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## Targeted inhibition of BRAF kinase: opportunities and challenges for therapeutics in melanoma

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### Synopsis

Malignant melanoma is the most aggressive form of skin cancer and its incidence has increased dramatically in the past two decades. Even with a high rate of success in the treatment of early stages of this malignancy, currently there are no effective strategies for the treatment of advanced metastatic melanoma. Much effort has been put into the use of different target-specific drugs amongst which BRAF kinase-specific small molecule inhibitors have rendered promising results as therapeutic agents in metastatic melanoma. Nonetheless, some side effects, such as development of squamous cell carcinoma, as well as tumor resistance and recurrence are common limitations of this therapeutic strategy. The use of combination treatments in which different regulatory pathways or the immunological response are targeted seems promising in the future of melanoma therapeutics.

### Keywords

BRAF inhibitors; Combination therapy; MAP kinases; Melanoma; Tumorigenesis

### INTRODUCTION

Melanoma is a major form of skin cancer and arises from malignant transformation of melanocytes, specialized black-pigmented cells producing melanins [1, 2]. In general, skin cancer is the most common cancer in the United States and melanoma, which accounts for about 5% of all the cancers of the skin, is the most aggressive form. Its incidence is increasing faster than that of any other cancer and has more than doubled in the past 20 years. The lifetime risk for melanoma is one in 68. The American Cancer Society predicted 68,130 new cases of melanoma and 8,700 consequential deaths in 2010 while no more than 0.1% of the more common non-melanoma skin cancers will result in death. Risk factors for melanoma include sun exposure, tanning salon use, pale skin, red-hair, inability to tan, tendency to sunburn, increased numbers of freckles and moles, and the presence of dysplastic nevi [2–4]. If detected and surgically removed at an early stage, the cure rate for melanoma is approximately 90%. However, there is no effective treatment for melanoma metastases, and patients with this metastatic disease have a short life expectancy with a 5-year survival rate of 11% and a median survival of 6 to 12 months [5]. The high propensity to metastasize in addition to the rapidly rising incidence and morbidity rate of melanoma underscores the urgency to better understand its pathogenesis and identify effective therapeutic targets and strategies.

## BRAF KINASE: A POTENTIAL THERAPEUTIC TARGET IN MELANOMA

The RAS-RAF-MEK-ERK MAP kinases pathway has a central role in the biology of various cell types, including melanocytes, the cell type from which a melanoma arises. Stimulation of different membrane-bound receptors, mainly tyrosine kinases and G-protein coupled receptors, promotes activation of RAS and this activates RAF kinases (ARAF, BRAF and CRAF). Activated RAF then sequentially activates MEK and phosphorylates ERK, which in turn targets different cytoplasmic and nuclear molecules involved in proliferation, differentiation and cell survival [6–8]. Davies and coworkers [9] reported a high frequency of BRAF somatic missense mutation within the kinase domain in melanoma tumor cells. A single amino acid substitution, V600E, accounts for at least 80% of those BRAF mutations [9–11] and results in a protein with RAS-independent elevated kinase activity by mimicking BRAF phosphorylation in the activation segment [12]. This mutation also confers on BRAF the ability to transform cells, which are tumorigenic *in vivo* [9]. In addition to Val to Glu substitution, other mutations at this position such as, V600K and V600E have also been reported with variable frequency [11, 13, 14]. In addition to melanoma, mutations in BRAF are also quite frequent in thyroid (40–70 %) and colorectal cancers (5–20%) [9, 15, 16].

Melanoma cells harboring BRAF mutations depend on activated BRAF for their growth and maintenance. It has been shown that silencing BRAF activity by RNA interference blocks ERK activity and inhibits DNA synthesis causing reduced growth and increased apoptosis of melanoma cells *in vitro* [17–20]. Moreover, this siRNA-mediated block of BRAF<sup>V600E</sup> inhibits tumor development in xenograft models [20]. In addition, silencing of mutant BRAF inhibits melanoma cell extravasations in an *in vitro* flow migration model and the development of lung metastases *in vivo* [19].

