

# Overcoming resistance to BRAF inhibitors

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**Abstract:** The discovery of activating mutations in the serine/threonine (S/T) kinase BRAF followed by a wave of follow-up research manifested that the MAPK-pathway plays a critical role in melanoma initiation and progression. BRAF and MEK inhibitors produce an unparalleled response rate in melanoma, but it is now clear that most responses are transient, and while some patients show long lasting responses the majority progress within 1 year. In accordance with the key role played by the MAPK-pathway in BRAF mutant melanomas, disease progression is mostly due to the appearance of drug-resistance mechanisms leading to restoration of MAPK-pathway activity. In the present article we will review the development, application and clinical effects of BRAF and MEK inhibitors both, as single agent and in combination in the context of targeted therapy in melanoma. We will then describe the most prominent mechanisms of resistance found in patients progressed on these targeted therapies. Finally we will discuss strategies for further optimizing the use of MAPK inhibitors and will describe the potential of alternative combination therapies to either delay the onset of resistance to MAPK inhibitors or directly target specific mechanisms of resistance to BRAF/MEK inhibitors.

**Keywords:** Melanoma; BRAF; MEK; targeted therapy; resistance; PI3-kinase; MITF

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## Introduction

Before the discovery of activating mutations in the *BRAF* gene in melanoma, metastatic melanoma was considered a cancer with the direst prognosis. Classical chemotherapy regimens and the prodrug dacarbazine provided little therapeutic opportunities for clinicians trying to manage this deadly form of skin cancer. In 2002, a seminal study performed by the Cancer Genome Project at the Sanger Institute identified BRAF mutations in over 60% of melanomas (1). This groundbreaking discovery initiated great scientific efforts to dissect the role of BRAF and the MAPK-pathway in melanoma initiation and progression.

Today we know that BRAF is mutated in around 50% of melanomas (2) and, by activating the MAPK-pathway, is the main driver of melanoma development. The substitution of a valine for a glutamic acid at position 600 is the most common mutation found in BRAF (2,3). BRAFV600E strongly activates the MAPK-pathway in melanocytes in culture (4), where it eventually induces senescence (5). Intriguingly, this mutation is also found in up to 80% of human benign moles or nevi (6), which are considered senescent clones of melanocytes. In conjunction with INK4A inactivation BRAFV600E transforms melanocytes *in vitro* (4). Targeted expression of BRAFV600E in the melanocyte lineage *in vivo* induces melanoma formation,

and its onset can be accelerated by INK4 or PTEN deficiency or concomitant UV exposure (7-9).

While the detection of BRAF mutations majorly influenced the approach to melanoma therapy, the recognition of the importance of the MAPK-pathway for melanoma biology actually preceded the discovery of BRAF as an oncogene. Indeed, studies into cell signaling in a fish model for melanoma (10,11) and the use of early-stage MEK inhibitors such as PD908059 and U0126 (12,13) demonstrated the early activation of the MAPK-pathway during melanoma development *in vivo*, as well as its relevance for melanoma cell proliferation and its potential as therapeutic target. In 2002, Cohen *et al.* reported constitutive ERK-phosphorylation in >20% of benign nevi and >80% of primary melanoma (14), and hence confirmed activation of MAPK-signaling as an early event in human melanoma development. Pre-clinical studies developed over the following 15 years have underscored the importance of the BRAF-MAPK pathway in a multitude of processes involved in melanoma development and progression and BRAFV600E has been established as central for the control of melanoma cell proliferation and cell cycle progression, apoptosis, migration, invasion, glucose metabolism, adaptation to hypoxia or angiogenesis (3,15-22). Indeed it has now become well accepted that melanomas harboring BRAF mutations are addicted to the MAPK-pathway, as BRAFV600E cells show significant sensibility towards either genetic or chemical inhibition of this pathway.

