RESISTANCE TO TARGETED THERAPIES IN MELANOMA: NEW INSIGHTS

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ABSTRACT

Several molecular mechanisms are involved in melanoma genesis and progression. Molecular targets for effective therapeutic intervention have been identified within the RAS-RAF-MEK-ERK and, to a less extent, PI3K-AKT pathways. The development of inhibitors of key effectors (mainly BRAF mutant, MEK, and KIT) into such pathways has significantly improved the treatment of patients with advanced melanoma. However, emerging data indicate that a large variety of acquired and intrinsic mechanisms may drive resistance to the main targeted inhibitors. All the evidence suggests that in melanoma, as probably in all types of cancer, it is unlikely that targeting a single component in pathogenetic signalling pathways could yield significant antitumour responses. Therefore, knowledge of the multiple altered signalling events involved in response and resistance to targeted treatments will allow for the development of more effective combination therapies, which may represent the next challenge for the management of patients with such a disease.

Keywords: Malignant melanoma, molecular pathogenesis, targeted therapy, BRAF/MEK/KIT inhibitors, drug resistance.

INTRODUCTION

Complex molecular mechanisms are involved in the development, progression, and resistance-to-therapy of melanoma. Although the majority of such pathogenetic mechanisms are still largely unknown, several genes and cell-signalling pathways have been implicated.1 Among them, the mitogen-activated protein kinase (MAPK; including the cascade of NRAS, BRAF, MEK1/2, and ERK1/2 proteins) - a major signalling pathway involved in the control of cell proliferation - has been reported to play a crucial role in melanoma pathogenesis.2 Indeed, the ERK1/2 proteins have been found to be constitutively activated in melanoma, mostly as a consequence of mutations in upstream components of the pathway, and their increased activity has been implicated in rapid cell growth as well as enhanced cell survival and resistance to apoptosis.2 About half of melanomas harbour a driver mutation in BRAF; whereas one-fifth of cases present an oncogenic mutation in NRAS.3 Since BRAF and NRAS mutations have been found mutually exclusive,4,5 about two-thirds of patients present a melanoma carrying a mutated BRAF or NRAS gene.

In the treatment of patients with advanced melanoma, the availability of either targeted T cell immunotherapy (the anti-CTLA4 agent ipilimumab and the anti-PD-1 and anti-PD-L1 agents [nivolumab, lambrolizumab, MPDL3280A]) or inhibitors of key effectors into the MAPK pathway (BRAF-mutant inhibitors [vemurafenib,
dabrafenib] MEK inhibitors [trametinib], and their combination) is allowing for the ineffectiveness of the conventional therapies to be overcome. Vemurafenib and dabrafenib have been successfully introduced into the clinical practice and have been demonstrated to achieve rapid tumour shrinkage in the majority of cases. Treatments with both these drugs improve response rates and progression-free survival (PFS), with a favourable impact on overall survival (OS). Analogously, MEK inhibitors as well as the combination of a BRAF inhibitor along with a MEK inhibitor have been recently demonstrated to exert a similar clinical efficacy (in the latter case, with a reduced incidence of both keratoacanthomas and squamous cell carcinomas as cutaneous adverse effects).

Although the vast majority (up to 80%) of melanoma patients carrying BRAF mutations show clinical and pathological response to therapy - with different rates of tumour reduction - when treated with either a BRAF inhibitor or a MEK inhibitor (this latter agent exerts a more limited antiproliferative effect in NRAS-mutated tumours) most of them develop resistance within 6-8 months after treatment initiation as a consequence of reactivation of the MAPK pathway or activation of alternative signalling pathways. Nevertheless, a fraction of them are primarily refractory due to an intrinsic resistance to such inhibitors. Here we summarise the main results with inhibitors of the MAPK components in melanoma patients and present the known mechanisms of resistance to such targeted therapies.