The high frequency of BRAF mutations in melanoma as well as the critical role of BRAF in tumor proliferation, survival and malignancy suggested that BRAF is a potentially valuable molecular target and has led to the development of BRAF kinase inhibitors for targeted therapy particularly in the treatment of metastatic melanoma.

## PRECLINICAL STUDIES ON USING BRAF-SPECIFIC INHIBITORS IN MELANOMA

One of the first attempts targeting the serine-threonine protein kinase BRAF pathway as a therapeutic intervention in melanoma was the development of the small molecule multikinase inhibitor sorafenib which inhibits ERK activation, cell proliferation and induces apoptosis in cultured cells [18, 21]. This drug was originally designed as a C-RAF kinase inhibitor; however it was demonstrated that it also inhibits the B-RAF kinase as well as VEGFR-2, PDGFR- and c-Kit receptor tyrosine kinases (RTK) among others [21, 22]. When tested *in vitro* in an extensive panel of melanoma cell lines, no correlation was observed between sensitivity to sorafenib and BRAF mutation status [23]. Besides, it has been unequivocally demonstrated that its antitumor effects are not due to specific inhibition of oncogenic BRAF [24], suggesting that the down regulation of the RAF/MEK/ERK pathway and the anti-tumoral effects are probably due to inhibition of various RTK targets or CRAF [21–23].

Based on the above-described high frequency of activating V600E mutations in the BRAF kinase and the so-called “BRAF addiction” in melanoma, different small molecule BRAF-specific inhibitors have been developed based on co-crystallography and chemical scaffolding technology which seems especially well-suited for kinase inhibitor design due to the conserved conformation of the kinase domain [25]. Among these small molecule BRAF-

kinase specific inhibitors, PLX4720 and its homologue PLX4032 (also known as RG7204) as well as GDC-0879, GSK2118436 and AZ628 are specific inhibitors of BRAF<sup>V600E</sup> kinase activity at significantly lower concentrations than their inhibitory effect in wild-type (WT) BRAF [26–30]. Treatment of an extensive collection of melanoma cell lines with these BRAF inhibitors has shown a consistent inhibition of cell viability and cell growth with selectivity for the BRAF<sup>V600E</sup> mutant exceeding 100-fold over the WT BRAF, suggesting that these drugs have anti-melanoma activity only against cells that harbor BRAF<sup>V600E</sup> [26, 28–33]. Upon treatment with PLX4720, PLX4032 or GDC-0879, BRAF<sup>V600E</sup> mutant cells show a decrease in phosphorylation of ERK [29, 31, 34–36] and MEK [33, 36, 37] that indicates inactivation of the MAPK pathway [26, 28, 32]. The effect of GDC-0879 on global gene expression in A375 cells, particularly on those involved in cell proliferation, has been shown to be very similar to that observed with BRAF blockade by siRNA [29]. PLX4720/PLX4032 treated BRAF mutant melanoma cells undergo cell cycle arrest in G1 phase with a reduction in cyclin D1 expression and increase in p27 expression. These changes do not occur in WT BRAF or NRAS mutated melanoma cells [32, 35, 36], regardless of zygosity [37]. Furthermore, cells more sensitive to PLX4032 growth inhibitory effects are affected in a cytotoxic manner as demonstrated by an increase in apoptosis and cleavage of PARP after treatment with this drug [34–36]. Interestingly, PLX4032 treatment was shown to induce the expression of melanocyte-specific genes (*TYR*, *TYRP1* and *MITF* among others) as well as genes associated with melanosome function in BRAF-mutated cell lines, such as *RAB27A*, *MYO5* and *RILP* [37]. Therefore PLX4032 not only inhibits proliferation and survival but also may lead to resumed melanin production by counteracting the mutant BRAF-induced melanocytic differentiation arrest. Thus the inhibition of pERK may relieve the inhibition on melanogenesis and explain why differentiation markers specific to melanin production and transport are increased after treatment with PLX4032 [37].

