Review

Molecular Cancer Therapeutics

BRAF^{V600E}: Implications for Carcinogenesis and Molecular Therapy

Emma R. Cantwell-Dorris, John J. O'Leary, and Orla M. Sheils

Abstract

The mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway is frequently mutated in human cancer. This pathway consists of a small GTP protein of the RAS family that is activated in response to extracellular signaling to recruit a member of the RAF kinase family to the cell membrane. Active RAF signals through MAP/ERK kinase to activate ERK and its downstream effectors to regulate a wide range of biological activities including cell differentiation, proliferation, senescence, and survival. Mutations in the v-raf murine sarcoma viral oncogenes homolog B1 (BRAF) isoform of the RAF kinase or KRAS isoform of the RAS protein are found as activating mutations in approximately 30% of all human cancers. The BRAF pathway has become a target of interest for molecular therapy, with promising results emerging from clinical trials. Here, the role of the most common BRAF mutation *BRAF*^{V600E} in human carcinogenesis is investigated through a review of the literature, with specific focus on its role in melanoma, colorectal, and thyroid cancers and its potential as a therapeutic target. *Mol Cancer Ther; 10(3); 385–94. ©2011 AACR.*

Introduction

The mitogen-activated protein kinase (MAPK) kinase (MEKK)/extracellular signal-regulated kinase (ERK) pathway is a conserved kinase cascade involved in the regulation of cell proliferation, differentiation, and survival in response to extracellular signaling. The most potent activator of MAP/ERK kinase (MEK) is the v-raf murine sarcoma viral oncogenes homolog B1 (BRAF). The RAS/BRAF/MEK/ERK pathway is mutated in an estimated 30% of all cancers (1), with mutations in the *braf* gene found in approximately 7% of cancers (2). The predominant mutation in the *braf* gene involves a thymidine to adenosine transversion at nucleotide 1,799, accounting for greater than 90% of the observed mutations in *braf* (2). This results in an activating mutation due to the substitution of valine with glutamic acid at amino acid (aa) 600. Significant progress has been made in understanding the carcinogenic role of the BRAF^{V600E} mutation (3). BRAF^{V600E} is an attractive target for molecular therapy. Inhibitors targeted against BRAF and its primary downstream target MEK are producing interesting results in early-phase clinical trials. In this article, the molecular aspects of BRAF^{V600E} and the consequences of this activating mutation are reviewed. The carcinogenic effect of BRAF^{V600E} is discussed through its effect on 3 cancers types with the highest incidence of *braf* mutation: melanoma, colorectal, and thyroid cancers. Furthermore, the clinical application of molecular inhibitors targeting BRAF^{V600E} and its downstream effector MEK is reviewed.

Authors' Affiliation: Department of Histopathology, University of Dublin, Trinity College, Dublin, Ireland

Corresponding Author: Emma R Cantwell-Dorris, Department of Histopathology, Trinity College, Sir Patrick Dun Research Laboratory, Pathology Building, St. James' Hospital, Dublin 8, Ireland. Phone: 0035318963289; Fax: 0035314542043. E-mail: cantweer@tcd.ie

doi: 10.1158/1535-7163.MCT-10-0799

The Role of BRAF in the MAPK/ERK Pathway

BRAF is a member of the RAF family of serine/threonine protein kinases. This family consists of 3 kinases, ARAF, CRAF (RAF-1), and BRAF, of which the latter has the highest basal kinase. BRAF functions to regulate the MAPK/ERK pathway, a pathway that is conserved in all eukaryotes (4). The RAS/RAF/MEK/ERK pathway acts as a signal transducer between the extracellular environment and the nucleus. Extracellular signals such as hormones, cytokines, and various growth factors interact with their receptors to activate the small G-proteins of the RAS family. Active RAS acts via adaptor proteins to activate and recruit RAF proteins to the cell membrane where they are activated (1). Active BRAF signals through MEK to activate ERK, which, in turn, activates downstream transcription factors to induce a range of biochemical processes including cell differentiation, proliferation, growth, and apoptosis.

BRAF is the most potent activator of MEK. Active RAS induces conformational changes in RAF that allows its recruitment to the cell membrane, promoting changes in the phosphorylation status and triggering its kinase activity. Unlike BRAF, ARAF and CRAF require an additional phosphorylation in the N-region of their

kinase domain for full activation (5), likely contributing to the predominance of the BRAF isoform in the activation of MEK.

BRAF phosphorylates and activates MEK1/2, which initiate a kinase cascade that acts through ERK1/2 to signal for ligand- and cell-specific responses. MEK1/2 contain proline-rich segments in the carboxy-terminal domains. These segments are not found in other MEK family members (4). It is postulated that these segments are required for MEK activation by RAF, supported by the observation that deletion of the proline insert from MEK-1 impairs its activation by RAF in transfected cells (6). RAF/MEK coupling is required for the downstream phosphorylation of ERK1/2. Regulation of this pathway is crucial for the maintenance of homeostasis in response to extracellular signaling. It has been shown that hyperactivation of this pathway can induce cell-cycle arrest whereas aberrant regulation of the pathway can initiate tumorigenesis (2).

The *braf* Oncogene

More than 40 different mutations have been identified in the *braf* gene in human cancer (Fig. 1). Ninety percent of *braf* mutations are accounted for by a thymine to adenine single-base change at position 1,799. This missense mutation, located in exon 15, results in a change at residue 600 that substitutes glutamine for valine (V600E; ref. 2). $BRAF^{V600E}$ can gain 500-fold increased activation, stimulating the constitutive activation of MEK/ERK