**TARGETED THERAPIES AGAINST MAPK PATHWAY COMPONENTS**

Despite the huge amount of knowledge implicating RAS in tumour initiation and promotion, RAS itself has not become a successful target of therapy. The strategies used to develop drugs able to inhibit the RAS activity are aimed at preventing its interaction with several components of the upstream or downstream signalling pathways regulated by this protein. In this sense, the block of prenylation (farnesylation) markedly impairs the functioning of active RAS protein. While a good *in vitro* antitumour activity has been reported in human melanoma cell lines (with downregulation of ERK and/or AKT and induction of apoptosis), farnesyltransferase inhibitors have always failed to be effective in melanoma patients (even if all cohorts treated with these agents were never selected for the RAS status). A recently discovered farnesyltransferase inhibitor, lonafarnib, exhibited to enhance the antitumour activity of the pan-RAF inhibitor sorafenib by exerting downregulation of the antiapoptotic signals and inhibition of cell proliferation; however, this agent alone lacked any capability of inhibiting tumour growth. Therefore, a combination of farnesyltransferase inhibitors with other pathway-targeted drugs or, alternatively, a more stringent selection of the patients’ cohorts could be helpful to increase the clinical efficacy of such compounds. Therapeutic strategies have thus been focused on inhibiting downstream effectors of the RAS-driven pathways, MAPK and PI3K-AKT.

The first drug developed against BRAF was the BAY 43-9006 or sorafenib, which is however unspecific for mutated BRAF and suppresses activity of several different kinases (indeed, it is recognised as a multikinase inhibitor). In advanced melanoma, the combination of sorafenib with the chemotherapeutic agents carboplatin and paclitaxel has failed to show any efficacy in terms of either PFS or OS compared to the same regimen plus an oral placebo in a Phase III trial, despite an initial encouraging improvement in PFS by the addition of sorafenib to dacarbazine in a previous Phase II study.

Thereafter, a second generation anti-BRAF compound (vemurafenib, also known as PLX4032 or RO5185426), which instead acts a potent and selective inhibitor of the mutated BRAF kinase, has been demonstrated to be highly effective in melanoma patients carrying the V600E BRAF mutation. A Phase III study comparing vemurafenib with dacarbazine in 675 previously untreated BRAF-mutant patients revealed OS to be 84% (95% CI: 78-89) in the vemurafenib group and 64% (95% CI: 56-73) in the dacarbazine group. In this study, patients treated with vemurafenib presented a relative reduction of 63% in the risk of death and of 74% in the risk of either death or disease progression, as compared with those undergoing dacarbazine treatment. An analogous clinical activity has been demonstrated for an additional BRAF inhibitor compound, dabrafenib (previously known as GSK2118436), which significantly improved PFS compared with dacarbazine.
Interestingly, this molecule seems to be equally active on different mutations at codons 600 of the BRAF gene (V600E/K/D/R).\textsuperscript{22-25}

In addition to the inhibitory activity in cells with a mutant BRAF (which is revealed by the decreased levels of phosphorylated ERK1/2 proteins and subsequent growth arrest), vemurafenib and dabrafenib also induce MAPK pathway activation in cells with a wild-type BRAF through RAF-mediated induction of ERK1/2 phosphorylation.\textsuperscript{26-27} BRAF inhibitors seem to paradoxically stimulate ERK signalling through activating dimerisation of the different RAF isoforms (see below); such conformational effects may explain either the high frequency of keratoacanthomas and squamous cell carcinomas among patients treated with BRAF inhibitors or the development of an acquired resistance to these drugs.\textsuperscript{26-28} Overall, a clinical benefit has been reported up to an unprecedented 80% rate of BRAF-mutated patients treated with vemurafenib or dabrafenib; response to each of these oral agents occurs within few days or weeks.\textsuperscript{29} Since reactivation of the downstream MEK-ERK pathway seems to represent the main mechanism of resistance to BRAF inhibitors (see below), a promising strategy for overcoming such a limited persistence of the antiproliferative effects was to include new compounds blocking MEK1/2 proteins into the treatment options.