### BRAF inhibitors as single agent

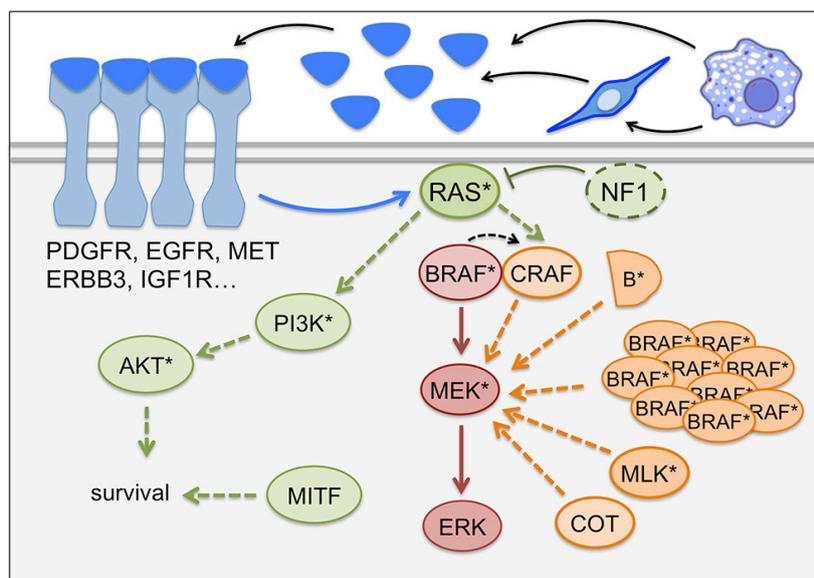
A few years after the seminal study by the Sanger institute the serine/threonine (S/T) kinase inhibitor sorafenib (BAY 43-9006), which possesses activity against CRAF (23) was trialed in solid tumors including melanoma (24). Dose limiting toxicity and probably due to its low specificity towards mutant BRAF, limited clinical effect, and led to sorafenib being discarded as an anti-melanoma treatment although its application has shown certain success against other neoplastic diseases such as renal cancer (25,26). The first drug developed to specifically target BRAFV600E was Vemurafenib (PLX4032). Vemurafenib is a small molecule reversible inhibitor with specific affinity for the ATP-binding pocket of the constitutively active mutant form of BRAFV600E, where it binds to the active site of the kinase domain in its “DGF-in” conformation to block access to ATP (27,28). In early phase I and II trials (BRIM-1 and BRIM-2) vemurafenib showed unprecedented clinical

responses (56% and 53%, respectively) and complete response in up to 6% of cases with 15.9 months of median overall survival (OS) and a median progression-free survival (PFS) of 6.7 months (29,30). BRIM-3, a phase III trial of vemurafenib extended to patients with mutations V600E, V600K and V600D demonstrated a median OS and PFS of 13.6 and 6.9 months respectively (31). More recently another potent inhibitor against mutated BRAF, Dabrafenib (GSK2118436), was shown to mirror the effects observed with vemurafenib (32). The unprecedented efficacy of these drugs changed the way clinicians managed melanoma patients, but soon the long-term suitability of these drugs to treat melanomas harboring activating mutations in the BRAF gene was questioned. Apart from the appearance of a wide range of adverse effects, the more concerning fact was the appearance of skin-related effects within the first 3 months that included the development of squamous cell carcinomas and keratoacanthomas in 10–20% of melanoma patients treated with BRAF inhibitors (29-31,33-35). These secondary cancers were subsequently found to be induced by the “paradoxical activation” of CRAF by BRAF inhibitors in pre-existing keratinocytic lesions harboring wild-type BRAF, but activated RAS (36,37). While clinically manageable, these discoveries prompted further studies, which found that indeed, BRAF inhibition can accelerate the development of pre-existing RAS mutant malignancies including NRAS mutant leukemias or K-RAS mutant pancreatic or colon adenocarcinomas (38-41).

Despite great initial responses the main problem regarding the use of BRAF inhibitors remains the fact they are short lived because within a year the majority of patients no longer respond to treatment and relapse (31,42). In addition, ~20% of patients harboring activating mutations in BRAF present intrinsic resistance and do not respond to BRAF inhibitors (30,31,43), which limits the effectiveness of this therapeutic approach.

### Mechanisms of resistance to BRAF inhibitors

Work into understanding the mechanisms by which melanoma cells evade BRAF inhibitor therapies revealed that the majority of resistances are centered around the reactivation of the MAPK-pathway (*Figure 1*), which further highlights the central role played by this signaling cascade in BRAF mutated melanomas. Alternatively, mechanisms of escaping BRAF inhibitor induced cytotoxicity have been described that are independent of re-activation of the MAPK-pathway (*Figure 1*).



**Figure 1** Resistance to BRAF inhibitors can occur through stromal extracellular signals (blue) leading to ERK reactivation upstream of MEK (orange) or to enhanced survival signaling (green). Pathway re-activation occurs through either activating *NRAS* mutations (\*), loss of the RAS suppressor *NF1*, *BRAF* amplification or alternative splicing leading to *BRAF* truncations, overexpression or mutation of the MEK activators *CRAF*, *MLK* or *COT/TP2/MLK3K8* as well as MEK mutations (\*). Enhanced survival signaling is brought about by the PI3K pathway [mutations in *PI3K* or *AKT* (\*)] or for instance by over-expressed *MITF*. Stromal derived signals can emanate from fibroblasts or macrophages; thereby macrophages can release factors that directly act on melanoma cells or factors that activate fibroblasts.

