

Resistant mechanisms to BRAF inhibitors in melanoma

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Abstract: Patients with advanced melanoma have traditionally had very poor prognosis. However, since 2011 better understanding of the biology and epidemiology of this disease has revolutionized its treatment, with newer therapies becoming available. These newer therapies can be classified into immunotherapy and targeted therapy. The immunotherapy arsenal includes inhibitors of CTLA4, PD-1 and PDL-1, while targeted therapy focuses on BRAF and MEK. BRAF inhibitors (vemurafenib, dabrafenib) have shown benefit in terms of overall survival (OS) compared to chemotherapy, and their combination with MEK inhibitors has recently been shown to improve progression-free survival (PFS), compared with monotherapy with BRAF inhibitors. However, almost 20% of patients initially do not respond, due to intrinsic resistance to therapy and, of those who do, most eventually develop mechanisms of acquired resistance, including reactivation of the MAP kinase pathway, persistent activation of receptor tyrosine kinase (RTKS) receptor, activation of phosphatidylinositol-3OH kinase, overexpression of epidermal growth factor receptor (EGFR), and interactions with the tumor microenvironment. Herein we comment in detail on mechanisms of resistance to targeted therapy and discuss the strategies to overcome them.

Keywords: Melanoma; MAP kinase; resistance; biomarkers; targeted therapy

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Introduction

Melanoma is the most aggressive form of skin cancer, representing over 10% of all skin cancers, but responsible for more than 80% of skin cancer-related deaths (1).

The mitogen-activated protein kinases (MAPK) pathway is a key oncogenic signaling system of a relay of kinases that culminate in cell proliferation, differentiation and survival. Genomic classification of cutaneous melanoma proposed four subtypes: BRAF mutations, NRAS mutation, loss of NF-1 and triple wild-type.

The discovery of hotspot mutations in BRAF V600E, a

key serine-threonine kinase in the RAS-RAF-MEK-ERK (MAPK pathway) signaling pathway, led to development of molecular targeted therapies for melanoma (2). Activating BRAF mutations harbor 50% of cutaneous melanoma with non-chronic sun damage (involves another tumor as colorectal cancer, ovarian, thyroid) (*Figure 1*). In other clinical subtypes of melanoma, BRAF mutations are present in 10–20% of mucosal or acral melanoma, but absent in uveal melanoma (3,4). The most common mutation is a substitution of valine to glutamic acid (V600E) or lysine (V600K) at codon 600 in 20% of BRAF-mutants patients (5). The BRAF V600R occurs in 7% of patients, presents a

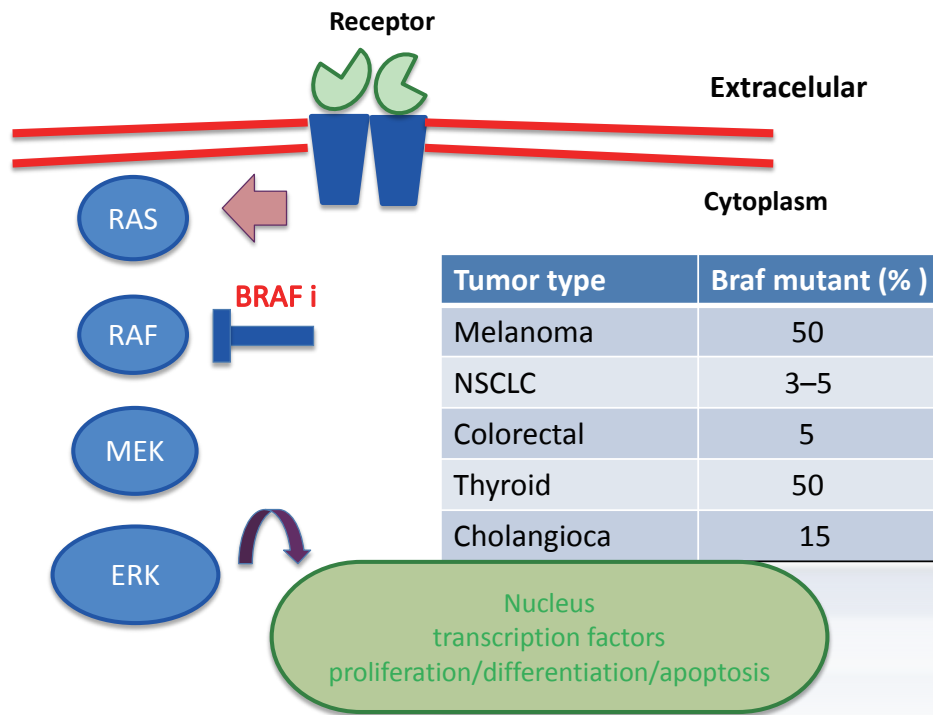


Figure 1 MAPK pathway. NSCLC, non-small-cell lung carcinoma; MAPK, mitogen-activated protein kinases.