Several MEK inhibitors have been introduced in clinical trials. Unlike the BRAF inhibitors which are highly selective for the mutated protein, MEK inhibitors are targeted against the wild-type gene product. As single agents, these compounds (AS703026, AZD6244, E6201, GSK1120212, GDC0973, MEK162) have shown a markedly high activity in patients carrying tumours with constitutive activation of the RAS-BRAF-MEK-ERK signalling cascade. Detection of RAS mutations in primary tumours seems to represent the strongest marker for selecting patients with the highest chance to respond to MEK inhibitors; AS703026 and AZD6244 have activity in KRAS mutant colon cancer cell lines/xenografts in combination with cetuximab,\textsuperscript{30,31} whereas GSK1120212 (also known as trametinib) has been found to be effective in NRAS-mutated melanoma.\textsuperscript{32} In melanoma patients carrying BRAF mutations, the response to MEK inhibitors seems to be partially dependent on exposition to prior BRAF inhibitor therapy (for GSK1120212, a significant clinical activity was observed in BRAF inhibitor-naive patients only\textsuperscript{33}) or status of the PI3K-AKT pathway (for selumetinib [previously known as AZD6244] and E6201, a significantly low responsiveness to MEK inhibitors was found in BRAF mutant melanomas expressing high levels of phosphorylated MEK or presenting PTEN inactivation with subsequent stimulation of downstream PI3K signalling,\textsuperscript{35} respectively). In other words, coexistence of an unaffected PI3K-AKT status may contribute to increased sensitivity to MEK inhibitors in melanomas whose MAPK pathway is activated through oncogenic mutations in BRAF gene. Finally, the MEK inhibition has been demonstrated to abrogate the CRAF-dependent activation of ERK in wild-type BRAF cells, contributing to reduce the chances of cutaneous adverse events.\textsuperscript{36}

Current clinical investigations have shown great promise with the combination of targeted therapies as a new effective strategy of melanoma treatment. A combined treatment with MEK and BRAF inhibitors in BRAF mutated metastatic patients showed a significant improvement of the PFS rates,\textsuperscript{37} providing further support to the hypothesis that this could be the way for a better management of such melanoma cases. Actually, a number of clinical trials of trametinib in combination with other targeted drugs, whose activity is somehow interfering with the MAPK-driven tumour growth, are underway and expected to show great promise. As an example, it has been recently demonstrated that MEK inhibitors may enhance the ability of histone deacetylase (HDAC) inhibitors to induce apoptosis in tumour cells with constitutive activation of the BRAF-MEK-ERK signalling cascade both \textit{in vitro} and \textit{in vivo}.\textsuperscript{38}

Specific mutations within the kinase domain of the KIT gene may also cause an uncontrolled melanoma cell proliferation; such mutations are less frequent than those in BRAF/NRAS genes among cutaneous melanomas (overall, 2-3% of total cases; about 20% in acral lentiginous melanomas and 3-5% in melanomas from chronic sun-damaged skin).\textsuperscript{39,40} Overall, 30% of melanomas with KIT mutations also show increased copy number/amplification of the gene; all KIT aberrations do not typically coexist with BRAF or NRAS mutations.\textsuperscript{39,40} For the limited cohort of cutaneous melanomas carrying KIT mutations, several small tyrosine kinase inhibitors have been shown to induce cell cycle arrest and apoptosis
with significant inhibition of migration and invasion of melanoma cells. Promising results concerning the clinical responses have been registered for these compounds, though on limited subsets of melanoma patients harbouring KIT aberrations (mainly, those carrying some gene sequence variants - such as K642E and L576P - which are highly responsive). In particular:

- imatinib, formerly known as STI571, has been demonstrated to be effective in patients with metastatic melanoma harbouring KIT mutations, but not in cases with KIT amplification only. Therefore, the National Comprehensive Cancer Network (NCCN) guidelines have included imatinib as an effective treatment option for KIT-mutated tumours;

- nilotinib (AMN107) inhibits both wild-type and mutant (in exons 11, 13, and 17) KIT as well as imatinib-resistant KIT mutant tumours. This drug has been reported to present a very favourable toxicity profile with durable response in metastatic melanoma patients with KIT mutations;

- dasatinib inhibits both wild-type and mutant KIT in a dose-dependent manner, causing inhibition of cell migration and invasion through reduction of the phosphorylation of either Src kinase or FAK pathway. Dasatinib in combination with dacarbazine appears to be more active than either agent alone.

**RESISTANCE TO MAPK-TARGETED THERAPIES**

Intrinsic and acquired resistance to targeted therapy agents have been reported to play a role in the treatment of advanced melanoma patients. In this regard, it is to be underlined that the vast majority of data about such an issue are related to the resistance to BRAF inhibitors, since vemurafenib and dabrafenib have been the most extensively studied, both preclinically and clinically.

