and amenable to local resection with no reports of metastasis [75].

More worrisome than the cutaneous toxicities are the possibility that BRAF inhibitors may induce the proliferation of pre-malignant cells that harbour RAS mutations in non-cutaneous tissues, such as the lung, colon or pancreas. The ability of BRAF inhibitors to promote the growth of pre-malignant precursor lesions along the path to adenocarcinoma is of considerable concern. Indeed, a recent report described a patient with metastatic BRAF-V600K melanoma who developed NRAS-mutant chronic myelomonocytic leukaemia during the course of vemurafenib treatment. Upon withdrawal of vemurafenib, the leukaemic cell count of the patient dropped, thereby necessitating an empirically determined intermittent dosing schedule of vemurafenib and cobimetinib (a MEK

inhibitor) to simultaneously control the metastatic melanoma and to prevent the outgrowth of leukaemia. A second case report described a patient presenting with BRAF-mutated metastatic melanoma who had undergone a prior successful surgical resection of a KRAS-G12D colorectal cancer. Although the combination of dabrafenib and trametinib caused a partial response of the patient's melanoma, at 12 weeks the patient presented with a brain metastasis that, upon resection, was characterized as originating from a KRAS-mutated colon cancer. After progressive metastatic colon cancer necessitated cessation of dabrafenib and trametinib combination therapy, the patient received trametinib monotherapy and experienced a brief improvement in performance status. *In vitro* studies that were carried out using tissue that was derived from the aforementioned patients suggested that MEK inhibitors suppress the growth of BRAF-V600E melanoma and also prevent expansion of the RAS-mutated malignancies. These case reports not only emphasize the caution that must be exercised when considering the use of RAF inhibitors as an adjuvant therapy but also demonstrate the importance of determining which node of the MAP kinase pathway is most appropriate to therapeutically target in patients with BRAF-mutated melanoma [76].

Primary resistance to RAF inhibition

BRAF mutation is not always prognostic of responsiveness to BRAF inhibitor therapy. Although BRAF-mutated melanoma is heralded as the malignancy in which BRAF inhibitors prove to be most beneficial, primary resistance occurs even in BRAF-mutated melanoma. In the dabrafenib Phase II clinical trial, 16% of patients with BRAF-V600E melanoma and 31% of patients with BRAF-V600K melanoma experienced progressive disease despite treatment with dabrafenib. In some cases, inhibition of the BRAF oncoprotein may activate signalling through wild-type RAF by relieving feedback mechanisms and increasing RAS–GTP levels. Moreover, results from the use of BRAF inhibitors in non-melanoma malignancies show that the cell of origin of the cancer — and thus the signalling pathways that are inherently available to the cancer cell — predicts the efficacy of BRAF inhibition. For example, although BRAF-V600E is expressed in ~10% of patients with metastatic colorectal carcinomas (mCRCs), a Phase I clinical trial of vemurafenib in patients with BRAF-mutated mCRC was associated with only a 3.7-month median PFS — about one-half of the PFS observed in BRAF-mutated advanced melanoma.