substitution of valine to arginine. Mutations in NRAS are the second most common genetic alteration, being present in 20% of melanomas, and always exclusive to BRAF mutations (6). Twenty to thirty percent of mucosal melanomas harbor mutations or genomic amplification of cKIT (7). These are infrequently altered in cutaneous melanoma, while more than 85% of uveal melanomas contains mutations in GNAQ and GNA11; these mutations are also rarely present in cutaneous melanoma (8,9). BRAF inhibitors (vemurafenib, dabrafenib) and MEK inhibitors (trametinib) have been approved for treatment of unresectable or metastatic BRAF mutated melanoma, since they have shown improved progression-free survival (PFS) and overall survival (OS) compared to chemotherapy (10). Although responses and tumor control with BRAF inhibitors are impressive, durability of response is limited due to resistance, and evidence of disease progression can be seen within 6 to 8 months of starting therapy due to development of resistance mechanisms (11,12). Combined therapy with BRAF and MEK inhibitors has shown benefit in PFS and response rate, compared with monotherapy, delaying the appearance of alterations involved in resistance (13).

Mechanisms of resistance to MAPK pathway inhibition can be subdivided in two groups: MAPK-dependent and MAPK-

independent. Into BRAF/MEK/ERK dependent reactivation, mechanisms of resistance including: amplification of BRAF, splicing BRAF, NRAS mutation, MEK mutation, loss of NF1. MAPK-independent includes: up-regulated receptor tyrosine kinases (RTKS), overexpression COT.

Primary and acquired resistance, tumoral heterogeneity

Numerous mechanisms of resistance have been detected using *in vitro* and *in vivo* models, and many have been observed in pre- and post-treatment tumor samples. It is very difficult to explain the behavior of neoplastic cells, but melanoma cells are highly heterogeneous, regardless of their mutational and epigenomic profile. Resistant melanoma cells may become so under selective pressure from therapies from preexisting resistant clones, or secondary as an evolving process during treatment. Melanoma cells do not show arrangement, but express great plasticity, with several tumor subclones sustained by the microenvironment. This microenvironment supports tumor growth and the maintenance of two populations, slow-cycling tumor cells, and cells with epithelial to mesenchymal transition (EMT). Plasticity supports organization within the tumor, and

Table 1 Mechanisms of resistance

Intrinsic
RAC1 mutations
Stromal secretion HFG/cMET
Aberration CDK4, cyclin D1
HOXD8 mutation
Acquired
Splicing BRAF
Copy gain or amplification BRAF
Upregulation TK receptors
ERK feedback
Intrinsic/acquired
MITF
Aberrations of PI3K/AKT pathway
Loss PTEN
Mutations MEK
COT expresión
Loss of NF1
NRAF mutations

CDK4, cyclin-dependent kinase 4; HFG, hepatocyte growth factor; MITF, microphthalmia-associated transcription factor.

survival during treatment with BRAF inhibitors *in vitro* and *in vivo* (3–6% complete response) (14).

These mechanisms are known as primary or intrinsic resistance when no clinical benefit is achieved, and as secondary or acquired when progressive disease is seen after clinical benefit. Mechanisms of primary resistance include mutations in RAC1, loss of PTEN, amplification of cyclin D; secondary resistance mechanisms include alternative splicing of BRAF, BRAF copy number amplification and alterations in PI3K (Table 1).