Although small molecule BRAF-kinase specific inhibitors were promising as a therapeutic alternative for metastatic melanoma in BRAF<sup>V600E</sup> bearing melanoma cell lines, it was unexpectedly found that PLX4720, PLX4032 and GDC-0879 cause an increase in viable cell numbers in WT BRAF or NRAS mutant cell lines that is associated with a markedly increased MAPK pathway activation [28, 31, 34, 38, 39]. The molecular mechanisms involved in this increased activation of the MAPK pathway in WT BRAF or NRAS mutant melanoma cell lines remain to be fully elucidated. Nonetheless, it has been suggested that selective BRAF inhibition induces RAS-dependent dimerization of CRAF with WT BRAF, but not BRAF<sup>V600E</sup>, as well as the formation of CRAF homodimers, leading to subsequent activation of CRAF and downstream MEK-ERK signaling (Figure 1) [8, 28, 38, 39].

While characterizing the biological responses associated to these completely opposed biochemical and proliferative responses to PLX compounds between BRAF<sup>V600E</sup> bearing cell lines and those with a WT gene, Halaban *et al.* [31], demonstrated that PLX4032 induces proliferation in WT BRAF melanoma cells treated after culture in suboptimal conditions. Furthermore, WT melanoma cells show a time-dependent enhanced detachment, reduced cell adhesion and enhanced motility after treatment with PLX4032 for up to 24h hours (with 99% of viability), effects that correlate with increased phosphorylation of focal adhesion kinase at the ERK phosphorylation site S910 [31]. This suggests that PLX4032 actually may confer an advantage for proliferation and enhanced metastatic capability to cells bearing non-mutated BRAF.

In different animal models of melanoma, mostly based on orthologous growth of human malignant melanoma cell lines in immunocompromised mice (xenografts), PLX4720, PLX4032 or GDC-0879 treatment have resulted in a significant reduction in tumor growth and in all cases this correlated with a high percentage of inhibition of ERK phosphorylation

[26, 28, 29, 33]. At higher doses, PLX4720 induces regression of these grafted melanoma tumors. Conversely, WT BRAF bearing tumors are unaffected by the treatment or even exhibit accelerated tumor growth [26, 28]. Importantly, in these xenograft models using highly aggressive melanoma cells, the oral administration of a crystalline form of PLX4032 has proven to be of low efficacy while a microprecipitated-bulk powder (MBP), formulation that increases drug exposure, has a dose-dependent positive effect as an anti-tumor drug [36]. In addition, no toxic effects were seen in these animal models. Low doses induce regression but recurrence, an effect that was not seen when a higher dose was used, suggesting that the maximum tolerated dose in the clinical settings should be used to achieve the most beneficial result [36, 40].

## THE CLINICAL TRIALS WITH BRAF-SPECIFIC INHIBITORS IN METASTATIC MELANOMA: THE PROMISE

Based on the successful results derived from preclinical studies using BRAF kinase specific inhibitors, several clinical trials have been carried out with these potential therapeutics. Among them, PLX4032 and GSK2118436 have shown strong promise in the early stages of clinical development [30, 41–45].