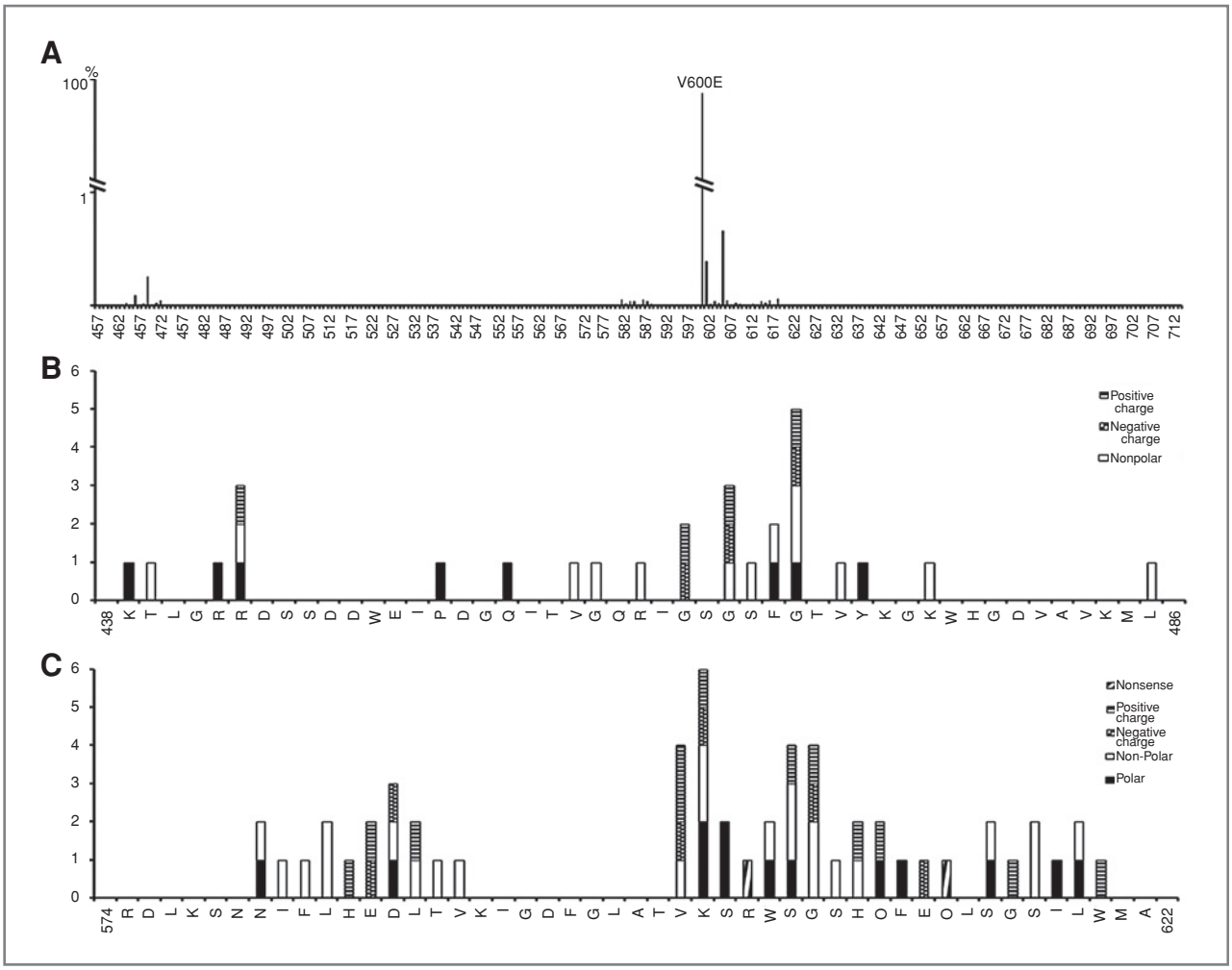

Figure 1. Somatic mutations of the *braf* gene identified in human tumor samples. A, the V600E mutation accounts for greater than 95% of the somatic mutations described in human tumor samples. The graph illustrates the locations and relative frequency of somatic mutations within the kinase domain of the *braf* gene identified in human tumor samples ($n = 11,860$). B, somatic mutations cluster close to the P-loop of the N-terminus. The graph illustrates the amino acid substitutions (grouped on the basis of side-chain properties) identified as arising from somatic mutations within the region aa438–486. C, somatic mutations cluster close to the activating segment of the kinase domain. The graph illustrates the amino acid substitutions (grouped on the basis of side-chain properties) identified as arising from somatic mutations within the region aa574–622. Note: The mutation data was obtained from the Sanger Institute Catalogue of Somatic Mutations in Cancer web site (7).

signaling in tumor cells. Furthermore, it allows activation of this signaling cascade in the absence of any extracellular stimuli, allowing the cell to become self-sufficient in growth signals within this pathway. The structure of the BRAF protein gives insight into how this constitutive activation is initiated.

The BRAF kinase domain has characteristic bilobal architecture, with small and large lobes separated by a catalytic cleft. In the inactive BRAF conformation, the conserved aspartate, phenylalanine, and glycine (DFG) motif found in the activation segment displays a flipped conformation that orients the region of activation toward the P-loop of the N-lobe. This brings the residues' glycine-rich segments G595-V600 near to the G463-V470, which allows hydrophobic interactions to be formed between these segments. This results in a conformational orientation that renders the catalytic cleft inaccessible. In this inactive conformation, the residues required for phosphotransfer reactions are aligned but the ATP and peptide substrate recognition segments are partially disorganized. Thus, all that is required for transition to an active state is a change in position of the DFG motif/activation segment (3). Phosphorylation of the activation segment results in the destabilization of the hydrophobic interactions between the activation segment and the P-loop, resulting in a flip of the DFG segment to its active state, aligning the ATP and peptide recognition segments, and allowing access to the catalytic cleft.

Activating mutations tend to cluster to the glycine-rich loop and activation segments (Fig. 1B and C) and are often located in residues that normally stabilize the interactions between these regions (1). The BRAF^{V600E} mutation replaces V600 valine, a medium-sized hydrophobic side chain that interacts with F467 phenylalanine of the P-loop, with E600 glutamine, a larger, charged side chain. V600 is located in the activation segment close to the DFG motif (3). V600E substitution disrupts the hydrophobic interaction, destabilizing the conformation that maintains the inactive orientation of the DFG motif, resulting in the restitution of DFG motif to its active state and thereby restoring the activation segment to its active orientation. This phosphomimetic mutation renders BRAF^{V600E} in a constitutively active state (1, 3).

BRAF Mutation in Carcinogenesis

BRAF has been shown to be mutated in a wide range of cancers including 40% to 70% of malignant melanomas, an average of 45% of papillary thyroid cancer, and 10% of colorectal cancers (CRC) and has also been identified in ovarian, breast, and lung cancers (2, 8, 9). Germline mutations in *braf* and *craf* are associated with LEOPARD syndrome, a developmental disorder with an increased incidence of multiple granular cell tumors (10). Mutations in *braf* commonly occur in the same cancer types that harbor *ras* mutations. Mutations in *braf* and *ras* generally occur in a mutually exclusive fashion, suggesting that aberrant regulation of the RAS/BRAF/MEK/ERK pathway may be the pathogenesis of these tumor types (2), which can be achieved at different levels of the pathway.

BRAF Mutation in Melanoma

Melanoma arises from melanocytes, the specialized pigment cells found in the epidermis, meninges, inner ear, and eye. The RAS/RAF/MEK/ERK pathway is frequently mutated in melanoma, with *ras* mutations, predominantly of the *nras* isoform, found in 15% to 30% and *braf* mutations found in up to 70% of melanomas (2, 8). BRAF^{V600E} is the most frequent (>90%) *BRAF* mutation in melanoma (2). It is associated with nonchronic sun-induced damage (non-CSD) melanomas, indicating a different pathology of disease than that by the CSD-induced p53 loss of function progression to disease associated with long-term UV radiation (UVR; ref. 11). BRAF^{V600E} is associated with low UVR dose, younger presentation, and melanocortin-1 receptor (MC1R) variants (12). The relationship between *braf*-mutated melanoma and sun exposure is complex. Mutations in *braf* occur at a very low rate in melanomas located at mucosal lining and areas of low sun exposure such as the palms and the soles of the feet, indicating that sun exposure is required for the development of *braf*-mutated disease (13). However, *braf* mutation has a very low frequency in CSD melanoma associated with long-term sun exposure (12). Thus, the link between UVR and *braf* mutation does not seem to be straightforward.