Mechanisms of primary resistance

Loss of PTEN

Loss of PTEN occurs in 10–35% of melanomas, is mutually exclusive to NRAS mutations and coexists with BRAF mutations. PTEN is lost in the most of melanomas by loss of heterozygosity, mutations, and methylation. PTEN serves a tumor suppressor and major regulator of PI3K (15,16). Deletions or mutations in PTEN are associated with shorter PFS in patients treated with BRAF inhibitors. It is known, that PTEN loss alone is not sufficient to

confer resistance to BRAF inhibitors other concurrent alterations, such as activation of AKT, are necessary. Cell lines with inactivation of PTEN are less sensitive to BRAF inhibitors than wild-type PTEN melanoma cells (17). In clinical practice, patients with wild-type PTEN treated with BRAF inhibitors had longer PFS than patients with mutated PTEN (18) (32.1 *vs.* 18 weeks; $P=0.066$), and a weak association was seen between low expression of PTEN and lower response rates in patients treated with BRAF inhibitors (19). Dual inhibition BRAF and PI3K has been studied as a means of overcoming this resistance and restoring apoptosis in deleted PTEN cells (20).

Dysregulation of cyclin-dependent kinase 4 (CDK4)

In the cell cycle, cyclin D1 regulates proliferation through binding to CDK4 and CDK6, which activate retinoblastoma protein and lead to cell cycle progression. CDK4 mutations and cyclin D1 amplification confer strong resistance to therapy with BRAF inhibitors (21). Cyclin D1 amplifications are found in about 20% of BRAF mutated melanomas. CDK4-6 inhibitors (key regulators of G1-S transition of the cell cycle) alone failed to decrease tumor size, but when BRAF and MEK inhibitors were combined, complete responses were achieved in 30% of mouse models (22).

Hepatocyte grow factor (HGF) and microenvironment

Stromal cells secrete several factors such as HGF receptor c-MET, able to activate tumor cell growth in a paracrine form upregulating PI3K, thus conferring resistance to BRAF inhibitors or combinations of BRAF and MEK inhibitors (23). It was reported in cell lines that the combination of BRAF and AKT inhibitors or anti-MET therapies can lead to overcoming resistance of this pathway (24).

Loss of NF1

NF1 is a tumor suppressor of RAS; mutations of NF-1 are present in 14% of melanomas. Inactivation of NF1 leads to activation of RAS, PI3K-AKT-mTOR and MAPK pathways. NF1 mutations prevents under BRAF inhibition senescence of melanoma cells and too, NF1 mutations and NRAS mutations coexist in the inactivating BRAF, were been required RAS isoforms for the pro-tumorigenic activity of these cells. In this scenario, one means of overcoming resistance to BRAF inhibition is the combination of MEK and mTOR inhibitors (25).

RAC1 mutations

RAC1 is a key regulator of motility and proliferation cells and a GTPase effector of RAS. RAC mutations are present in 9% of melanomas, coexisting with BRAF and NRAS mutations. In clinical practice, from a cohort of 45 patients treated with BRAF inhibitors, 14 showed primary resistance, three had RAC1 mutations, any of the which no patients with reached response to therapy (26,27).

Mechanisms of secondary resistance

Acquired resistance mechanisms are associated mainly with the reactivation of the MAP kinase pathway (>70%), sometimes it coexists with the reactivation of the PI3K-AKT pathway, in a less percentage of patients it depends in exclusive on the AKT reactivation in parallel (PI3K-PTEN-mTOR).

BRAF variants

BRAF inhibitors drive suppression MEK/ERK signaling, although they activate MEK/ERK signaling in RAS mutant cells. In the presence of oncogene RAS, BRAF inhibitors lead to the formation of CRAF-BRAF heterodimers or homodimers. One part of BRAF inhibitors bound to hetero/homodimer, and another part that is drug-free. The BRAF inhibitor bound leads to activation of the drug-free, and through conformational changes, activating CRAF, and finally MEK-ERK activation. To overcome this resistance have been tested the combination targeting of BRAF and MEK inhibitors.

To date, no secondary gatekeeper BRAF mutations have been found. Two aberrations have been described affecting BRAF gene: gene copy number gain or amplification of BRAF, and alternative splicing of BRAF. Amplification of BRAF is a copy gain of the mutant allele of BRAF, resulting in overexpression and leading to reactivation of ERK independently of RAS (28). This aberration has been detected in about 20% of melanomas after treatment with BRAF inhibitors. ERK reactivation could be blocked with higher doses of BRAF inhibitors or with the combination of BRAF and MEK inhibitors. However, BRAF amplification also has been detected in patients treated with the combination of MEK and BRAF inhibitors. BRAF splicing is present in 32% of melanomas (29). The combination of BRAF and MEK or single therapy with ERK inhibitors should prevent this phenomenon, although BRAF splicing has been also detected in patients treated with the

combination of BRAF and MEK inhibitors.