Despite the very similar preclinical results between PLX4032 and its analogue PLX4720, the former has been chosen due to more favorable pharmacokinetics properties [40]. In agreement with the preclinical data [36], in a dose-escalation study using an MBP formulation, PLX4032 proved to be more effective and had a much higher bioavailability than the original formulation [40, 43, 45]. About 69% and 81% of the patients with BRAF<sup>V600E</sup> bearing melanomas treated with PLX4032 showed at least partial objective responses (based on RECIST, Response Evaluation Criteria in Solid Tumors) in the Phase I dose-escalation and extension phases, respectively, with latency in a range between 2 to more than 18 months. The estimated median progression-free survival among these patients is estimated to be at least 7 months [43, 45]. In agreement with the preclinical studies, this positive response correlates with inhibition of the MAPK kinase pathway that induces a decrease in cyclin D1 levels and ultimately decreased proliferative responses within the tumors. Moreover, since the inhibition of cytoplasmic pERK is greater than 80% in patients with tumor regression, it seems that near-complete inhibition of ERK signaling may be needed to achieve a significant anti-tumor response [40, 46]. Together, these *in vitro* and *in vivo* mechanistic data predict that BRAF kinase inhibitors will not inhibit ERK signaling in normal cells and therefore the toxicity associated to MAPK inhibition would be low. Thus, it seems possible to reach greater clinical efficacy based on the possibility of the use of doses that are high enough to reach more complete ERK signaling inhibition with lower toxic effects [34]. Furthermore, these studies demonstrated again the high specificity of PLX4032 since patients with metastatic melanoma without BRAF mutations did not respond to the treatment, and even had progression of this disease during the treatment [40, 43, 45]. Currently, a phase III clinical study of PLX4032 is ongoing to assess the overall survival (OS) and progression-free survival (PFS) in melanoma patients with BRAF mutations, as compared to patients treated with the current standard of care.

GSK2118436 is another BRAF inhibitor in active clinical development. Recent data from a phase I clinical trial of GSK2118436 revealed a > 20% tumor decrease by RECIST at 8 to 9 weeks in about 60% of patients with BRAF<sup>V600E</sup> melanomas [30, 41]. Preliminary data from an ongoing phase I/II trial of this drug suggests that GSK2118436 may be effective against brain metastases in patients with tumors bearing mutant BRAF, rendering a promising therapy for metastatic melanoma patients [42].

Despite the positive responses and therefore encouraging results of the treatment with PLX4032, some adverse effects, which were proportional to the dose and exposure to the drug, were observed at high doses [43, 45]. The most common being arthralgia, rash, nausea, phosphosensitivity, fatigue, pruritus and palmo-plantar dysesthesia. Similar adverse effects were also commonly seen during the clinical trial with GSK2118436 [41]. Nevertheless, none of these adverse effects prompted the discontinuation of treatment [43, 45]. Importantly, 31% of the patients treated with PLX4032 at higher yet well-tolerated doses, developed cutaneous squamous cell carcinoma (SCC) mainly of the keratoacanthoma type within a median time of 8 weeks of treatment initiation [43, 45, 47]. The molecular mechanisms underlying the development of SCC in these PLX4032-treated melanoma patients are under active investigations. It has been speculated that the paradoxical activation of RAF-MEK-ERK signaling pathway by PLX4032 in the WT BRAF cells might be involved [15, 43]. Activating RAS mutations and hyperactivation of the ERK signaling pathway are known to play a very important role in the initiation of SCC [48–50]. As discussed above, when WT BRAF cells bearing activating RAS mutations are treated with BRAF-specific inhibitors, a BRAF/CRAF heterodimerization induces activation of the MAPK pathway via CRAF [8, 28, 38, 39] (Figure 1). This could result in an increased proliferative response in the epidermis leading to the development of SCC. Therefore it seems possible that some pre-existing oncogenic mutations in keratinocytes of the skin, such as those in RAS proteins, can potentiate the effect of BRAF inhibitors leading to SCC development [15, 51]. Cutaneous SCC as a result of melanoma therapy with BRAF inhibitors are considered innocuous and are surgically removable. They are confined to the skin, mainly in areas that are subjected to sun exposure, and no metastatic evolution has been reported [15, 43]. However, the potential for SCC development in other locations or perhaps other cancer types during long-term treatment must be considered. Therefore, it is necessary to further investigate this toxicity to fully understand the mechanisms involved in its development and ultimately to identify therapeutic strategies to prevent it.