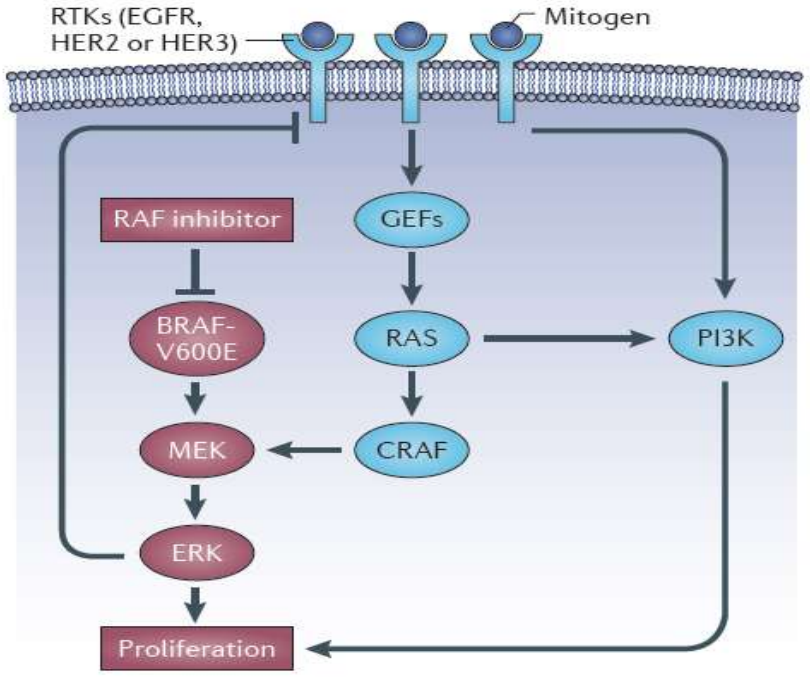

Figure 4: Loss of feedback inhibition and activation of the PI3K pathway mediates primary resistance to BRAF-V600E inhibition.

Resistance to BRAF inhibitors in metastatic colorectal and papillary thyroid cancers is mediated by loss of a feedback loop (epidermal growth factor (EGF)–EGF receptor (EGFR) signalling in colorectal cancers or neuregulin 1 (NRG1)–human epidermal growth factor receptor 2 (HER2) and NRG1–HER3 signalling in papillary thyroid cancers), whereby MAP kinase pathway activity inhibits mitogen-dependent signalling (shown in red). The negative-feedback loop is disrupted upon treatment with a RAF inhibitor, and proliferation is restored through receptor tyrosine kinase (RTK), RAS and PI3K signalling (shown in blue). GEFs, guanine nucleotide exchange factors.

Subsequent studies suggested that the decreased efficacy of vemurafenib in BRAF-mutated mCRC can be attributed to increased epidermal growth factor receptor (EGFR) signalling in these cells in response to vemurafenib. At baseline, BRAF-V600E–MEK–ERK signalling activates a negative-feedback loop that functions to attenuate RTK–EGFR signalling. However, BRAF inhibition relieves this negative feedback, and this leads to increased signalling through EGFR; in summary, the on-target activity of BRAF inhibitors activates a rapid, adaptive mechanism of chemoresistance. In contrast to mCRC, basal EGFR levels are low in melanoma, thereby allowing for evasion of this resistance mechanism. Thus, the cellular context in which BRAF inhibitors are used is highly relevant to therapeutic efficacy [77].

Papillary thyroid cancer is another malignancy for which BRAF mutational status does not predict the response to BRAF-V600E inhibition. By a mechanism that is conceptually concordant with that observed in mCRC, the inhibition of BRAF-V600E in papillary thyroid cancer causes a relief of negative feedback, leading to an induction of human epidermal growth factor receptor 2 (HER2; also known as ERBB2) and HER3 signalling. Fagin and colleagues showed that the BRAF inhibitor-mediated induction of HER2 and HER3 signalling in thyroid cancer is dependent on the secretion of the HER2 and HER3 ligand neuregulin 1 (NRG1). This ligand is not expressed in melanoma or colorectal cancer cells, thereby allowing the evasion of HER2-and HER3-mediated *de novo* resistance in these tissues [78].

The prognostic value of BRAF mutation for pathway-targeted therapy in NSCLC or ovarian cancer remains mostly unknown at this time but, at least with NSCLC, grounds for optimism are offered by the response of BRAF-V600E-initiated NSCLCs to pathway-targeted blockade in GEMMs and by a case report on the response of a patient with ARAF-mutated lung cancer to sorafenib.