### MAPK pathway reactivation

The detailed analysis of melanomas from relapsed patients found that in 70% of cases a plethora of different mechanisms allow melanoma cells to bypass BRAF inhibition by maintaining MEK activation in a BRAF independent manner, which will ultimately restore ERK dependent signaling (44,45).

Upstream of BRAF the overexpression of *NRAS*, activating mutations in the *NRAS* protein or loss of the GTPase-activating protein *NF1* leads to BRAF independent MEK activation (*Figure 1*) (46,47). Furthermore, increased activity of MEK emanating from several receptor tyrosine kinases (RTKs) such as EGFR, IGF-1R, or PDGFR has also been found to be present in relapsed melanomas (*Figure 1*) (46,48,49). At the level of the RAF kinases, *CRAF* overexpression, *BRAF* gene amplification and the presence of *BRAF* truncations (as a consequence of alternative splicing) permit maintenance of ERK activation (50,51), while downstream of BRAF, overexpression or mutation of MEK itself or its activators, *COT/TP2/MLK3K8* or *MLKs*, have also been described to reactivate the pathway (*Figure 1*) (52-57).

### MAPK-reactivation independent mechanisms of resistance to BRAF inhibitors

Apart from reactivation of ERK through the RAS-CRAF-MEK-ERK route, RAS dependent parallel activation of the PI3K/AKT pathway downstream of IGF-R or MET receptors can also contribute to limit cytotoxicity resulting from RAF inhibition (49,58). Moreover mutations in this pathway, notably activating mutations in *AKT* and loss of function mutations in *PTEN* (observed in 6–7% of melanomas and leading to PI3K activation) have been described in melanomas resistant to BRAF inhibitors (*Figure 1*) (44,59). Upon BRAF inhibitor treatment, ERBB3 receptor hyper-phosphorylation also promotes survival of BRAF mutated melanoma in an AKT dependent manner (60,61). Indeed the activation of the PI3K pathway seems to act as a cell death-evading mechanism through regulation of expression of apoptosis regulators such as *BCL2* or *BIM* (58,62,63).

Another regulator of anti-apoptotic survival genes such as *BCL2A1* and *BCL2* is *MITF*, the master regulator of melanocytes and melanoma biology (17,64). High *MITF* levels allow melanoma cells to evade cell death triggered

by BRAF (and MEK) inhibitors even when the MAPK pathway appears fully blocked (65,66). Accordingly, MITF gene amplification is found in a small percentage of progressed melanomas (45). Furthermore, MITF is upregulated by BRAF/MEK-inhibitor treatments through a MAPK-dependent re-wiring of the transcriptional control of MITF expression (67). This rewiring appears to happen in the initial phases of patient treatment, because ~80% of melanomas show a significant induction of MITF expression, and this allows melanoma cells to enter a drug-tolerance phase in the early steps of MAPK inhibitor treatment (67).

Another type of resistance is linked to increased RTK signaling, which is correlated with high expression of the RTK AXL (65,68). While RTK dependent melanoma cells are less frequently found before treatment with BRAF inhibitors, high expression of AXL is observed in ~50% of relapsed melanomas (69). Intriguingly, high AXL expression in melanoma cells is accompanied with low expression of MITF thus defining an  $AXL^{high}/MITF^{low}$  phenotype. This  $AXL^{high}/MITF^{low}$  phenotype does not only display innate resistance to BRAF inhibitors and increased invasiveness, but also represents a more de-differentiated state (65). Thus the role of MITF in the response of melanoma cells to MAPK inhibitors is complex: the presence of MITF is a marker for responsiveness to treatment, but when MITF expression is upregulated, it confers resistance to MAPK inhibitors. On the other hand very low levels of MITF when co-existing with high levels of the RTK AXL protect melanoma cells from BRAF inhibitor induced cytotoxicity. Taken together it seems that the responsiveness of melanoma cells to BRAF inhibitors lies between two different “MITF-states” with the plasticity of tumors cells to switch from one state to the other being a determining factor to adapt to MAPK pathway inhibitors (64). It would be of interest to assess in patient samples whether the relative proportion of  $AXL^{high}/MITF^{low}$  and  $AXL^{low}/MITF^{high}$  might have predictive value towards the determination of patients responsiveness to BRAF inhibitor based therapies.