The *mc1r* gene is a key determinant of pigmentation. It is highly polymorphic in humans, and specific variants are linked to the distinctive red hair, pale skin, and freckles phenotype, the same phenotype that is associated with increased melanoma risk in the Celtic population (14, 15). MC1R variants have been shown to be associated with *braf* mutation in non-CSD melanomas in both Italian and American populations (12, 16). MC1R is a G-protein–coupled receptor, and its variant isoforms can affect signaling through this pathway. MC1R binds α-melanocyte–stimulating hormone (α-MSH), a crucial regulator of melanocyte homeostasis. In response to UVB radiation, α-MSH binds MC1R signaling for increased proliferation and melanogenesis. The binding of α-MSH to MC1R upregulates cyclic AMP that results in a signaling cascade that acts through BRAF to signal to ERK. ERK activation induces proliferation or differentiation, depending on microenvironment-specific signals. α-MSH signaling is also extremely important for the reduction of free radical formation in response to UV and is involved in inhibition of UV-induced apoptosis (17).

Variant isoforms of MC1R display reduced function of the receptor due to an inadequate ability to respond to their ligands, such as α-MSH (17, 18). Wild-type MC1R signals for the production of eumelanin over pheomelanin in response to UVR. However, variant isoforms of MC1R are unable to signal for this switch in melanin

production, which can lead to an accumulation of pheomelanin. Pheomelanin can increase the production of free radical in response to UVR and thus can be regarded as a carcinogen. It is unclear whether the association between MC1R variants and BRAF is due to a direct relationship or is caused indirectly via the accumulation of pheomelanin, which can increase oxidative stress and thus induce DNA instability.

Human melanocytes are dependent on adhesion to the extracellular matrix (ECM) for efficient activation of ERK1/2 (19). ERK1/2 signaling is required for cell-cycle progression through the G_1/S phase, as both the upregulation of cyclin D1 and the downregulation of cyclin-dependent kinase inhibitors (cdk) p27kip1 are ERK1/2 dependent. To progress through the G_1/S phase, growth factors and integrin-mediated signaling from the ECM are required to induce hyperphosphorylation of the retinoblastoma protein via the downregulation of cdk inhibitors and the expression of D-type cyclins. This allows the derepression of the E2F transcription factor, thereby inducing its binding to promoter sites of genes involved in S-phase entry. It has been shown that loss of adhesion in melanocytes impairs the growth factor–mediated ERK1/2 activation, including the activation of cyclin D1 (19).

$BRAF^{V600E}$ mutation in melanoma cells results in the activation of ERK1/2 without the need for signaling from the ECM. Constitutive activation of BRAF results in the upregulation of cyclin D1 in the absence of extracellular signaling. The cdk inhibitor p27kip1 is downregulated in mutant BRAF melanoma via the derepression of its transcription but also via the upregulation of the proteasomal proteins involved in the p27kip1 protein degradation. The S-phase kinase-associated protein 2 (SKP2) and its cofactor Cdc kinase subunit 1 (CKS1) are members of an ubiquitin-dependent proteasomal ligase complex involved in the degradation of p27kip1. SKP2 is dependent on adhesion in melanocytes and has been inversely correlated to p27kip1 levels (20). Knockdown studies of BRAF or cyclin D1 have shown a decrease in SKP2 and CKS1. Regulation of these proteasomal proteins involved in p27kip1 degradation is dependent on the expression of BRAF and cyclin D1. Thus, BRAF mutation subverts the adhesion and growth factor requirements for ERK1/2 signaling in melanoma cells, thereby allowing unregulated progression through the G_1/S checkpoint.

It has been shown that BRAF is an upstream signaling component of a principal signaling pathway in melanocytes (21). This is a melanocyte-specific pathway that regulates differentiation and proliferation, which is reliant on BRAF activation. The high frequency of *braf* mutation in melanoma relative to other cancers may be partly explained by the role of BRAF in melanocyte-specific biology (2).

BRAF Mutation in CRC

CRC is highly prevalent within the Western world and is 1 of 4 most prevalent cancer types worldwide (22). The most prevalent CRC type follows the Kinzler–Vogelstein model, whereby a stepwise accumulation of mutations involving the adenomatous polposis coli gene *p53* and members of the β-catenin signaling pathway (23, 24) leads to adenocarcinoma development. However, an alternative serrated pathway to CRC has recently been highlighted, involving mutations of RAS/RAF/MEK/ERK pathway and microsatellite instability (MSI; refs. 25, 26). This sporadic CRC frequently contains activating mutations in *kras* and *braf* (51% and 10% respectively; ref. 25). These mutations are found in a mutually exclusive fashion (2, 24, 25) and *kras* mutations tend to display a more aggressive phenotype and less favorable clinical outcome (27, 28). $BRAF^{V600E}$ mutation is associated with CRC tumors that also display deficiency in mismatch repair (MMR). The prevalence of *braf* mutation in MMR-deficient tumors has been shown to be 3-fold greater than in MMR-proficient tumors, whereas *kras* mutation was equally prevalent (25).

braf mutation is tightly associated with a CpG island methylation phenotype (CIMP; ref. 29), which is characterized by methylation of the MMR gene *mlh1* and associated high MSI (MSI-H). MHL1 is involved in the MMR system, in which it is part of a protein complex that introduces single strand breaks close to the mismatch, thereby creating new entry points for exonucleases. It is also implicated in DNA damage signaling that can lead to the induction of cell-cycle arrest or apoptosis in response to major DNA damage. *mlh1* has a large CpG island within its promoter region. Methylation of a small proximal region (region C) close to its transcriptional start site has been correlated with loss of gene expression (30). The association between *braf* mutation and CIMP is unclear. However, it has been shown that *braf* mutations are also found in the precursor lesions (31–33), indicating that it is an early event in the development of these tumors.

The mutant *braf* CIMP tumors tend to exhibit a serrated morphology. This serrated morphology has been postulated to be as a result of the inhibition of apoptosis (34). Mutant BRAF can act as a potent inhibitor of apoptosis. Erhardt (35) showed that overexpression of BRAF can inhibit apoptosis via postmitochondrial regulation of apoptosis. Acting through MEK, overexpression of BRAF works at the level of cytosolic caspase activation to inhibit cytochrome *c*–induced apoptosis. BRAF mutations in colorectal tissue have also been associated with significant decreases in the rates of apoptosis in tumor cells (36). Thus, it has been postulated that BRAF mutation introduces a microenvironment resistant to apoptosis, which allows extensive DNA damage, such as that found in CIMP tumors, to be tolerated (37).

The high correlation of BRAF and CIMP (29) suggests that the association is causal. Forced overexpression of $BRAF^{V600E}$ induced the methylation of the promoter region of *mlh1* in a colon cell line (37). DNA methyltransferase and DNA methylation can be regulated via the RAS/RAF/MEK/ERK pathway (38). This has lead to the hypothesis that BRAF mutation induces a microenvironment of apoptosis resistance and predisposition to

promoter hypermethylation, as exemplified by the high rate of *mlh1* promoter methylation. This leads to a state permissive to MSI-H due to MMR deficiency, which gives rise to the CIMP CRC (37). This hypothesis explains the progression of the serrated pathway but has yet to be validated.