Acquired resistance to RAF inhibition

Although the reported sample sizes remain small, the survival benefit in patients with BRAF-mutated melanoma treated with BRAF inhibitors seems to be limited to less than 1 year, with the durability of patient responses to RAF inhibitors being limited by the onset of drug-resistant disease. Initial attempts to anticipate resistance mechanisms were informed by experience with acquired resistance to imatinib in BCR–ABL-mutated leukaemia, which occurs when mutation of the 'gatekeeper' threonine prevents the binding of the drug without substantially affecting normal kinase activity. Surprisingly, although engineering of mutations at the analogous gatekeeper residue (T529) in BRAF confers vemurafenib resistance *in vitro*, T529 mutations have never been reported in tumour samples from BRAF inhibitor-resistant patients. One possible explanation for the failure to find such an obvious and highly predicted mechanism of resistance may be that cells expressing BRAF that is doubly mutated at amino acids 529 and 600 are not viable in the absence of the drug. Hence, there would be no reservoir of such cells prior to drug treatment and, therefore, this would not occur as a mechanism of drug resistance. Although Marais and colleagues have shown that a myeloid cell line (Ba/F3) remains viable in the absence of BRAF inhibition when transfected with *BRAF* that is doubly mutated at codons 529 and 600, this observation is probably cell type-specific and may also reflect the outgrowth of cells that are transduced with lower — and, thus, non-toxic — levels of doubly mutated *BRAF*.

Despite the absence of T529 mutations in BRAF, numerous other mechanisms of acquired resistance to BRAF inhibitors that contribute to clinical drug resistance have been described. Most resistance mechanisms promote reactivation of the MAP kinase signalling pathway in the presence of BRAF inhibition. For example, mutational activation of NRAS, MEK1 or MEK2 can reactivate the MAP kinase pathway in the presence of BRAF inhibition.

and increased CRAF protein levels have also been shown to confer resistance to BRAF inhibition in cell culture models of melanoma. Increased levels of CRAF protein have yet to be identified in clinical samples of BRAF inhibitor resistance, and it has been shown that, in some contexts, CRAF negatively regulates BRAF-V600E so further analysis is necessary to determine the clinical relevance of increased levels of CRAF protein as a *bona fide* BRAF inhibitor resistance mechanism. Through an unbiased screen, the serine/threonine MAP kinase kinase kinase COT (also known as MAP3K8) was shown to activate MEK in the presence of BRAF inhibition. Elevated *COT* copy number and mRNA expression was identified in biopsy specimens of metastatic melanoma following vemurafenib treatment. Overexpression of the mutant BRAF protein itself has also been reported, which further emphasizes the importance of increased expression of the drug target as a relevant mechanism of cancer drug resistance [79]. Additionally, BRAF-V600E splice variants, which endow the proteins with the ability to dimerize in a RAS-independent manner and which increase the kinase activity of the proteins, represent the only resistance mechanism that involves a structural change to BRAF itself and one of the first examples of how altered mRNA splicing can render an oncoprotein resistant to a drug.

Multiple mechanisms of acquired resistance (shown in blue) to BRAF-V600E inhibitors lead to reactivation of the MEK–ERK pathway (shown in red) in the presence of a RAF inhibitor. The mechanisms of resistance include mutational activation (asterisks) of NRAS, MEK1 and MEK2, overexpression (↑) of CRAF, of BRAF-V600E itself or of COT, alternative splicing of *BRAF* that renders the protein immune to inhibitors (p61 BRAF-V600E), ligand-dependent RAS signalling through either of the receptor tyrosine kinases (RTKs) platelet-derived growth factor receptor-β (PDGFRβ) or insulin-like growth factor 1 receptor (IGF1R), or stromal cell secretion of hepatocyte growth factor (HGF) to activate MET (also known as HGF receptor) signalling. GEFs, guanine nucleotide exchange factors [80].