NRAS mutations

NRAS mutations (Q61, Q12, Q13) occurring at either codon 12 or 61, and with mutations of NF1 drive MAPK activation in 30% of melanomas. BRAF and NRAS mutations are considered to be mutually exclusive. NRAS mutations not only activate MAPK pathway, is thought activate the PI3K pathway.

NRAS mutations are the second most common oncogenic alteration in melanoma (20%) and represent a clinical problem since they are associated with more aggressive tumors and shorter survival in early and late stage melanoma (30,31). The mutation of NRAS, activates transduction signals through CRAF in patients treated with BRAF inhibitors, resulting in a paradoxical transactivation of MAPK signaling via dimerization of BRAF and CRAF (32). Preclinical data in NRAS mutated patients supported the use of MEK, ERK and Pan-RAF inhibitors due to their high level of activity. In clinical trials, a MEK inhibitor (binimetinib) achieved 20% of response rate in NRAS mutant melanoma (33). Two trials have now completed enrollment, one phase II comparing pimasertib versus dacarbazine, and another phase III comparing a MEK inhibitor (binimetinib) versus dacarbazine in an NRAS mutated population. Data presented, but not published of this phase III of binimetinib, showed significant benefit in PFS. Preclinical data are interesting, although the benefit from MEK inhibitors is transient (34). Therefore, one possibility is to look for the last effector of this pathway, in this case blocking CDK4. Clinical trials are ongoing with the combination of MEK and CDK4-6 inhibitors (35). One clinical trial of MEK inhibitor with CDK4/6 inhibitor, binimetinib with LEE011 combination, showed 33% response rate in NRAS mutant population, with good tolerability.

Hyperactivation of RTKS

Overexpression or hyperactivation of RTKs could drive resistance by activation of parallel pathways or by direct induction of the RAS pathway (36). The most frequently involved receptors are platelet derived growth factor receptor beta (PDGFR β) and insulin-like growth factor I receptor (IGF-1R) (37,38). The activation of these receptors is due to epigenetic changes. The activation of RTKs induces additional activation of the PI3K pathway in patients treated with BRAF or MEK inhibitors, therefore leading

Table 2 Clinical trials in progress

Phase	Drugs	Targets
II	Vemurafenib followed ipilimumab	Immunotherapy plus BRAFi
II	LGX818 + MEKi/CDK4,6/FGFRi/PI3Ki/METi	Sequential TKi
I/II	Pembrolizumab + dabrafenib + trametinib	Anti-PD1 + BRAFi + MEKi
I/II	MEDI4736 + dabrafenib+ trametinib	Anti-PDL1 + BRAFi + MEKi
I	Dabrafenib ± trametinib + ipilimumab	Anti-CTL4 + BRAFi/MEKi
I/II	Vemurafenib + BKM120	BRAFi + PI3Ki
I/II	Dabrafenib + GSK2141795	BRAFi + AKTi
I/II	Vemurafenib + P1446A-05	BRAFi + CDKi
I	Dabrafenib, trametinib, ipilimumab sequent	BRAFi, MEKi, anti-CTL4
I	GDC-0941 + cobimetinib	PI3Ki + MEKi
I	BKM120 + MEK162	PI3Ki + MEKi

to resistance (39). The epidermal growth factor receptor (EGFR) gene is not normally expressed in non-treated melanoma, but in some patients that develop resistance to BRAF or MEK inhibitors overexpression of EGFR is induced by negative feedback. In this case, it is possible restoring sensitivity by inhibiting EGFR (40).

Aberrations in PI3K-PTEN-AKT pathway

MAPK pathway is deregulated in more than 70% of melanomas, but the PI3K/AKT/mTOR pathway is deregulated in more than 50% of melanomas.

It has been found that in 10–20% of cases that develop early resistance, or are intrinsically resistant to the MAPK inhibition, there is a loss of PTEN, or mutations in PI3K or AKT. Experiments in melanoma cell lines supports combined treatment with BRAF/MEK plus PI3K/AKT inhibitors to overcome resistance. Although the results in preclinical models are promising, there is currently limited clinical data (41,42).