**Intrinsic Resistance**

About one-fifth of patients treated with vemurafenib or dabrafenib are not responsive to the treatment from the beginning. As shown in Figure 1, the molecular events underlying such an intrinsic resistance are various:

- loss of PTEN tumour suppressor protein, with increased basal levels of AKT signalling;

- gene amplification and/or overexpression of cyclin D1, which contrasts the activity of the cyclin-dependent kinase inhibitor p16CDKN2A and stimulates the cyclin D1-RB pathway. In this regard, inactivation of FBXO4, encoding an enzyme involved in cyclin D1 proteolysis, has been recently demonstrated to induce cyclin D1 accumulation in melanoma cells;

- silencing of the NF1 gene, which either promotes RAS activation or impairs the mechanisms regulating the senescence process and controlling the cell proliferation;

- increased activity of protein kinase D3 (PRKD3), with activation of the PI3K-AKT signalling in presence of a specific inhibition of the oncogenic BRAF.

To better understand the reasons why all these apparently different molecular alterations are implicated in conferring resistance to BRAF or MEK inhibitors in melanoma cells, it is necessary to keep in mind the relationship between RAF-MEK-ERK activation and melanomagenesis. Oncogenic BRAF mutant strongly stimulates cell cycle progression by activation of the downstream MEK-ERK pathway. However, the BRAF-driven melanocytic proliferation needs the coexistence of alterations in additional cell-cycle factors (such as p53 deficiency, genetic or epigenetic inactivation of p16CDKN2A gene with subsequent preponderant activity of cyclin D1-CDK4/6-phospho-RB complex, increased levels of active AKT) in order to promote the melanoma growth and progression. In a subset of melanomas, such additional pathogenetic alterations acquire a prevalent role, and tumour cell proliferation becomes independent or less dependent on activation of the BRAF-MEK-ERK pathway. In these cases, treatment with BRAF inhibitors may be ineffective due to existence of such alternative proliferation drivers.

The elevated intracellular concentration of cyclin D1 - often related to the amplification of the gene locus at chromosomal level - may represent a strong stimulus to cell proliferation, independently from the functional status of the RAF-MEK-ERK pathway, since it determines a marked increase in activating bind to the CDK4/6 kinases and, sequentially, in phosphorylation of the RB protein. As a confirmation of the role of cyclin D1 overexpression in promoting MAPK-independent cell proliferation, cytostatic effects
of BRAF (as well as MEK) inhibitors have always been associated with diminished levels of both cyclin D1 and phospho-Rb.58,59 Analogously, activated AKT has been indicated to promote cell proliferation through the downregulation of the p27 cyclin-dependent kinase inhibitor and, mostly, the upregulation of cyclins E and D1.60,61 Activation of AKT is almost entirely determined by an upstream PI3K activation, since activating mutations of AKT are nearly absent in melanoma (rare mutations in AKT1 and AKT3 genes have been reported in a limited number of melanomas and melanoma cell lines).62,63 The intracellular accumulation of active AKT does result in the suppression of apoptosis and induction of cell survival,61 through inactivation of many pro-apoptotic proteins, such as BAD (Bcl-2 antagonist of cell death64) and MDM2 (that lead to increased p53 degradation65,66). A member of the Bcl-2 family, BCL2A1, has been recently found amplified in ~30% of melanomas and overexpression of the corresponding gene product associated with poorer clinical responses to BRAF inhibitors.67 Moreover, silencing of PTEN and subsequent activation of the PI3K-AKT pathway participate, in conjunction with the activation of the BRAF-MEK-ERK pathway, in regulating the expression levels of the BIM protein, a pro-apoptotic member of the Bcl-2 protein family.68 The presence of PTEN inactivation may therefore interfere with the BRAF inhibition by reducing the levels of BIM protein and, thus, the extent of apoptotic induction; as a confirmation of this, a simultaneous treatment with BRAF and PI3K inhibitors has been reported to enhance BIM expression and increase the level of apoptosis.52 Alternatively, the PI3K signalling may be directly increased by the occurrence of activating mutations in its kinase domain.69

Figure 1. Mechanisms of intrinsic resistance to BRAF-MEK inhibitors.
Coexistent molecular features (pink balloons) are found to impair the antitumour activity of BRAF and/or MEK inhibitors by interfering with the key effectors of the two major pathways involved in melanoma pathogenesis. Arrows represent activating signals and interrupted lines represent inhibiting signals.