BRAF Mutation in Thyroid Cancer

Thyroid cancer is the most common endocrine malignancy and is the most rapidly increasing cancer type amongst women. Up to 90% of all thyroid cancers are papillary thyroid cancer (PTC; ref. 39), of which an average of 45% display mutant *braf* (9). Rearrangements of *ret* are found in up to 30% of PTC (*ret*/PTC; ref. 40) in a mutually exclusive fashion with *braf* and *ras* (41). RET is a tyrosine kinase receptor not usually expressed in thyroid follicular cells, but the chimeric RET/PTC is driven by the promoter of the partner gene, of which more than 10 genes have been identified. RET/PTC signals through the RAS/RAF/MEK/ERK pathway, indicating that it is overactivation of the pathway itself that is required for tumor pathogenesis in PTC.

RET/PTC is common among radiation-induced tumors and sporadic tumors in juveniles (42), whereas BRAF^{V600E} is found at a higher rate in adults. BRAF^{V600E} has also been associated with more aggressive characteristics (42), which has also been shown in animal models (43). BRAF has been shown to be necessary for RET/PTC-induced activation on the ERK pathway and its regulation of downstream effectors relevant to tumor pathogenesis (43).

BRAF^{V600E} is associated with silencing of multiple thyroid-specific iodine-metabolizing genes (43, 44). Iodine is important in the synthesis of thyroid hormones. Inorganic iodine is actively transported into the thyroid cells and then, in turn, it is transported into follicular cells where it is oxidized and incorporated into thyroid hormones. BRAF^{V600E} has been associated with the decreased gene expression of several of the genes involved in this process (43, 45, 46). Thyroid DNA methylation of the promoter regions of iodine-metabolizing genes have been linked with BRAF^{V600E} PTC but not with RET/PTC rearrangement-induced PTC. The function of these genes could be restored in a thyroid cell model carrying BRAF^{V600E} via the inhibition of MEK (43, 44). As previously discussed, DNA methyltransferase is regulated by the BRAF pathway (38). Promoter methylation seems to play a substantial role in the silencing of iodine-metabolizing genes in BRAF^{V600E} PTC (43, 44). It has been postulated that the decreased expression of iodine metabolism–associated genes may explain the more aggressive phenotype exhibited in BRAF^{V600E} PTC.

BRAF as a Therapeutic Target

BRAF-mutated tumors have been correlated with poor response to traditional chemotherapy and poor prognosis in melanoma, thyroid, and colon cancers (43, 47). Targeted therapies are of great interest for these cancer types and the elucidation of the structure and functions of the BRAF kinase is the subject of a lot of ongoing research. The approach of targeting oncogenic kinases has been successful in the treatment of cancers, with activating mutations in the kinase gene that drives their progression (48). Imatinib (Gleevec; Novartis) is the original success story for small-molecule kinase inhibitors. Imatinib is used in the treatment of chronic myeloid leukemia, which is driven by a characteristic mutant fusion protein Bcr-Abl, resulting in constitutive activation of the Abl kinase. Imatinib effectively blocks the Abl kinase producing an effective clinical response that can be correlated to the presence of the activating kinase mutation.

BRAF-specific inhibitors such as GDC-0879 and PLX4720 effectively block BRAF^{V600E} and thus block BRAF^{V600E}-induced ERK activation (Fig. 2). PLX4720 was designed according to the atomic structure of BRAF^{V600E} and as a result has a 10-fold increased potency for BRAF^{V600E} over the wild-type kinase (49). These first-generation BRAF inhibitors were found to be effective in preclinical trials but underwhelmed in clinical trials. The emergence of second-generation inhibitors, such as the PLX4720 analogue PLX4032, is proving much better in clinical trials. The details of a phase I trial for PLX4032 announced an 80% response rate (as determined by complete or partial tumor regression) among patients with BRAF^{V600E} mutations whereas patients without the mutation did not respond (50). This 80% response rate is the highest response rate to date for a melanoma drug. This was a phase I trial and therefore is based on a small number of patients. It does however provide proof of concept for small BRAF^{V600E} inhibitors and provides hope for their use in the clinic.

Increasing research of BRAF-specific inhibitors has highlighted several mechanisms of interest that may negate their use in the clinic. It has been shown that mutant BRAF cell lines can acquire resistance toward BRAF-specific inhibitors through an increase in CRAF expression (51). In the presence of oncogenic or growth factor–activated RAS, BRAF-selective inhibitors can induce BRAF binding to CRAF. This can lead to transactivation of CRAF and consequent elevation of MEK and ERK signaling (Fig. 2C; ref. 52). This results in the cell regaining autonomy and hyperactivation of the MEK/ERK pathway through CRAF in a RAS-dependent manner (53). The genotyping of tumors is therefore critical to ensure not only that an activating mutation is present in *braf* but also that activating mutations of *ras* are not present. This reactivation of the pathway can occur with chemical or genetic (short hairpin RNA inhibition) inhibition of BRAF but does not seem to occur with pan-RAF drugs.

Pan-RAF drugs (e.g., sorafenib and XL281) target all the RAF isoforms. It has been shown in mice and human xenographs carrying BRAF^{V600E} that pan-RAF inhibitors hyperactivate CRAF through the inhibition of BRAF but