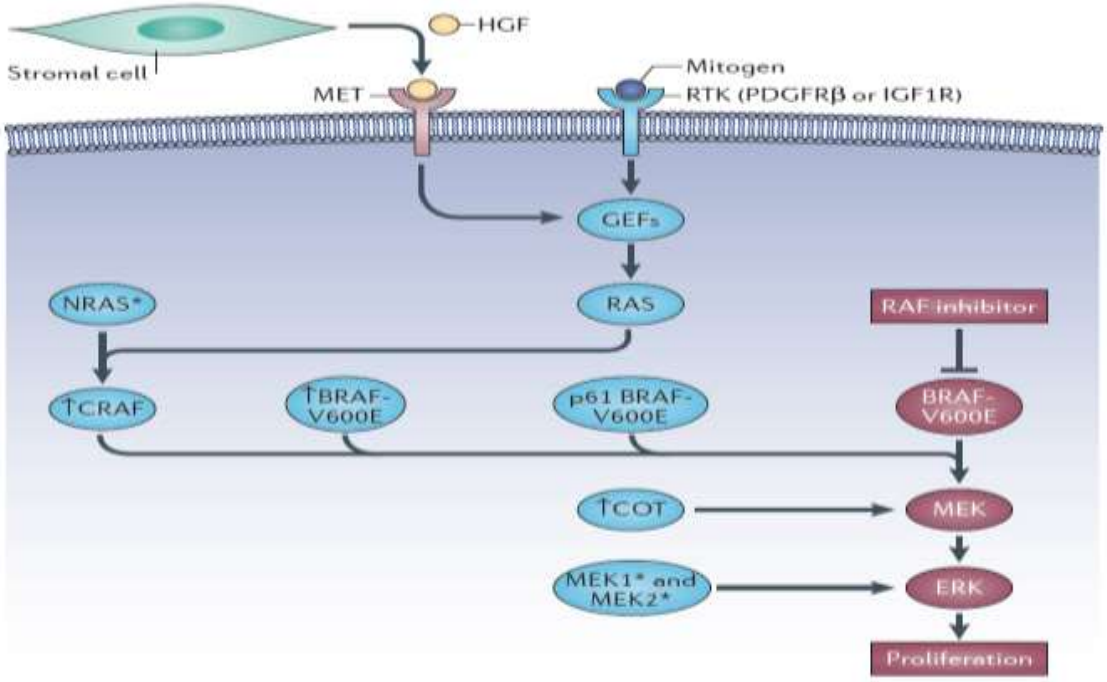

Figure 5: Mechanisms of acquired resistance to BRAF-V600E inhibition that led to reactivation of the MAP kinase pathway.

In addition to MAP kinase pathway reactivation in the presence of BRAF inhibition, it has been suggested that about 30% of patients develop MAP kinase pathway-independent mechanisms of resistance. Initial reports propose that most MAP kinase pathway-independent mechanisms of resistance involve alterations that lead to upregulation of the PTEN–PI3K–AKT signalling axis. Increased expression of either PDGFRβ or insulin-like growth factor 1 receptor (IGF1R) was identified in cultured cells and in specimens from patients with vemurafenib-resistant melanomas. It is

claimed that PDGFRβ or IGF1R signalling allows for the activation of MAP kinase pathway-independent pro-survival signalling pathways, such as the PI3K–AKT axis, which render cells resistant to the effects of BRAF inhibition. Furthermore, activation of HER3 signalling has been identified as an adaptive mechanism of resistance in a subset of patients with melanoma. It is thought that MAP kinase pathway inhibition promotes the upregulation of the transcription factor forkhead box protein D3 (FOXD3), which in turn directs increased expression of *HER3* and allows for enhanced HER2–HER3 signalling. In addition, amplification of the melanocyte lineage-specific transcription factor *MITF* or its effector, BCL2-related protein A1 (*BCL2A1*), has been shown to confer resistance to BRAF and MEK inhibitors in cell culture models, and upregulation of *MITF* occurs in a minority of patients with BRAF-mutated melanoma. Secretion of growth factors by the tumour stroma has also been shown to confer resistance to BRAF inhibition. Specifically, stromal cell-directed secretion of hepatocyte growth factor (HGF) has been shown to signal through its receptor, MET (also known as HGF receptor; which is expressed on the surface of melanoma cells) and thereby reactivate MAPK and PI3K–AKT signalling pathways in the presence of BRAF inhibition [81].

Although numerous on-pathway or off-pathway mechanisms of resistance have been identified through preclinical studies, the challenge lies in characterizing which of these resistance mechanisms are clinically relevant and in determining the relative frequencies of the resistance mechanisms observed in the clinic. The development of second-generation BRAF inhibitors has definitively shown the potential of pathway-targeted therapeutics in the treatment of BRAF-mutated melanoma. However, the emergence of drug-resistant disease stands as a formidable obstacle that remains to be addressed to enhance the durability of patient responses [82].