#### *Non-cell autonomous derived resistance to BRAF inhibitors*

Tumors are heterogeneous and not only harbor cancer cells, but also stromal cells that can both positively and negatively impact on tumor growth, metastatic potential and importantly, on response to therapies. As such fibroblasts can protect melanoma cells from BRAF inhibition. Thereby, Hepatocyte Growth Factor (HGF)

secreted from stromal fibroblast can restore ERK activity through CRAF by stimulating the MET tyrosine kinase receptor (58). Tumor associated fibroblasts can also provide resistance by modifying the extracellular matrix (ECM), which induces integrin mediated Focal Adhesion kinase activation (70). Apart from fibroblasts, macrophages by secreting Tumor Necrosis Factor, can activate NF $\kappa$ B signaling in melanoma cells to upregulate the expression of MITF and allow melanoma cells to evade MAPK inhibitor induced cytotoxicity (71). Macrophages have also been shown to produce VEGF, which can reactivate ERK in the presence of BRAF inhibitors (72). Macrophages and fibroblasts can also act together in “inflammatory niches”, whereby macrophages secrete IL1, which stimulates the production of GRO by fibroblasts. GRO activates CXCR2 signaling in melanoma cells, which overcomes BRAF inhibition as well as BRAF/MEK combination therapies (73). BRAF inhibition shows also efficacy in brain metastases (74), but it has been suggested that ERK- and PI3K-activating extrinsic factors contained in cerebrospinal fluid might contribute to BRAF inhibitor resistance in this setting (75). Finally, upon BRAF inhibitor treatment, specific soluble factors can sustain the proliferation of innate resistant cells that normally present as slow cycling cells in therapy naïve tumors (76,77).

The observed side effects and most prominently, the plethora of intrinsic/innate and acquired resistance mechanisms described above limit the efficacy of BRAF inhibitor based therapies in melanoma patients.

#### **Combinatorial BRAF and MEK inhibitor therapies**

Prior and during the development of BRAF inhibitors, MEK, acknowledged as the only effector of BRAF had attracted interest as therapeutic target. Soon it was realized that melanomas harboring mutations in *BRAF* were significantly more sensitive to MEK inhibition than melanomas expressing mutant *NRAS*, which resulted in PI3K activation providing pro-survival signals (78,79). Indeed MEK inhibitors such as selumetinib (AZD6244), binimetinib (MEK162) or trametinib have shown clinical efficacy as single agents in melanomas with BRAF mutations (80-85). Nevertheless insufficient efficacy has hindered the application of MEK inhibitors as single agents. On the other hand the combination of BRAF and MEK inhibitors has shown great success in BRAF mutant patients and as a result, between 2014 and 2015 the FDA approved the use of trametinib/dabrafenib and cobimetinib/

vemurafenib as standard of care for BRAF-mutant advanced melanoma. In these trials it was seen that in patients who previously developed resistance to dabrafenib the follow-up treatment with a dabrafenib/trametinib showed limited clinical efficacy with an overall response rate (ORR) of around 10% and modest increases in OS and PFS, possibly dependent on the duration of previous BRAF inhibitor exposure (86). On the other hand, in BRAF inhibitor naïve patients BRAF/MEK-inhibitor combinations increase both overall OS and PFS when compared with the clinical effect of BRAF inhibitors only. Indeed, the cobimetinib/vemurafenib combination increased median OS, which at the final analysis was 22.3 months (95% CI, 20.3–not estimable) for cobimetinib and vemurafenib versus 17.4 months (95% CI, 15.0–19.8) for placebo and vemurafenib (HR 0.70, 95% CI, 0.55–0.90;  $P=0.005$ ) (87–89). Similar improvements were also observed in patients treated with trametinib/dabrafenib, and treatment with dabrafenib 150 mg twice daily plus trametinib 2 mg daily achieved a median OS of 27.4 months (95% CI, 12.9 to not reached), with an OS at 1, 2, and 3 years of 72%, 60%, and 47%, respectively (42,90,91). Despite slight differences on clinical endpoints both combinations provide equally evident benefit, and as previously mentioned have now become the standard of care for BRAF mutant melanomas (92,93). Combinations of BRAF and MEK targeting drugs provide further clinical benefits as the occurrence of cutaneous adverse events such as squamous cell carcinoma or keratoacanthoma is significantly diminished (94), positively impacting on patient's health-related quality of life (95,96).