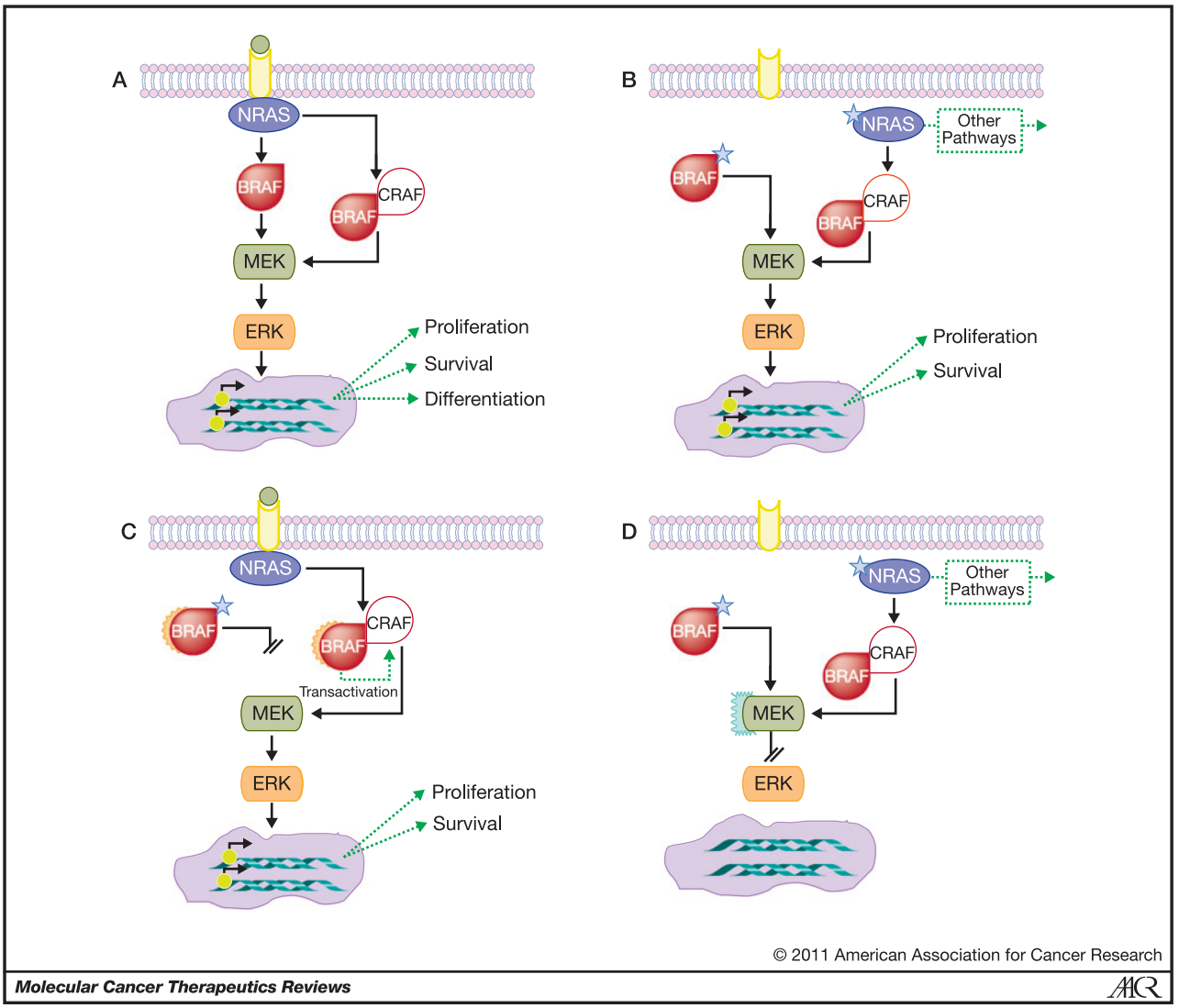

Figure 2. Targeted therapy in the BRAF pathway. A, the BRAF/MEK/ERK pathway. Extracellular signaling acts through RAS to activate BRAF, which signals through MEK to ERK culminating in activation of transcription factors involved in cell fate determination. B, mutations in the pathway lead to constitutive activation of the pathway. Activating mutation in $BRAF^{V600E}$ activates the MEK/ERK pathway in the absence of extracellular signaling. Activating mutations in RAS can stimulate MEK/ERK signaling through CRAF in the absence of extracellular signaling. BRAF and RAS mutations occur in a mutually exclusive fashion. (Note: Whereas BRAF relies on the MEK/ERK pathway for signaling, mutant RAS can act through a number of diverse pathways.) C, BRAF-specific inhibitors prevent BRAF signaling. Small-molecule BRAF-specific inhibitors block the kinase activity of BRAF, preventing it from activating its downstream targets. However, in the cells with wild-type BRAF or activating mutations in RAS, BRAF inhibition can result in the transactivation of CRAF, which can signal to MEK, resulting in reactivation of the pathway and acquired resistance to BRAF-specific inhibitors. D, targeting downstream of BRAF may circumvent the pathway reactivation associated with BRAF inhibition. Targeting MEK may be useful in tumors with activating BRAF or RAS mutations due to its strategic position in the pathway. However, the reliance of BRAF on the MEK/ERK pathway makes mutant BRAF tumors more sensitive to MEK inhibition than mutant RAS tumors, which can signal through multiple pathways.

do not activate the MEK/ERK pathway because they concurrently inhibit CRAF. Thus, a major distinction between BRAF-specific and pan-RAF drugs is that in the presence of increased RAS expression, melanoma cell lines treated with BRAF-selective drugs result in pathway reactivation but this is less likely to occur with pan-RAF drug treatment as the CRAF isoform is also inhibited. However, the increase in CRAF expression in response to pan-RAF drugs can mediate acquired resistance to the drugs in mutant BRAF melanoma cells lines (51). Interestingly, separate phase I clinical trials with BRAF-specific inhibitors both noted that 10% to 15% of patients treated with BRAF inhibitors developed squamous cell carcinoma, 22% of which harbored RAS mutations (50, 54). It has been postulated that BRAF-specific inhibitors may act as tumor promoters in premalignant skin cells with underlying RAS mutations (53). The release of results from phase II and III trials will aid in elucidating the role of BRAF in these subtle pathway regulations.

Inflammation is also a potential factor that needs to be accounted for the successful use of BRAF inhibitors. TNF-α is a pleiotropic cytokine that has been shown to block apoptosis in melanoma cells when BRAF signaling is inhibited (55). Macrophage infiltration has been correlated with tumor stage, progression, and angiogenesis in human malignant melanoma. Activated macrophages are strong secretors of TNF-α. The effect of TNF-α on melanoma cells depends on the context in which it acts. Epithelial malignancies have been associated with chronically produced TNF-α, whereas it can be tumor destructive in mice if high doses of TNF-α are administered therapeutically (56). Autocrine production of TNF-α in malignant melanoma has been associated with resistance to the cytolytic effects of TNF-α, a response that could have clinical implications (57). Melanoma cells treated with BRAF^{V600E} inhibitors resulted in both cytostatic and cytotoxic effects. Subsequent treatment with TNF-α resulted in apoptosis being blocked and recovery of the oncogenic cells to reenter the cell cycle (55). This effect was TNF-α specific and mediated by NF-κB signaling. NF-κB depletion blocks the ability of TNF-α to rescue BRAF-inhibited cells. The relations between the BRAF and NF-κB signaling pathways is not fully understood, nor is the interaction that leads to the specific rescue that allows TNF-α to override the effect of BRAF inhibition. However, the observation that TNF-α can rescue cells undergoing BRAF inhibition may be important if the microenvironment has a high degree of macrophage infiltration.

Targeting MEK in Mutant BRAF Tumors

The effectiveness of a targeted agent depends on where in the pathway the activating mutant occurs and what other pathways the aberrant protein is channeling. As outlined earlier, targeting BRAF can lead to pathway reactivation under certain genetic conditions. Thus, it may be of greater benefit to target downstream in the BRAF pathway. Because of the strategic position of the MEK1/2 kinases in the RAS/RAF/MEK/ERK pathway, it is a very promising target for new drug development (Fig. 2D). Although MEK is downstream of both RAS and RAF, Solit and colleagues showed that *braf* mutation, but not *ras* mutation, could predict sensitivity to MEK inhibition, indicating that *braf* mutations are more reliant on MEK/ERK signaling than *ras* mutants. The reliance of BRAF on the MEK/ERK pathway seems to make it more sensitive to MEK inhibition (58). Preclinical trials have shown that small-molecule MEK1/2 kinase inhibitors can significantly reduce the proliferation of a variety of cancer cell lines via the induction of apoptosis and cell-cycle arrest (58, 59). Thus, MEK seems to be a logical target for targeted therapy.

The first small-molecule MEK inhibitor to enter clinical trials was CI1040, which is highly selective for MEK1/2. This molecule exerts its effect by binding a hydrophobic pocket adjacent to the Mg-ATP binding site of the kinase. This results in the active site of the kinase becoming locked into a catalytically inactive conformation. The binding pocket is located in a region of low-sequence homology to other kinases, resulting in a high selectivity and noncompetitive kinetics of inhibition (60). However, it was found in phase II trials that CI1040 had poor pharmacodynamics and its development was stopped.