Targeted therapy in non-cutaneous melanoma

Uveal melanoma (5% of all melanomas) has mutations in GNAQ/GNA11 (codon 209 or 183) in more than 80% of cases, and result in partial or complete loss of GTPase activity, thereby leading to constitutive activation effector pathways. This aberration activates the MAPK or PI3K pathways or protein kinase C. This activation can be suppressed by PKC inhibitors In a phase II clinical trial, a MEK inhibitor (selumetinib) as monotherapy was compared

with chemotherapy. . The study showed a benefit in terms of response rate and PFS, but there was no improvement in terms of OS (43). There are ongoing clinical trials testing the combination of a MEK inhibitor (trametinib) with an AKT inhibitor (GSK2141795), the PKC inhibitor AEB071 as single agent or the combination of combined MEK or PI3K inhibitors.

Mucosal or acral melanomas (3% of all melanomas) harbor mutations or amplifications in cKIT (20–30% of these melanomas) Activating KIT mutations lead to activation of KIT tyrosine kinase activity, stimulate the MAPK and PI3K/AKT pathway; and mutation non amplification predict response to TK inhibitors. The cKIT inhibitor imatinib was tested in in three clinical trials, demonstrating a response rate around 30%. Clinical trials with other ckit inhibitors (nilotinib, dasatinib, sunitinib) have been completed and results are pending (44,45) (*Table 2*).

Strategies to overcome resistance

Currently, the combination of BRAF and MEK inhibitors represents the gold standard of targeted therapy in BRAF mutated melanoma. However, even with this combination, efficacy is limited due to development of resistance.

There are several strategies for overcoming such resistance, as combination with other targeted therapies, sequential/intermittent treatment schedules, and the combination of this targeted therapy and with immunotherapy.

The addition of a third drug might help to overcome resistance and several trials are ongoing testing the triple combination of MEK plus BRAF inhibitors with MET,

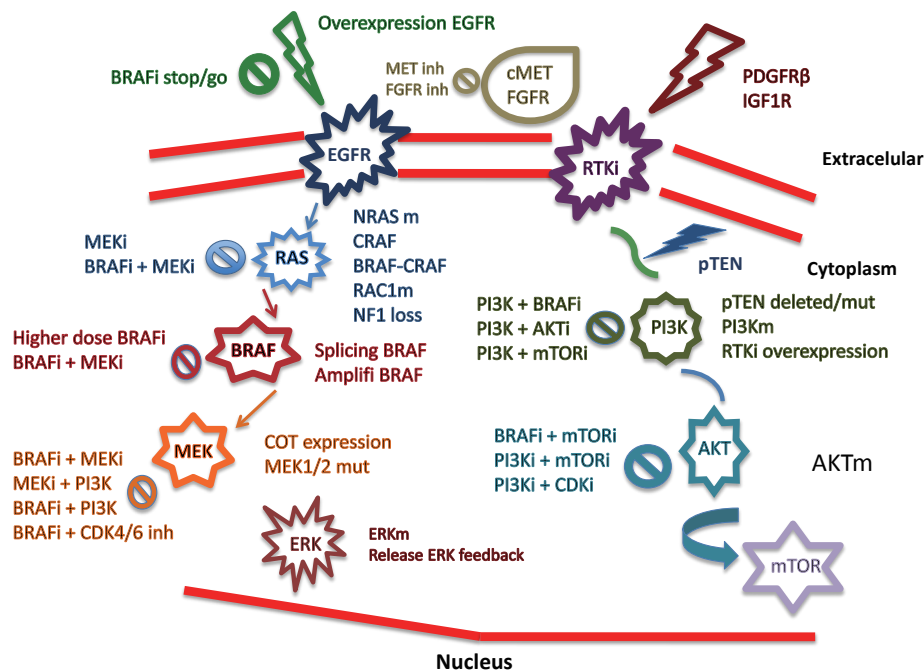


Figure 2 Strategies to overcome resistance. CDK4, cyclin-dependent kinase 4.

FGF, CDK, VEGF, or mTOR inhibitors (46,47).

It has been demonstrated that in resistant melanoma cell lines harboring BRAF splicing forms or BRAF amplifications stopping BRAF inhibition, leads to melanoma regression (48,49).