## ACQUIRED AND INTRINSIC DRUG RESISTANCE TO BRAF INHIBITORS: A MAJOR DRAWBACK

As discussed, a high percent of patients with melanomas carrying activating BRAF<sup>V600E</sup> mutations treated with PLX4032, currently the most promising BRAF kinase inhibitor under clinical trials, respond to treatment at least partially at all sites of metastasis with as yet unknown durability of the response [40, 45]. Even when these clinical results are very encouraging, one must remember the high specificity of PLX4032 and other BRAF inhibitors for BRAF<sup>V600E</sup> bearing tumors. In addition to WT BRAF bearing tumors, there is a fraction (around 20%) of patients with BRAF mutated melanoma tumors that are not responsive at all due to an intrinsic resistance, and another population in which tumors reappear due to the generation of acquired resistance during the course of treatment [35, 36, 45]. The mechanisms for intrinsic and acquired resistance to BRAF inhibitors therapy are currently under intense investigation.

In order to understand the intrinsic resistance to BRAF-specific inhibitors in mutant BRAF cells and tumors, one must consider that tumor cells are heterogeneous entities and particular attention should be paid to the fact that there may be different genetic alterations in cell proliferation pathways that can bypass BRAF inhibition. Genomic alterations in the PI3K/Akt pathway, including deletions in *PTEN* and increase of Akt3 activity have been described in resistant cell lines and tumors [35, 52, 53]. Moreover, it has been proposed that overexpression of cyclin D1, probably due to *CCND1* amplification, in combination with activating mutations in *CDK4* may contribute to resistance to BRAF-specific inhibitors in BRAF<sup>V600E</sup> bearing melanoma cells [54].

Acquired resistance to BRAF-specific inhibitors is one of the greatest challenges for these targeted therapies. Emerging evidence point toward the reactivation of the RAF-MEK-ERK pathway in an “oncogene bypass” manner as a major mechanism, with no secondary BRAF mutations involved [32, 55–57]. However, it is likely that there are a number of mechanisms involved in the recovery of the MEK-ERK activation during the development of resistance to BRAF inhibitors (Figure 2). Amongst these mechanisms, it has been shown that in some PLX4032-resistant melanoma cell lines as well as in cells derived from a subset of resistant tumors from treated patients which resist PLX4032-induced downregulation of the MEK-ERK pathway, this resistance is dependent on activating NRAS mutations [56]. On the other hand, resistance to BRAF inhibitors may be associated with elevated ARAF and CRAF protein levels and pERK activity in some cases. When this occurs, the BRAF dependence is lost and tumor cells switch their oncogenic addiction to another RAF kinase [8, 58, 59]. This is indeed in line with the hypothesis that cells only require one active RAF isoform to activate the MAPK pathway [59]. Lastly, acquired gain-of-function mutations in MEK1 have been reported as a result of the treatment with AZD6244 and also confer cross-resistance to PLX4720 [60].

Negative regulators of the RAF-MEK-ERK pathways might be also involved in the development of acquired resistance to BRAF inhibitors. For example, the genetic signature in several cell lines showing acquired resistance revealed inhibition of expression of the MAPK phosphatases (DUSP) 4, 5 and 6 and Sprouty proteins (SPRY) 2 and 4 [37]. These proteins are important negative regulators of the RAS/RAF signaling pathway. Thus MAPK activity in melanoma cell lines that are resistant to BRAF inhibitors may be insensitive to the physiologic negative feedback inhibition provided by DUSP and SPRY [37, 61].

More recently, yet another potential mechanism to reactivate the RAF-MEK-ERK pathway for acquired resistance to BRAF inhibitors in melanoma cell lines has been described. This mechanism involves COT protein kinase (also known as MAP3K8 or Tpl2) [62], which is a MAPK agonist that activates ERK in a MEK-dependent, but RAF-independent manner [63] (Figure 2). Therefore it is possible that BRAF inhibition potentiates the outgrowth of COT-expressing cells during the course of treatment [62]. In fact, there is a correlation between COT expression and acquired resistance to BRAF inhibitors in tissue from patients with relapsing tumors [62].