CDK: cyclin-dependent kinase; ERK: extracellular-related kinase; MEK: mitogen-activated protein kinase-extracellular-related kinase; PI3K: phosphatidylinositol 3 kinase; PTEN: phosphatase and tensin homologue are reported.
Hence, the occurrence of a p53 deficiency or, more generally, a status of apoptosis escape, with an unbalanced ratio between pro and anti-apoptotic effectors - all events found to cooperate with BRAF mutations in driving the melanoma progression⁷⁰,⁷¹ - may induce a MAPK-independent tumour growth.⁷² Inactivation of AKT by targeting PI3K has also been demonstrated to effectively inhibit cell proliferation.⁵²,⁷³ The combination of a BRAF or MEK inhibitor with a PI3K/mTOR inhibitor was found to enhance cell growth inhibition through achievement of ERK hypophosphorylation, reduced cyclin D₁ levels, and increased p27 levels, overcoming the resistance encountered by the use of a single anti-BRAF or anti-MEK agent.⁵⁸,⁷⁴ Amplification of cyclin D₁, allelic deletions downregulating p16⁰⁶⁰⁴⁰⁻⁰⁶⁰⁴⁰²⁰, and alterations inactivating PTEN have all been associated with a poorer PFS after treatment with dabrafenib in patients with BRAF-mutant metastatic melanoma.⁷⁵

Finally, loss of NF1 and activation of PRKD3 - the other two molecular events mentioned previously - contribute to the resistance to such target therapies by also stimulating the PI3K-AKT pathway directly (PRKD3) or indirectly, through activation of RAS (NF1).⁵⁵,⁶⁶ Inactivation of NF1 by genetic or epigenetic impairments has been described in BRAF-mutant melanoma cells that are intrinsically resistant to BRAF inhibition as well as in melanomas developing resistance to vemurafenib.⁵⁵,⁷⁶ For PRKD3, gene silencing has been reported to enhance cell growth arrest by BRAF and MEK inhibitors, and enforce cell sensitivity to these agents.⁵⁶ The NF1 loss and the PRKD3 activation can be considered as key mediators of both acquired and intrinsic BRAF inhibitor resistance (increased activity of PRKD3 seems to however confer resistance to RAF265 rather than approved BRAF inhibitors⁵⁶).

**Acquired Resistance**

In the vast majority of patients with BRAF-mutated melanomas, response to BRAF inhibitors is not durable and resistance to treatment develops in 6-8 months from the initiation of therapy. The mechanisms for this acquired resistance have proven to be highly heterogeneous.⁷⁷ Figure 2 summarises the different events involved in such a drug resistance. At a glance, two separate scenarios may be depicted.

The first scenario includes mechanisms underlying reactivation of the RAS-RAF-MEK-ERK pathway through induced alterations in components of this signalling cascade: activation of RAS signalling,⁷⁸ activating mutations in MAP2K1 (encoding MEK1 protein) or MAP2K2 (encoding MEK2 protein) genes,⁷⁹,⁸⁰ activation of MAPK pathway agonists such as COT kinase,⁸¹ occurrence of alternative splicing of the mutated BRAF mRNA,⁸² BRAF-mutated gene amplification.⁸³ In this case, the cell proliferation/tumour growth is still depending on RAS-BRAF-MEK-ERK cascade activity and BRAF inhibition is overcome with alternative changes within this same pathway (real failure of BRAF inhibitors).

The second scenario is represented by reactivation of the suppressed ERK signalling through induced alterations in components of cell proliferation-controlling pathways different from the BRAF-MEK-ERK one: upregulation of the receptor tyrosine kinase (RTK) effectors - such as the platelet-derived growth factor receptor β (PDGFRβ),⁸⁴ activation of the MET-HGF system,⁸⁵ amplification of the CCND1/cyclin D₁ gene or lack of PTEN function with subsequent activation of the PI3K-AKT pathway,⁵⁹ enhancement of the IGFR-IR/PI3K signalling,⁸⁶ upregulation of the signal transducer and activator of transcription 3 (STAT3)-paired box homeotic gene 3 (PAX3)-signalling pathway.⁸⁷,⁸⁸ In this case, BRAF inhibition is still effective, but the tumour is not dependent upon RAF-MEK-ERK signalling for growth and survival (paradoxical failure of BRAF inhibitors).