Improving responses to RAF inhibition with other therapeutic targets

It is currently unclear whether survival benefit alone will cause RAF inhibitors to be used as single agents, but preclinical and early clinical data strongly suggest that some combination of inhibitors of RAF and either MEK or, conceivably, ERK will have the greatest efficacy. FDA approval was recently granted for the combination of dabrafenib and trametinib in advanced melanoma, and detailed results of the Phase III trial are forthcoming. Phase I/II results demonstrated that the combination increased median PFS from 5.8 months for single agent treatment to 9.4 months in the combination group, and the ORR increased from 54% to 76%. Perhaps unexpectedly, the tolerability of either agent improved in the combination group. Acneiform dermatitis, which is the most common dose-limiting toxicity that is associated with trametinib (8%), was not observed in the combination group. Likewise, the frequency of hyperkeratosis, cutaneous SCCs and papillomas, which are all RAF inhibitor class effects, each decreased when dabrafenib was co-administered with trametinib. Mechanistically, this most probably occurs because the paradoxical activation of the MEK–ERK MAP kinase pathway that is induced by RAF inhibitors is suppressed by MEK inhibition [83].

Tumours that have acquired on-pathway mechanisms of resistance to BRAF inhibitors constitute an important group in which to test the efficacy of second-line MEK or ERK inhibitors. Unfortunately, early clinical data suggest a limited efficacy of MEK inhibitor monotherapy for patients with BRAF-mutated melanoma who have drug-resistant disease. Within a cohort of 40 patients who had progressed on either vemurafenib or dabrafenib monotherapy, subsequent treatment with trametinib gave only a 1.8-month median PFS and a 5.8-month median overall survival. Only two patients within the cohort had a complete or partial response, and both of these patients had discontinued BRAF inhibitor therapy owing to adverse events rather than the onset of drug-resistant disease [84]. Thus, second-line MEK inhibitor monotherapy to treat BRAF inhibitor-resistant disease seems limited. These results may be partially explained by a recent report showing that oncogenic *BRAF* overexpression or alternative splicing — previously known to confer resistance to BRAF inhibitor monotherapy — is also a resistance mechanism among patients who receive first-line BRAF and MEK combination therapy. Another tactic to target tumours that have acquired MAP kinase pathway-dependent resistance mechanisms is the use of ERK inhibitors. Although ERK inhibitors have only recently entered clinical trials, preclinical data suggest that cells that have developed resistance to RAF and MEK inhibitors remain sensitive to a selective ERK1 and ERK2 inhibitor. The acquisition of MAP kinase pathway-independent mechanisms of resistance may also sensitize tumours to other pathway-targeted

therapies. For example, increased PDGFRβ or IGF1R signalling might be combated with RTK or PI3K inhibitors [85].

BRAF-V600E: 'oncogene overdose' and intermittent drug dosing

Although BRAF-V600E constitutively activates the MEK–ERK pathway, many cancer cells remain sensitive to the quantity of MAP kinase pathway signalling (as measured by ERK phosphorylation or transcription of target genes). More than ten years ago, it was shown that mouse or human fibroblasts display a peak proliferative response with an intermediate amount of RAF–MEK–ERK pathway activation [86]. Indeed, in non-immortalized human IMR-90 fibroblasts, sustained RAF activation induced an irreversible cell cycle arrest with features of cellular senescence. This further substantiated the hypothesis that RAF-induced transformation is determined not only by activation of the MAP kinase pathway but also by the quantity of pathway activation, as reviewed by Marshall [87].