The second-generation MEK inhibitor PD0325901 is a structural analogue of CI1040. This MEK inhibitor had 100-fold greater activity than that of CI1040 and preclinical trials showed its ability to inhibit the growth of human tumor xenographs bearing mutant *braf* (58, 61). Phase I clinical trials seemed promising, but phase II trials displayed increased toxicity, more severe than that of CI1040. PD0325901 development was terminated because of these unacceptable toxicities (62, 63).

A second-generation inhibitor that is showing very promising results in clinical trial is the benzimidazole derivative AZD6244. This small-molecule inhibitor is selective for MEK1/2 by a mechanism that is not fully elucidated but occurs in a manner noncompetitive for ATP. Phase I trials produced very promising results, with one patient with malignant melanoma showing 70% tumor shrinkage (64). A phase II trial comparing AZD6244 to the standard treatment temozolomide in melanoma showed a survival advantage favoring AZD6244 in the subset of tumors with *braf* mutation, although there was no difference in the total progression-free survival between treatments. In this trial, the AZD6244 group had 6 confirmed responses, of which 5 carried mutant *braf* (65). In another phase I trial of AZD6244-based combination therapy, a positive trend was identified between *braf* mutation and clinical benefit (66). No such trend was identified for *nras* mutations. In this small study, all responders [as defined by RECIST (Response Evaluation Criteria in Solid Tumors) criteria] carried *braf* mutation and none of the subjects with *braf* mutation had early disease progression. There are currently more than 30 clinical trials ongoing (www.clinicaltrials.gov), involving AZD6244 in a variety of tumor types.

MEK inhibitors are very promising as targeted therapies in tumors with aberrant RAS/RAF/MEK/ERK pathway activation. MEK inhibitors are highly selective for their targets and those with improved pharmacodynamics are under development. Determining whether BRAF and/or MEK are a clinically viable level in the pathway to target will become clear as results from the ongoing and future trials are released. It is likely that any targeted therapies will be susceptible to the development of resistance due to secondary mutations in kinase domains that reduce drug binding while preserving catalytic function, activation of redundant signaling pathways, or upregulation or activation of a ligand or regulator of an alternative signaling pathway (67). Thus, it is likely that MEK inhibition, or indeed BRAF inhibition, will be less important as a single chemotherapeutic agent than it will be as a combination therapy. To this effect,

Merck and AstraZeneca announced a collaboration to test AZD6244 and the AKT inhibitor [phosphoinositide 3-kinase (PI3K) pathway] MK-2206 in 2009. It has been shown in preclinical trials for MEK inhibitors that PI3K activation can significantly decrease the response of *KRAS* mutants to MEK1/2 inhibition (68). Thereby, targeting both pathways should reduce the ability of the tumor to gain resistance. This unusual collaboration between pharmaceutical companies is more evidence of the expectation for the usefulness of these molecules in combination chemotherapy.

Conclusions

Combination therapy is likely to be the most effective management plan for the treatment of *braf*-mutated tumors. Many BRAF-specific inhibitors display a cytostatic response inducing senescence and are susceptible to acquired resistance (69, 70). Therefore, combination with traditional chemotherapeutic agents seems to be more effective than either treatment alone. Multiple signaling pathways are involved in tumor progression. BRAF mutation may be the activating mutation, but mutations subsequently acquired may also need to be targeted for successful treatment (71). For example, combined therapy blocking both the ERK and the PI3K pathways has been shown to be significantly more effective than blocking either pathway alone in a mouse model of melanoma (72). The strategy for treating mutant *braf* tumors will also rely on the tumor stage. Early tumor stage may be susceptible to BRAF-specific inhibition alone. However, as tumor stage progresses, as does the accumulation of mutations. Therefore, multiple pathways will need to be targeted in later-stage tumors (73).

Genotyping of tumors is of high importance when using molecular therapy. The *braf* mutation can predict sensitivity of a tumor to BRAF inhibition and to downstream MEK inhibition (58, 69). Correct use of molecular therapy is dependent on tumor genotyping not only for the identification of mutations that are susceptible to therapy but also for mutations that may confound therapeutic use of the inhibitor. The genotyping of tumors is also extremely important for clinical trials to ensure that the correct cohort is being assessed for drug response and to control for any underlying mutations that would confound results.

In summary, recent clinical trial results indicate that targeted therapy for mutant BRAF is a promising strategy (50, 54, 74). Both mutant $BRAF^{V600E}$ and its downstream target MEK seem to be effective targets for tumors with activating *braf* mutations. However, acquired resistance through subsequent reactivation of the pathway or activation of redundant pathways is a major issue that must be overcome before these inhibitors will be ready for routine use in the clinic.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

E.R Cantwell-Dorris is funded by the Health Research Board of Ireland's PhD Scholars Programme.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received August 25, 2010; revised November 15, 2010; accepted December 8, 2010; published online March 9, 2011.

References

1. Garnett M, Marais R. Guilty as charged: B-RAF is a human oncogene. Cancer Cell 2004;6:313–9.
2. Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, et al. Mutations of the BRAF gene in human cancer. Nature 2002;417:949–54.
3. Wan PT, Garnett MJ, Roe SM, Lee S, Niculescu-Duvaz D, Good VM, et al. Mechanism of activation of the RAF-ERK signaling pathway by oncogenic mutations of B-RAF. Cell 2004;116:855–67.
4. Robinson M, Cobb M. Mitogen-activated protein kinase pathways. Curr Opin Cell Biol 1997;9:180–6.
5. Mason CS, Springer CJ, Cooper RG, Superti-Furga G, Marshall CJ, Marais R. Serine and tyrosine phosphorylations cooperate in Raf-1, but not B-Raf activation. EMBO J 1999;18:2137–48.
6. Catling A, Schaeffer H, Reuter C, Reddy G, Weber M. A proline-rich sequence unique to MEK1 and MEK2 is required for raf binding and regulates MEK function. Mol Cell Biol 1995;15:5214–25.
7. Bamford S, Dawson E, Forbes S, Clements J, Pettett R, Dogan A, et al. The COSMIC (Catalogue of Somatic Mutations in Cancer) database and website. Br J Cancer 2004;91:355–8. Available from: http://www.sanger.ac.uk/cosmic.
8. Dhomen N, Marais R. BRAF signaling and targeted therapies in melanoma. Hematol Oncol Clin North Am 2009;23:529–45, ix.
9. Xing M. BRAF mutation in thyroid cancer. Endocr Relat Cancer 2005;12:245–62.
10. Gunson T, Hashim N, Sharpe G. Generalized lentiginosis, short stature, and multiple cutaneous nodules—quiz case. LEOPARD syndrome (LS) associated with multiple granular cell tumors (GCTs). Arch Dermatol 2010;146:337–42.
11. Urano Y, Asano T, Yoshimoto K, Iwahana H, Kubo Y, Kato S, et al. Frequent p53 accumulation in the chronically sun-exposed epidermis and clonal expansion of p53 mutant cells in the epidermis adjacent to basal cell carcinoma. J Invest Dermatol 1995;104:928–32.
12. Landi M, Bauer J, Pfeiffer R, Elder DE, Hulley B, Minghetti P, et al. MC1R germline variants confer risk for BRAF-mutant melanoma. Science 2006;313:521–2.
13. Curtin JA, Fridlyand J, Kageshita T, Patel HN, Busam KJ, Kutzner H, et al. Distinct sets of genetic alterations in melanoma. N Engl J Med 2005;353:2135–47.
14. Sturm R, Duffy D, Box N, Chen W, Smit DJ, Brown DL, et al. The role of melanocortin-1 receptor polymorphism in skin cancer risk phenotypes. Pigment Cell Res 2003;16:266–72.
15. Gerstenblith M, Goldstein A, Fargnoli M, Peris K, Landi M. Comprehensive evaluation of allele frequency differences of MC1R variants across populations. Hum Mutat 2007;28:495–505.