### Drug tolerance, acquired resistance and scheduling

Despite the immediate and, sometimes, long-lasting results achieved with combinatorial targeted therapies (42,88,97), most patients treated with combination therapies still relapse due to the development of resistance mechanism similar to those described above. The appearance of BRAF amplifications, BRAF splice variants and mutations in MEK1/2 have been described as mechanisms of cross-resistance to both mono and combination therapy that involve reactivation of the MAPK-pathway (98,99), while MITF overexpression, known to confer resistance to both BRAF and MEK inhibitors is found in ~80% of patients on treatment with BRAF/MEK-inhibitor (67). A common view of the phenomenon of acquired resistance is that

initially the presence of BRAF and MEK inhibitors induces a rewiring of the MAPK pathway allowing the tumors to develop drug tolerance (100,101). Following on from this, the responsiveness of the tumor to the kinase inhibitors eventually changes towards a permanent resistance program through either the selection for mutant clones or the establishment of new mutations in response to the signaling pressure exerted by the BRAF/MEK-inhibitor mediated MAPK blockade.

Recent studies suggest that by modulating the schedule of inhibitor administration in a way that prevents MAPK-pathway rewiring tumor responsiveness to BRAF and MEK inhibitors over time is maintained. Pre-clinical studies propose an on-off schedule or drug-holiday to break the rewiring of the MAPK pathway (102,103), and this concept has been taken on board in the currently ongoing study GEM015 (<https://www.clinicaltrialsregister.eu/ctr-search/trial/2014-005277-36/ES>). The theory of a long-term on-off scheduling is further supported by the exciting outcome of a phase II trial in patients that had progressed on BRAF inhibitor and had been taken off drugs for at least 12 weeks. Thirty percent of these patients had partial responses and 40% continued with stable disease (104). Intriguingly, while off BRAF inhibitors, all the patients in the trial had been treated with immunotherapies but had progressed, suggesting the potential of combining an on-off BRAF/MEK-inhibitor treatment with anti-PD-1 or PD-L1 antibodies (104).

### Potential for alternative combination therapies

As previously mentioned ~70% of mechanisms of resistance involve reactivation of the MAPK pathway and of these ~37% occur in a RAS-dependent manner (44). In addition, over 20% of patients relapse with activated PI3K signaling, be it through RAS or mutations in the PI3K pathway (44,45). Thus, in progressed tumors MAPK pathway activation is dependent on CRAF and not BRAF, and PI3K signaling is activated and provides pro-survival signals. With this in mind researchers have explored several approaches to prevent the development of resistance and/or provide therapeutic alternatives to progressed patients.

While in the post-sorafenib era the main goal had been to gain specificity towards mutated BRAF lately some effort has been put towards the development of small molecules directed to both BRAF and CRAF and capable to interfere with isoform hetero- and homo-dimerization thus preventing paradoxical activation of CRAF by BRAF

inhibitors (72,105-110). While this approach has provided promising results in pre-clinical models, their efficacy and the risk of adverse events will have to be tested in early phase trials (NCT02607813, www.clinicaltrials.gov). Another possibility to target RAF kinases is by means of interfering with the protein stability by using chaperone inhibitors. Heat Shock Protein 90 (HSP-90) regulates the stability and degradation of both BRAF V600E and CRAF (111,112) and HSP-90 inhibitors have been postulated as candidates to prevent BRAF inhibitor resistance (56,113,114). At present several trials are underway, which assess these compounds in combination with BRAF and MEK inhibitors in melanoma patients (NCT02097225, NCT02721459, NCT01657591, www.clinicaltrials.gov).

In line with the view of developing multi-targeted combination treatments there is a growing number of pre-clinical and even clinical studies underway testing the potential of broad spectrum RTKs and S/T kinase inhibitors as single agent or in combination with BRAF and/or MEK inhibitors. The rationale of these approaches is to prevent or overcome MAPK inhibitor dependent resistance mechanisms, although the appearance of systemic toxicities remains one of the main concerns when combining several small molecules (115-119). Other groups have attempted to tackle mechanisms of resistance related to the appearance of mutations in MEK1/2 and novel MEK inhibitors active in some of these mutants show interesting potential (120). An avenue of action expected to draw increasing attention is the use of ERK inhibitors (121-123). Whether in combination with BRAF or MEK inhibitors, small molecule inhibitors such as SCH772984 and GDC-0994 are being tested at present.