**Activation of RAS**

Inhibition of mutated BRAF leads to ERK hypophosphorylation; thus, ERK signalling is temporarily turned down after BRAF inhibition with subsequent relief of the physiological negative feedback on RAS (Figure 2). In melanoma with mutated BRAF, activation of the downstream MEK-ERK pathway is independent on the RAS-ligand activity, and BRAF mutant transmits continuous proliferation signals acting as a RAF-inhibitor-sensitive monomer. Vemurafenib and dabrafenib potently inhibit such BRAF mutant monomers, causing markedly decreased levels of ERK phosphorylation.⁸⁹ As a consequence, the ERK-dependent feedback is progressively turned off, RAS-driven signal transduction is restored with increasing levels of active RAS-GTP, and RAF-inhibitor-resistant RAF dimers are generated (Figure 3).
The RAF homodimers (CRAF-CRAF) or heterodimers (BRAF mutant-CRAF) are able to restimulate the MEK-ERK pathway, resulting in an increased activity of the ERK1/2 proteins. In preclinical models, increased CRAF activity was firstly identified in drug-resistant clones derived from cell lines undergoing BRAF inhibition. Occurrence of CRAF mutations has been also reported to contribute to reactivate the MEK-ERK axis - again, in a dimerisation-dependent manner - following exposure to RAF inhibitors. Alternatively, an enhanced activation of fibroblast growth factor receptor 3 (FGFR3) has been found to promote the RAS-driven signal transduction and confer resistance to vemurafenib in BRAF<sup>V600E</sup> melanoma cells (in vitro inhibition of the FGFR3/RAS axis indeed restores the sensitivity of vemurafenib-resistant cells to vemurafenib). Enhanced RAS-dependent RAF dimerisation has also been involved into the pathogenesis of squamous cell carcinomas, as a side-effect in subsets of patients treated with RAF inhibitors. These agents have been demonstrated to indeed activate MAPK pathway by inducing RAF dimerisation in cells lacking BRAF mutations, leading to increased keratinocyte proliferation. In addition to the important role played by the intracellular levels of RAS-GTP, activating mutations in NRAS have been described to be acquired after treatment with BRAF inhibitors. Again, such oncogenic mutations...
Figure 3. BRAF inhibitor resistance by qualitative and quantitative alterations of the target.
Mechanisms of acquired resistance to BRAF inhibition based on either generation of resistant RAF dimers or amplification of the BRAF-mutant monomers have been reported.

(usually, affecting the codon 61 of the NRAS gene) lead to activation of the RAS-dependent pathways: the MEK-ERK signalling, through dimerisation of RAF proteins and trans-activations of the RAF dimers, and the AKT signalling, through direct stimulation of the PI3K protein. Mutations in any of the three isoforms of RAS (with preponderance of those occurring in HRAS gene) may also contribute to the development of squamous cell carcinomas as adverse events during the treatment with BRAF inhibitors.\textsuperscript{74,84}

**Quantitative and Qualitative Changes in BRAF**

Resistance to either BRAF or MEK inhibitors has been reported in melanomas showing an increased copy number of the BRAF-mutant allele in a subset of melanomas\textsuperscript{83,99} (Figure 3). Gene mutations and copy number gains may occur independently of each other, since they are determined from different pathogenetic mechanisms: alterations affecting the molecular machinery that monitor the proper progression of the cell cycle seem to be responsible for the presence of gross genomic anomalies during the malignant progression (indeed, copy number gains are often the consequence of random genomic instability), whereas mutations usually occur in diploid karyotypes with few structural abnormalities during the initial phases of evolution of malignancies.\textsuperscript{100} However, in some cases gene amplifications tend to occur in the same cancers presenting oncogenic mutations as reported for EGFR in NSCLC or BRAF in colorectal carcinoma.\textsuperscript{101,102} In melanoma, BRAF amplification has been poorly detected as a pre-existing alteration in cell clones prior to BRAF or MEK inhibitor treatment, suggesting that it might be mostly an acquired phenomenon in response to target therapy.\textsuperscript{103}

A peculiar, qualitative mechanism of resistance is represented by the intracellular accumulation of a splice variant of the mutated BRAF mRNA.
A subset of melanoma cells resistant to BRAF inhibitors expresses a truncated form of BRAF<sup>V600E</sup>, p61BRAF<sup>V600E</sup>, which lacks a region that encompasses the RAS-binding domain. This leads to enhanced dimerisation of the truncated BRAF mutant, whose kinase remains constitutively activated. The final effect of such an alteration is a transactivation of the MEK-ERK pathways, with ERK signalling being resistant to the RAF inhibitors. Moreover, the vemurafenib-resistant melanomas presenting an enhanced transcription and translation of the mutated BRAF kinase may develop a drug dependency for their continued proliferation, such that cessation of BRAF inhibitor administration may lead to regression of non-lethal drug-resistant tumours. This evidence has suggested that a discontinued treatment with these agents may somehow prevent the emergence of lethal drug-resistant cell clones.