Treatment with MAPK inhibitors increases the expression of melanocytic antigens, and CD8 lymphocyte infiltration. This observation supports a possible synergism with the combination of targeted therapy with immunotherapy (50). An early attempt, combining a BRAF inhibitor with anti-CTLA4 antibody (ipilimumab), was failed due a high grade of hepatotoxicity in the phase I trial that led to an early stop of the study (51). Results of the clinical trial testing the sequential combination of dabrafenib plus ipilimumab are pending. New immunotherapeutic agents, such as anti-PD-1 antibodies (pembrolizumab, nivolumab) demonstrated much higher activity and less toxicity than anti-CTLA4 antibody.

The tumor infiltrating cytotoxic CD8 lymphocyte is a component of the adaptive immune response against melanoma associated antigens (after treatment with BRAF inhibitors), circulating CD8 cells sustain a strong inflammatory response with cytotoxic effects. In the exhaustion profile of CD8, leading to their incapacity to proliferate and produce cytokines (IL-2, INF), is mediated

up-regulation of inhibitory signaling pathways as PD-1, PD-L1 and CTLA4 (52,53). Clinical trials are underway to determine the clinical activity of the combination of BRAF inhibitors with anti PD-1 antibodies (*Figure 2*).

Preclinical studies have demonstrated that intermittent as opposed to continuous therapy with a BRAF/MEK inhibitor, may delay the development of acquire resistance (54). There are several studies assessing sequential or intermittent dosing of BRAF and MEK inhibitors are ongoing. In the phase II COMBAT study (CT.gov: NCT02224781), patients are randomized to the combination of dabrafenib and trametinib versus their combination, after 8 weeks of monotherapy with dabrafenib or trametinib. Serial biopsies on treatment and at progression are used, to assess biomarkers related to response or resistance. Another clinical trial, SWOG study S1320 (CT.gov: NCT02196181) is looking at intermittent schedule, with the combination with dabrafenib y trametinib during an 8-week lead in period; the patients without disease progression in the lead period, ongoing continuous dosing or to intermittent dosing during 5 weeks on with 3 weeks off. In this study includes serial biopsies to determinate resistance mechanisms.

Reactivation of MAPKinase pathway leads to a highly recurrent transcriptomic alterations across resistant tumors, in contrast to mutations, and were correlated with

differential methylation. The authors identified in the tumor: c-MET up-expression, LEF1 down-expression and YAP1 signature enrichment, as a drivers of acquired resistance. The authors observed high intra-tumoral cytolytic T cell inflammation, prior to BRAF inhibitor therapy preceded CD8 T cell exhaustion, and loss of antigen presentation in half of progressive melanomas, suggesting resistance to anti PD-1/PD-L1.

In the presence of BRAF/MEK inhibitor are the adaptive mechanisms of resistance. During the early phase, when the patients still respond to drug with inhibition MAPK pathway, adaptive resistance to BRAF inhibitors can occur, within the first 24–48 hours, leading to dampening of the inhibitor effect. Adaptive signaling seen involves: acquired EGFR and PDGFR expression, increase sensitivity to growth factors as EGF, FGF, HGF, neuregulin-1; increased phosphorylation AKT, up-regulation ERBB3 and enhanced MITF expression (55).

Recently, it has been published that an oncogene MITF is a driver of an early non-mutational and reversible drug tolerance state, which is induced by PAX-3-mediated up-regulation of MITF, before acquire resistance. Nelfinavir, HIV-1 protease inhibitor, was showed as a potent suppressor of PAX3 and MITF expression. Nelfinavir sensitizes BRAF, NRAS and PTEN mutant melanoma cells to MAPK inhibitors (56).

Conclusions

Targeted therapies are highly active drugs against metastatic melanoma. Different mechanisms of resistance have been described: epigenetic (57), genomic (58) and phenotypic (59) changes produces several alterations, leading to intrinsic, acquired or adaptive resistance. Tumor heterogeneity is a major driver of resistance in melanoma. In clinical practice, combination of BRAF and MEK inhibitors is the gold standard for metastatic BRAF mutant melanoma patients. The combination is highly active, but the duration of response is limited due to the development of acquired and adaptive resistance mechanisms. In order to overcome this phenomenon, there are different strategies, as the combination with other drugs—as CDK, PI3K, ERK and AKT inhibitors, intermittent schedules, and the combination with immunotherapy drugs.

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Footnote

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

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