The central role of the PI3K signaling not only in melanoma development, but also in the innate and acquired resistance to MAPK inhibitors makes the PI3K/AKT/mTOR axis the second most attractive target in melanoma (2,44,45). While *in vitro* studies clearly show the potential of using AKT, pan-PI3K or dual PI3K/mTOR inhibitors in combination with BRAF/MEK inhibitor, the translation into the clinical setting is troublesome due to enhanced systemic toxicities (124-128). The PI3K family of proteins is relevant in many physiological processes and malignant conditions but nevertheless this interest on the pathway is producing encouraging data advancing in the understanding of melanoma specific PI3K-signaling (129) and lately PI3K $\beta$  isoform specific inhibitors are being developed and trialed in the context of PTEN mutant melanoma patients

(130-132). Interestingly targeting the PI3K has not only been proposed as a strategy to overcome resistance to BRAF/MEK inhibitors, but also with the therapeutic goal of preventing the onset of MEK1/2 inhibitor resistance in BRAF-mutated melanoma (133).

The potential of extending the efficacy of MAPK-inhibitor based therapies by targeting other melanoma relevant proteins is shown in a recent study (67), where in the above mentioned “drug-tolerance phase” when acquired resistance has not yet arisen, up-regulation of MITF contributes to increased survival signals. As previously mentioned in patients on BRAF/MEK inhibitor treatment MITF expression is upregulated, and this occurs through a transcriptional process in which PAX3 takes over the regulation of MITF transcription (66,67). A screen using FDA-approved drugs identified the HIV protease inhibitor Nelfinavir as inhibitor of MITF up-expression on treatment, and consequently Nelfinavir synergizes with BRAF and MEK inhibitors, suggesting that this combination could improve the efficacy and extend the clinical use of these drugs in BRAF mutant melanoma cells. Most importantly this study provides evidence suggesting the potential use of Nelfinavir mesylate in combination with MEK inhibitors in NRAS mutant cells and, importantly, in melanoma cells that progressed on BRAF/MEK inhibitors due to the appearance of activating mutations in NRAS (67). This study emphasizes that a greater understanding of the biology of melanoma cells during the early response to MAPK inhibitors should still provide us with clues for a better use of these drugs.

## Conclusions

Despite its limitations, the application of targeted therapies to melanoma has been a historic milestone in cancer therapeutics. Indeed the unprecedented responses observed in BRAF mutant patients and the existence of a significant number of long-term responder highlights the success, but also reflects the paramount importance that the MAPK pathway plays in cancer in general and in melanoma in particular. Nevertheless the early appearance of resistance mechanisms once again demonstrates the relentless ability of cancer cells to adapt to cytotoxic challenges directly targeting driving signaling pathways. More recently the advent of immune-checkpoint therapies has opened an extremely promising therapeutic opportunity for melanoma patients, but this should not lead to a dismissal of targeted

therapy approaches. Indeed, the recent “re-treatment” trial and continuous reports of pre-clinical studies describing novel combination approaches demonstrate that there is clearly room to improve the efficacy of the current BRAF/MEK targeting therapies.

Nevertheless, there is still need to explore alternative avenues of action to provide new treatments based on targeted therapies tackling mechanisms of resistance to BRAF/MEK inhibitors. Considering this, there are aspects of melanoma biology that require attention from the scientific community to better use MAPK targeted therapies, but also to gain insight into yet unknown therapeutic opportunities. For instance there is a very thought-provoking report suggesting patient’s age as a factor influencing both responsiveness to BRAF inhibitor and metastatic potential (134). In this context, the role of the tumor stroma of melanoma response to targeted therapies is still understudied as it is the effect of targeted therapies in the metastatic niches in which secondary tumor arise: why should a pulmonary melanoma metastasis respond equally as a liver or brain metastasis? A more defined study of visceral metastases and the role of specific anatomic location-dependent stromal characteristics might provide useful insight into innate and acquired mechanisms of resistance/sensibility to BRAF/MEK inhibitors. The interplay between cancer cell metabolism and response to targeted therapies too warrants further research. A growing number of reports are linking nutrient metabolism (glucose, glutamine) and melanoma cells responsiveness to BRAF inhibition while at the same time it is clear that the MAPK pathway regulates glycolysis and oxidative phosphorylation (16,135-141). All these gaps in our understanding of melanoma biology and the potential of managing MAPK inhibitor schedules with drug-holidays or combination with immunotherapies shows that there is room for improvement to better manage advanced melanoma.

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### Footnote

*Conflicts of Interest:* The authors have no conflicts of interest to declare.

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