**ERK Activation Via Alternative Kinases**

In a fraction of BRAF-mutant melanoma cells resistant to BRAF inhibitors, resistance has been demonstrated to be maintained after downregulation of the kinase activities inducing RAF dimerisation, as a consequence of an alternative way of stimulation of the ERK signalling (Figure 2). In some of these cases, amplification of the receptor tyrosine kinase (RTK) MET as well as increased levels of the hepatocyte growth factor (HGF), which is the main ligand of the MET receptor, have been reported. HGF acts as a soluble factor which may be overexpressed by stromal cells of the tumour microenvironment and stimulates MET receptor in a paracrine manner. The HGF-MET interaction promotes transduction of the signals to the downstream PI3K effector with subsequent enhancement of the AKT activity. Hyperstimulation of MET by HGF seems to be involved in both intrinsic and acquired resistance to BRAF inhibition; consistently, simultaneous administration of BRAF and HGF or MET inhibitors has been found to reverse drug resistance to the BRAF inhibitor alone.

Activation of other RTKs has been proposed as contributing to anti-BRAF drug resistance, including IGF-1R-mediated mechanisms. The IGF-1R signalling cooperates with the MAPK pathway in regulating progression from benign nevi to malignant melanoma through sustainment of cell survival and dissemination.

Interruption of IGF-1R signalling has been shown to inhibit tumour growth and block metastasis formation in a wide variety of tumour models. The main target of the increased expression of IGF-1R is again the PI3K-AKT pathway, whose activation is responsible for the development of resistance to BRAF inhibitors. Dual inhibition of IGF-1R and MEK inhibitors has been demonstrated to induce growth arrest in BRAF inhibitor-resistant cells.

An additional RTK protein involved in resistance to both BRAF and MEK inhibitors is represented by the PDGFRβ receptor, whose upregulation improves cell survival and invasiveness in a manner that is independent of the activation of the MAPK pathway. In the presence of BRAF or MEK inhibition, the increased activity of PDGFRβ has been indicated to induce overexpression of the transcriptional activation factors STAT3 or PAX3 through stimulation of the Src/FAK signal transducers. Recent data have indicated that STAT3 protein can be activated by mutated BRAF and involved in stabilisation of the anti-apoptotic protein Mcl-1. Downregulation of STAT3 - induced by BRAF-MEK inhibition - is able to impair the Mcl-1 activity and reduce melanoma cell survival. Conversely, upregulation of STAT3 - exerted by increased levels of RTK activation - allows cells to become independent of the activity of the BRAF-MEK pathway and contribute to resistance to BRAF and MEK inhibitors (STAT3 expression is strongly enhanced in BRAF/MEK-inhibitor-resistant cells).

Nearly all results about the role of the RTK effectors in resistance to such targeted treatments have been obtained in studies on melanoma cell lines; therefore, significant data from analysis of clinical samples are not yet available.

**Reactivation of MEK-ERK Pathway**

Preclinical models have indicated that an increased expression of the COT kinase may strongly stimulate MEK and subsequently activate ERK signalling or directly promote the ERK activity, independently of the status of the upstream BRAF kinase. The overexpression of the COT kinase, which is encoded by the MAP3K8 gene, is induced by the treatment with BRAF or
MEK inhibitors in both melanoma cells and tissues, acting as an agonist of the MAPK pathway and leading to resistance to BRAF-MEK inhibition.46

Another mechanism of resistance to BRAF of MEK inhibitors in BRAF-mutated melanoma is represented by the occurrence of activating mutations in either MAP2K1 (encoding MEK1 protein) or MAP2K2 (encoding MEK2 protein) genes.12 In vitro models indicated that specific mutations in MAP2K1 (P124L and Q56P) may contribute to modify the allosteric pocket of MEK1 or disrupt the helix A conformation; such changes are able to make MEK1 protein either independent of stimulation by upstream oncogenic BRAF or insensitive to MEK inhibitors (through a block of their bind to the kinase).79