16. Fargnoli M, Pike K, Pfeiffer R, Tsang S, Rozenblum E, Munroe DJ, et al. MC1R variants increase risk of melanomas harboring BRAF mutations. J Invest Dermatol 2008;128:2485–90.
17. Abdel-Malek Z, Knittel J, Kadekaro A, Swope V, Starner R. The melanocortin 1 receptor and the UV response of human melanocytes—a shift in paradigm. Photochem Photobiol 2008;84:501–8.
18. Hacker E, Hayward N. Germline MC1R variants and BRAF mutant melanoma. J Invest Dermatol 2008;128:2354–6.
19. Conner SR, Scott G, Aplin AE. Adhesion-dependent activation of the ERK1/2 cascade is by-passed in melanoma cells. J Biol Chem 2003;278:34548–54.
20. Bhatt KV, Spofford LS, Aram G, McMullen M, Pumiglia K, Aplin AE. Adhesion control of cyclin D1 and p27Kip1 levels is deregulated in melanoma cells through BRAF-MEK-ERK signaling. Oncogene 2005;24:3459–71.
21. Buscà R, Abbe P, Mantoux F, Aberdam E, Peyssonnaux C, Eychène A, et al. Ras mediates the cAMP-dependent activation of extracellular signal-regulated kinases (ERKs) in melanocytes. EMBO J 2000; 19:2900–10.
22. Boyle P, Langman J. ABC of colorectal cancer: epidemiology. BMJ 2000;321:805–8.
23. Kinzler KW, Vogelstein B. Lessons from hereditary colorectal cancer. Cell 1996;87:159–70.
24. Fransén K, Klintenäs M, Osterström A, Dimberg J, Monstein H, Söderkvist P. Mutation analysis of the BRAF, ARAF and RAF-1 genes in human colorectal adenocarcinomas. Carcinogenesis 2004;25: 527–33.
25. Rajagopalan H, Bardelli A, Lengauer C, Kinzler K, Vogelstein B, Velculescu V. Tumorigenesis: RAF/RAS oncogenes and mismatch-repair status. Nature 2002;418:934.
26. Velho S, Moutinho C, Cirnes L, Albuquerque C, Hamelin R, Schmitt F, et al. BRAF, KRAS and PIK3CA mutations in colorectal serrated polyps and cancer: primary or secondary genetic events in colorectal carcinogenesis?BMC Cancer 2008;8:255.
27. Benhattar J, Losi L, Chaubert P, Givel J, Costa J. Prognostic significance of K-ras mutations in colorectal carcinoma. Gastroenterology 1993;104:1044–8.
28. Kikuchi H, Pino M, Zeng M, Shirasawa S, Chung D. Oncogenic KRAS and BRAF differentially regulate hypoxia-inducible factor-1alpha and -2alpha in colon cancer. Cancer Res 2009;69:8499–506.
29. Weisenberger D, Siegmund K, Campan M, Young J, Long TI, Faasse MA, et al. CpG island methylator phenotype underlies sporadic microsatellite instability and is tightly associated with BRAF mutation in colorectal cancer. Nat Genet 2006;38:787–93.
30. Deng G, Chen A, Hong J, Chae HS, Kim YS. Methylation of CpG in a small region of the hMLH1 promoter invariably correlates with the absence of gene expression. Cancer Res 1999;59:2029–3.
31. Park S, Rashid A, Lee J, Kim S, Hamilton S, Wu T. Frequent CpG island methylation in serrated adenomas of the colorectum. Am J Pathol 2003;162:815–22.
32. Yang S, Farraye F, Mack C, Posnik O, O'Brien M. BRAF and KRAS Mutations in hyperplastic polyps and serrated adenomas of the colorectum: relationship to histology and CpG island methylation status. Am J Surg Pathol 2004;28:1452–9.
33. Dong S, Lee E, Jeon E, Park C, Kim K. Progressive methylation during the serrated neoplasia pathway of the colorectum. Mod Pathol 2005;18:170–8.
34. Tateyama H, Li W, Takahashi E, Miura Y, Sugiura H, Eimoto T. Apoptosis index and apoptosis-related antigen expression in serrated adenoma of the colorectum: the saw-toothed structure may be related to inhibition of apoptosis. Am J Surg Pathol 2002;26:249–56.
35. Erhardt P, Schremser EJ, Cooper GM. B-Raf inhibits programmed cell death downstream of cytochrome c release from mitochondria by activating the MEK/Erk pathway. Mol Cell Biol 1999;19:5308–15.
36. Ikehara N, Semba S, Sakashita M, Aoyama N, Kasuga M, Yokozaki H. BRAF mutation associated with dysregulation of apoptosis in human colorectal neoplasms. Int J Cancer 2005;115:943–50.
37. Minoo P, Moyer M, Jass J. Role of BRAF-V600E in the serrated pathway of colorectal tumourigenesis. J Pathol 2007;212:124–33.
38. Pruitt K, Ulkü A, Frantz K, Rojas RJ, Muniz-Medina VM, Rangnekar VM, et al. Ras-mediated loss of the pro-apoptotic response protein Par-4 is mediated by DNA hypermethylation through Raf-independent and Raf-dependent signaling cascades in epithelial cells. J Biol Chem 2005;280:23363–70.
39. Hundahl S, Fleming I, Fremgen A, Menck H. A National Cancer Data Base report on 53,856 cases of thyroid carcinoma treated in the U.S. 1985–1995[see comments]. Cancer 1998;83:2638–48.
40. Santoro M, Melillo R, Carlomagno F, Fusco A, Vecchio G. Molecular mechanisms of RET activation in human cancer. Ann N Y Acad Sci 2002;963:116–21.
41. Soares P, Trovisco V, Rocha AS, Lima J, Castro P, Preto A, et al. BRAF mutations and RET/PTC rearrangements are alternative events in the etiopathogenesis of PTC. Oncogene 2003;22:4578–80.
42. Ciampi R, Nikiforov Y. RET/PTC rearrangements and BRAF mutations in thyroid tumorigenesis. Endocrinology 2007;148:936–41.
43. Tang K, Lee C. BRAF mutation in papillary thyroid carcinoma: pathogenic role and clinical implications. J Chin Med Assoc 2010; 73:113–28.
44. Liu D, Hu S, Hou P, Jiang D, Condouris S, Xing M. Suppression of BRAF/MEK/MAP kinase pathway restores expression of iodide-metabolizing genes in thyroid cells expressing the V600E BRAF mutant. Clin Cancer Res 2007;13:1341–9.
45. Durante C, Puxeddu E, Ferretti E, Morisi R, Moretti S, Bruno R, et al. BRAF mutations in papillary thyroid carcinomas inhibit genes involved in iodine metabolism. J Clin Endocrinol Metab 2007;92:2840–3.
46. Scipioni A, Ferretti E, Soda G, Tosi E, Bruno R, Costante G, et al. hNIS protein in thyroid: the iodine supply influences its expression and localization. Thyroid 2007;17:613–8.
47. Houben R, Becker J, Kappel A, Terheyden P, Bröcker EB, Goetz R, et al. Constitutive activation of the Ras-Raf signaling pathway in metastatic melanoma is associated with poor prognosis. J Carcinog 2004;3:6.
48. Sawyers C. Targeted cancer therapy. Nature 2004;432:294–7.
49. Tsai J, Lee JT, Wang W, Zhang J, Cho H, Mamo S, , et al. Discovery of a selective inhibitor of oncogenic B-Raf kinase with potent antimelanoma activity. Proc Natl Acad Sci U S A 2008;105: 3041–6.
50. Flaherty K, Puzanov I, Kim K, Ribas A, McArthur GA, Sosman JA, et al. Inhibition of mutated, activated BRAF in metastatic melanoma. N Engl J Med 2010;363:809–19.
51. Montagut C, Sharma S, Shioda T, McDermott U, Ulman M, Ulkus LE, et al. Elevated CRAF as a potential mechanism of acquired resistance to BRAF inhibition in melanoma. Cancer Res 2008;68:4853–61.
52. Poulikakos P, Zhang C, Bollag G, Shokat K, Rosen N. RAF inhibitors transactivate RAF dimers and ERK signalling in cells with wild-type BRAF. Nature 2010;464:427–30.
53. Heidorn SJ, Milagre C, Whittaker S, Nourry A, Niculescu-Duvas I, Dhomen N, et al. Kinase-dead BRAF and oncogenic RAS cooperate to drive tumor progression through CRAF. Cell 2010;140:209–21.
54. Schwartz GK, Robertson S, Shen A, Wang E, Pace L, Dials H, et al. A phase I study of XL281, a selective oral RAF kinase inhibitor, in patients (Pts) with advanced solid tumors. ASCO Meet Abstr 2009;27:3513.
55. Gray-Schopfer VC, Karasarides M, Hayward R, Marais R. Tumor necrosis factor-{alpha} blocks apoptosis in melanoma cells when BRAF signaling is inhibited. Cancer Res 2007;67:122–9.
56. Moore RJ, Owens DM, Stamp G, Arnott C, Burke F, East N, et al. Mice deficient in tumor necrosis factor-[alpha] are resistant to skin carcinogenesis. Nat Med 1999;5:828–31.
57. Bergenwald C, Westermark G, Sander B. Variable expression of tumor necrosis factor alpha in human malignant melanoma localized by *in situ* hybridization for mRNA. Cancer Immunol Immunother 1997;44:335–40.
58. Solit D, Garraway L, Pratilas C, Sawai A, Getz G, Basso A, et al. BRAF mutation predicts sensitivity to MEK inhibition. Nature 2006;439:358–62
59. Sebolt-Leopold JS, Dudley DT, Herrera R, Van Becelaere K, Wiland A, Gowan RC, et al. Blockade of the MAP kinase pathway suppresses growth of colon tumors *in vivo*. Nat Med 1999;5:810–6.