In addition to re-activation of RAF-MEK-ERK signaling pathway, it is likely that resistance acquisition is dependent on other signaling pathways that are involved in the regulation of cancer cell proliferation and survival. It has been proposed that acquired resistance to BRAF-specific inhibitors may be partially associated with the activation of the PI3K-PTEN-Akt pathway [37] (Figure 2). In this regard, it was shown that mutations in PTEN can affect responses to BRAF inhibitors. Elevated levels of Akt phosphorylation due to loss of PTEN are seen in melanoma cells resistant to PLX4720 whereas inhibition of class I PI3K enhances responses to BRAF inhibitors [37, 52, 59, 64]. Moreover, Receptor Tyrosine Kinase (RTK)-mediated activation of alternative survival pathways has been described as another important mechanism of acquired resistance to BRAF inhibitors [56, 59]. In this regard, it was shown that resistance to the BRAF inhibitor PLX4032, in cell lines and patient-derived tumor cells that do not exhibit reactivation of the RAF-MEK-ERK pathway, can instead be acquired through up-regulation of PDGFR [56]. In fact, induction of PDGFR RNA, as well as protein and tyrosine phosphorylation with no reactivation of the MAPK pathway is a dominant feature of these resistant cells [56]. In a different study examining the levels of various RTKs in BRAF inhibitor-resistant melanoma cells, it was described that these cells up-regulate IGF-1R surface expression and phosphorylation at the posttranscriptional level. Interestingly, pharmacological inhibition of IGF-1R abrogated viability in these melanoma cells that were resistant to BRAF inhibitors. Furthermore,

persistent IGF-1R signaling induces PI3K/Akt activation in these resistant cells whereas treatment with an IGF1R inhibitor blocked Akt phosphorylation with no inhibition of ERK [59]. Of note, it was found that increased expression on IGF-1R and pAkt correlated with resistance to BRAF inhibitors in one of five tissue samples from relapsed patients [52, 59]. Finally, as mentioned before, activating mutations in RAS may be at least partially responsible for resistance to BRAF inhibitors in melanoma cells [56]. However, the RAS-dependent reactivation of the MAPK pathway [56] may not be the only mechanism of resistance. Actually, RAS also signals through activation of PI3K [51, 65], an event that can be responsible for acquired resistance to BRAF specific inhibitors [51, 52, 59, 64].

## RATIONAL COMBINATORIAL THERAPY: THE FUTURE

Target-specific therapeutics for cancer represents a very useful weapon against different forms of malignancy. As described throughout this review, both preclinical and clinical studies targeting mutated BRAF have rendered encouraging information toward the future of melanoma treatment, especially the metastatic form for which there is no current effective therapeutic strategy. However, the toxicities associated with BRAF inhibitors therapy, such as the appearance of cutaneous SCC and, both intrinsic and secondary to exposure resistance, which seem likely to be mechanistically related, need to be overcome. Increased proliferative responses in WT BRAF melanoma and non-melanoma cells as well as resistance to the inhibitory effects of BRAF inhibitors are likely to be a multifactorial process in which different biochemical pathways may be involved. Rational combination strategies that target oncogenic pathways along with BRAF have been proposed to overcome limitations associated with BRAF inhibitor single agent therapy [5, 43]. For example, when acquired resistance to PLX4032 is at least partially dependent on restoration of the MAPK signaling, treatment with the MEK inhibitor U0126 together with PLX4032 will not only counteract this resistance but also prevents the development of resistance [29, 32, 61, 66]. In fact, a phase I clinical trial combining the BRAF inhibitor GSK2118436 and the MEK inhibitor GSK1120212 is underway (<http://clinicaltrials.gov/ct2/show/NTC01072175>). Similarly, because of the potential involvement of the PI3K-PTEN-AKT pathway in developing acquired resistance, it has been suggested that the PI3K-PTEN-AKT pathway could be one of the additional targets to be included in combination therapies [29, 31, 64]. It has been reported that simultaneous MEK and PI3K inhibition leads to cytotoxicity in certain melanoma cells that are resistant to BRAF inhibitors [59], providing a rational basis for this combination therapeutic strategy.