Most of the previously presented data are referred to mechanisms of resistance to inhibitors of mutated BRAF. Among them, several alterations are also involved in the acquired resistance to MEK inhibitors, including: amplification of BRAF mutant,102 upregulation of the STAT3 transcription activator,11 and driver mutations in MAP2K1 or MAP2K2 genes constitutively inducing the kinase activity of the MEK protein or the allosteric block of the binding of anti-MEK agents.79,113 Melanoma cells chronically exposed to a MEK inhibitor have been recently reported to show both MAP2K2 mutations and BRAF-mutant amplification, with a subsequent acquired resistance to BRAF-MEK inhibition.48 In preclinical studies, resistance to MEK inhibitors in BRAF-mutated melanomas has been correlated to activation of AKT; conversely, sensitive cell lines show upregulation of the PTEN tumour suppressor gene.84,115

For KIT, presence of some specific sequence variants within the coding regions of the gene have been found to render melanoma cells sensitive to KIT inhibition (see above). Acquisition of secondary mutations able to resume the gene signalling represents the main mechanism of resistance to KIT inhibitors (imatinib, nilotinib, dasatinib, sunitinib); different types of mutations have been reported to suppress the inhibitory activity of some or all of these agents.96 Moreover, NRAS mutations and KIT amplifications may cause resistance to imatinib in KIT mutant melanoma.46 In KIT-inhibitor resistant cells, simultaneous inhibition of the BRAF-MEK or PI3K-AKT pathways has been reported to induce apoptosis and growth arrest, suggesting that resistance is mediated by activation of these functional cascades.46,117

**FUTURE PERSPECTIVES**

Considering all the above-described molecular mechanisms underlying resistance to BRAF, MEK, and KIT inhibitors, it is evident that a crucial role in determining such a phenomenon is played by the increased activity of ERK or AKT signalling. In most cases, the addition of a compound directed against one of these latter activated effectors to the treatment with a targeted agent may contribute to overcoming resistance to single inhibitors.

Activation of the ERK1/2 proteins and, therefore, of the ERK-dependent nuclear transcription has been largely reported to significantly drive either the development of an acquired drug resistance or the occurrence of most of the side-effects in melanoma patients. In preclinical models, a selective, ATP-competitive inhibitor of ERK1/2 kinases has been described to resume growth suppression in melanoma cells whose resistance was determined by ERK reactivation.118 Moreover, discovery of a new RAF inhibitor, able to both inhibit ERK activity and protect ERK1/2 kinases from NRAS-driven reactivation in vemurafenib-resistant cells, further supports the hypothesis that a more efficient inhibition of ERK signalling in patients with activated MAPK pathway might represent a treatment option for avoiding or delaying the development of drug resistance.119 Similar results have been described for a combined inhibition of BRAF mutant and MEK, with enhanced suppression of ERK activity, increased levels of apoptosis, and sustained antiproliferative effects.120 A combination therapy based on the simultaneous use of MEK and BRAF inhibitors - therefore, targeting two effectors of the same pathway - has also been reported to achieve a clinical benefit.37

Nevertheless, preclinical data for the combination of MAPK signalling inhibitors and PI3K-AKT pathway inhibitors seem to suggest that such a treatment may become a winning therapeutic strategy to exert an effective antitumour outcome in melanoma patients. In this sense, combined treatment based on inhibition of BRAF and silencing of AKT3 was found to significantly increase suppression of tumour growth as compared to the result obtained by single agent...
Similarly, the synergistic use of MEK and PI3K inhibitors as well as the combinations of MEK inhibitors with agents inhibiting mTOR (the downstream effector of the PI3K-AKT pathway) have been reported to exert an effective antitumour response. In other words, suppression of AKT activity by inhibition of either upstream (PI3K) or downstream (mTOR) effectors of this signalling cascade may enhance the antitumour effectiveness of the MAPK-targeted therapies. Finally, combination of MEK inhibitors with CDK4/6 inhibitors is under investigation, particularly in NRAS mutant melanomas. Future efforts will be aimed at assessing composition and schedules of administration for such combined therapies in melanoma patients.

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