The notion that malignant cells might remain sensitive to the strength of MAP kinase pathway signalling was recently substantiated using a patient-derived xenograft model of melanoma. Upon transplantation into immunocompromised mice, a chemonaive, BRAF-mutated melanoma showed striking regression in response to treatment with vemurafenib. However, sustained drug treatment led to the emergence of lethal drug-resistant disease due to increased BRAF-V600E expression [88]. Remarkably, the drug-resistant tumours and cell cultures were not only drug-resistant but also drug-dependent for their peak proliferation. Moreover, when vemurafenib was removed from drug-resistant cells or tumours, striking anti-proliferative effects were observed. These data indicate that BRAF-V600E-'addicted' melanoma cells can remain sensitive to the magnitude of BRAF-V600E–MEK–ERK signalling pathway activation, such that too much of the oncoprotein to which they are addicted can lead to oncogene overdose [89]. The demonstration that vemurafenib-resistant melanoma cells can have a fitness deficit in the absence of drug has led to the design of clinical trials to test the efficacy of intermittent dosing to forestall the onset of drug-resistant disease and therefore enhance the durability of patient responses [90].

Conclusions

Development of BRAF inhibitors has changed preclinical understanding and clinical treatment of late-stage melanoma, and it offers a more hopeful option to patients with a historically devastating diagnosis. Despite the short duration of responses, BRAF inhibitor-treated patients have a high ORR and frequently experience marked tumour regression. Several complete responses have been observed (by Response Evaluation Criteria in Solid Tumours. Drug-resistant disease progression is observed in most cases, often occurring within a few months after initial response. Current efforts to co-target BRAF and MEK127 may prevent some of the identified mechanisms of resistance, although it is not clear to what extent. Identifying and understanding common resistance mechanisms in BRAF and MEK inhibitor-resistant melanomas will inform additional rational pathway-targeted therapeutic combinations, such as co-targeting PI3K signalling, as well as dosing strategies to prevent or delay drug resistance and achieve long-term survival benefit.

A more thorough understanding of the mechanism (or mechanisms) of oncogenesis has led to potent and selective RAF inhibitors that exploit the unique biology of BRAF-mutated melanomas. However, BRAF-mutated melanoma only represents a small subset of RAF-mutated cancers. Several RAF fusion proteins seem to be insensitive to the current generation of BRAF inhibitors and instead show paradoxical activation in a similar way to wild-type RAF alleles. Additionally, CRC and NSCLC comprise a large cohort of BRAF-mutated cancers that are currently ineffectively treated. The mechanisms of RAF-driven oncogenesis in RAF inhibitor-resistant settings may involve redundant pathways, additional biochemical functions or additional substrates of RAF on coproteins. In the same way as rational structure-based drug design has yielded selective and targeted treatments for BRAF-mutated melanomas, understanding the mechanisms of other *BRAF* oncogenes and *CRAF*-dependent tumours may lead to effective treatments in these malignancies. Similarly, although an understanding of rapid adaptive resistance in BRAF-mutated CRC led to hypotheses for rational combinations using RTK or PI3K pathway inhibitors, understanding BRAF inhibitor resistance in papillary thyroid and ovarian cancers will probably yield additional rational therapeutic approaches and predictive biomarkers for these indications.

Although BRAF-V600E and BRAF-V600K are *bona fide* drug targets in melanoma, it remains unclear whether *ARAF* and *CRAF* alterations constitute driver mutations, but there is reason to suspect that they are therapeutic targets. A recent case study indicates that ARAF mutations could be treated with currently available RAF inhibitors. A patient with NSCLC with an ARAF-S124C mutation and a lack of alterations in any other known oncogenes or tumour suppressor genes, achieved a sustained tumour remission (~5 years) with sorafenib treatment, and this gives some promise of treatment for cancer patients with these rare mutations. Interest has also been renewed in wild-type CRAF as a therapeutic target for KRAS-mutated lung cancer after demonstrations of CRAF dependence for the onset of *Kras*-mutated NSCLC in GEMMs. Interestingly, BRAF was dispensable, but concurrent loss of MEK1 and MEK2 or ERK1 and ERK2 also prevented tumour initiation, suggesting that CRAF but not BRAF is essential for tumour formation in *Kras*-driven oncogenesis. CRAF but not BRAF dependence was also shown in a SOS-F-induced model of skin cancer, further supporting CRAF as a therapeutic target in RAS-dependent cancers.

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