60. Fremin C, Meloche S. From basic research to clinical development of MEK1/2 inhibitors for cancer therapy. J Hematol Oncol 2010;3:8.
61. Sebolt-Leopold JS, Herrera R. Targeting the mitogen-activated protein kinase cascade to treat cancer. Nat Rev Cancer 2004; 4:937–47.
62. Wang J, Wilcoxen K, Nomoto K, Wu S. Recent advances of MEK inhibitors and their clinical progress. Curr Top Med Chem 2007; 7:1364–78.
63. LoRusso PM, Krishnamurthi SS, Rinehart JJ, Nabell LM, Malburg L, Chapman PB, et al. Phase I pharmacokinetic and pharmacodynamic study of the oral MAPK/ERK kinase inhibitor PD-0325901 in patients with advanced cancers. Clin Cancer Res 2010;16: 1924–37.
64. Adjei AA, Cohen RB, Franklin W, Morris C, Wilson D, Molina JR, et al. Phase I pharmacokinetic and pharmacodynamic study of the oral, small-molecule mitogen-activated protein kinase kinase 1/2 inhibitor AZD6244 (ARRY-142886) in patients with advanced cancers. J Clin Oncol 2008;26:2139–46.
65. Board RE, Ellison G, Orr MCM, Kemsley KR, McWalter G, D , et al. Detection of BRAF mutations in the tumour and serum of patients enrolled in the AZD6244 (ARRY-142886) advanced melanoma phase II study. Br J Cancer 2009;101:1724–30.
66. Patel SP, Lazar AJ, Mahoney S, Vaughn C, Gonzalez N, Papadopoulos NE, et al. Clinical responses to AZD6244 (ARRY-142886)-based combination therapy stratified by gene mutations in patients with metastatic melanoma. J Clin Oncol 2010;Suppl:abstr8501.
67. Janne PA, Gray N, Settleman J. Factors underlying sensitivity of cancers to small-molecule kinase inhibitors. Nat Rev Drug Discov 2009;8:709–23.
68. Wee S, Jagani Z, Xiang KX, Loo A, Dorsch M, Yao YM, et al. PI3K Pathway activation mediates resistance to MEK inhibitors in KRAS mutant cancers. Cancer Res 2009;69:4286–93.
69. Wellbrock C, Hurlstone A. BRAF as therapeutic target in melanoma. Biochem Pharmacol 2010;80:561–7.
70. Hatzivassiliou G, Song K, Yen I, Brandhuber BJ, Anderson DJ, Alvarado R, et al. RAF inhibitors prime wild-type RAF to activate the MAPK pathway and enhance growth. Nature 2010;464:431–5
71. Bollag G, Hirth P, Tsai J, Zhang J, Ibrahim PN, Cho H, et al. Clinical efficacy of a RAF inhibitor needs broad target blockade in BRAF-mutant melanoma. Nature 2010;467:596–9
72. Bedogni B, Welford S, Kwan A, Ranger-Moore J, Saboda K, Powell M. Inhibition of phosphatidylinositol-3-kinase and mitogen-activated protein kinase kinase 1/2 prevents melanoma development and promotes melanoma regression in the transgenic TPRas mouse model. Mol Cancer Ther 2006;5:3071–7.
73. Smalley K, Haass N, Brafford P, Lioni M, Flaherty K, Herlyn M. Multiple signaling pathways must be targeted to overcome drug resistance in cell lines derived from melanoma metastases. Mol Cancer Ther 2006;5:1136–44.
74. Gupta-Abramson V, Troxel AB, Nellore A, Puttaswamy K, Redlinger M, Ransone K, et al. Phase II trial of sorafenib in advanced thyroid cancer. J Clin Oncol 2008;26:4714–9.

Molecular Cancer Therapeutics

$BRAF^{V600E}$: Implications for Carcinogenesis and Molecular Therapy

Emma R. Cantwell-Dorris, John J. O'Leary and Orla M. Sheils

Mol Cancer Ther 2011;10:385-394.