We have recently discovered an intriguing molecular linkage between BRAF and the LKB1-AMPK (AMP-activated protein kinase) signaling pathway [67]. We found that BRAF<sup>V600E</sup> mutant suppresses LKB1 and AMPK through the BRAF-MEK-ERK signaling cascade, and that this regulation is critical for melanoma cell proliferation and anchorage-independent growth. The tumor suppressor LKB1 is a serine-threonine protein kinase mutated in Peutz-Jeghers syndrome and several sporadic cancers, including melanoma [68]. Its downstream kinase AMPK is an evolutionarily conserved energy sensor that regulates energy homeostasis by monitoring changes in the intracellular AMP and ATP concentrations [69]. Recent studies have shown that the LKB1-AMPK signaling pathway plays an important role in suppressing cell growth, proliferation and survival under energy stress [69]. These studies also raise interesting possibilities for pharmaceutical intervention to suppress tumor growth through activation of this pathway. Drugs that activate the LKB1-AMPK pathway, such as metformin and its analog phenformin, are being used clinically to treat type II diabetes and could be quickly adapted for cancer treatment. Indeed, recent preclinical studies have demonstrated the anti-tumor activities of metformin and phenformin, and metformin is being evaluated for the treatment of breast and prostate cancers as a single agent in several clinical trials [70–72]. The cross-talk between the LKB1-AMPK and BRAF signaling pathways

suggests that targeting the LKB1-AMPK pathway with metformin or other AMPK activators together with BRAF inhibitor could be another rational combinatorial strategy for melanoma therapy (Figure 2).

Finally, regulation of the immunological responses as a part of the anti-melanoma therapy is an attractive possibility [73]. In fact, activating BRAF<sup>V600E</sup> mutations increase inflammatory responses that ultimately contribute to an increased metastatic potential [19]. In addition, there is evidence of increased expression of melanocyte differentiation antigens upon BRAF inhibition, therefore increasing immune recognition and elimination of tumors [74]. Furthermore, it has been demonstrated that the BRAF inhibitor PLX4032 does not have negative effects in the viability or function of T cells and that peripheral blood mononuclear cells activated with anti-CD3/IL-2 are highly resistant to this inhibitor [75]. Thus it is possible that inhibition of BRAF signaling may increase the efficacy of immunotherapy in melanoma. Taken together, combination therapy with BRAF-specific inhibitors and drugs targeting other oncogenic pathways or with immunotherapy may be a great opportunity for the success of the next generation therapeutics of melanoma.

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## Abbreviations used

<b>AMPK</b>	AMP-activated protein kinase
<b>DUSP</b>	dual-specificity protein phosphatase
<b>ERK</b>	extracellular-signal-regulated kinase
<b>MAPK</b>	mitogen-activated protein kinase
<b>MBP</b>	microprecipitated-bulk powder
<b>MEK (MKK)</b>	MAPK kinase
<b>RECIST</b>	Response Evaluation Criteria in Solid Tumors
<b>RTK</b>	receptor tyrosine kinase
<b>SCC</b>	squamous cell carcinoma

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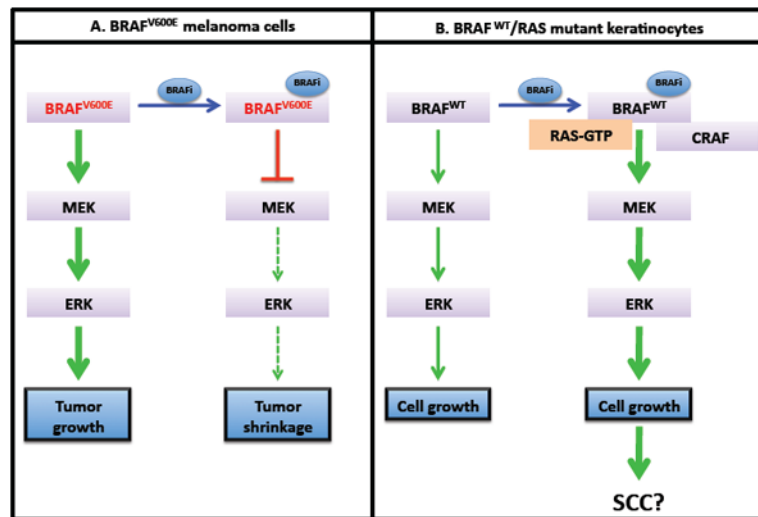
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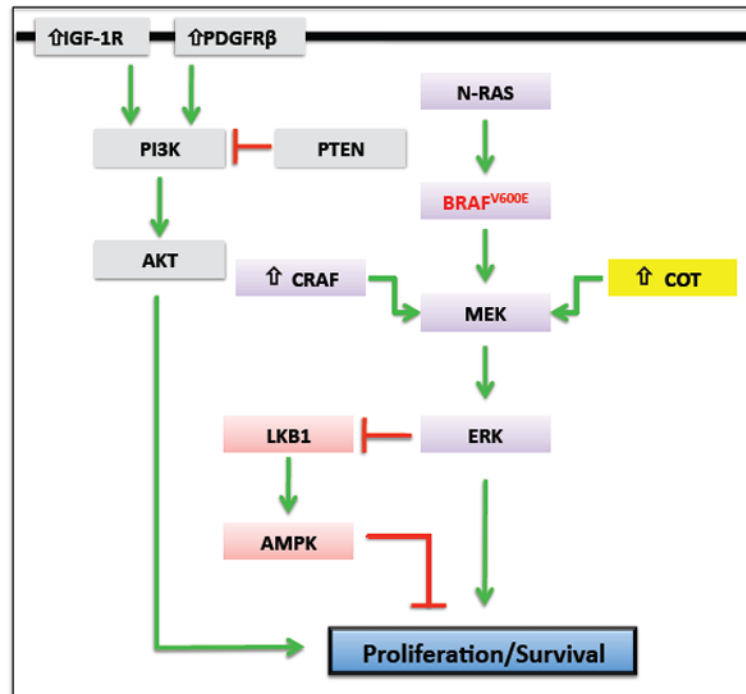
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**Figure 1. Tumor cells harboring BRAF<sup>V600E</sup> mutations and non-mutant BRAF cells respond differently to BRAF inhibitors**

**A.** BRAF<sup>V600E</sup> promotes tumor growth through hyperactivation of the MEK-ERK pathway. Treatment with BRAF inhibitors (BRAFi) such as PLX4032 blocks this signaling pathway, leading to tumor shrinkage. **B.** When cells bearing BRAF<sup>WT</sup> are treated with BRAF inhibitors, a BRAF/CRAF heterodimerization induces activation of the MEK-ERK pathway. This results in increased cell proliferation, which may contribute to the development of squamous cell carcinoma (SCC).



### Figure 2. Targeting the BRAF Signaling pathway in melanoma

BRAF-specific inhibitors block the RAS-RAF-MEK-ERK signaling pathway in BRAF<sup>V600E</sup> mutant cells. However, tumor cells acquire resistance to this therapy during the course of treatment. Several mechanisms that involve reactivation of ERK signaling through amplification or mutation of proteins in the RAS-RAF-MEK-ERK pathway (i.e. NRAS, BRAF, CRAF and COT1) have been recently described for the development of acquired resistance. In addition, hyper-activation of the PI3K-Akt signaling pathway (i.e. through upregulation of IGF-1R or PDGFR ) can also be involved in resistance by promoting survival or proliferation. BRAF<sup>V600E</sup> suppresses the LKB1 tumor suppressor and AMPK through the BRAF-MEK-ERK signaling cascade, and this regulation is critical for melanoma cell proliferation.