UNIVERSITÀ DEGLI STUDI DI MILANO

Scuola di Dottorato in Scienze Biologiche e Molecolari

XXIV Ciclo

BRAF AND MAPK PATHWAY MOLECULES FOR TARGETED THERAPY OF MALIGNANT MELANOMA

SDD: MED 16, BIO 11

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Anno Accademico 2010-2011

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Abbreviations

ADAM	ADAM metallopeptidase
AK	adenylate kinase
AKT	v-akt murine thymoma viral oncogene homolog
ANOVA	analysis of variance
ATP	adenosine triphosphate
BAD	BCL2-associated agonist of cell death
BCRP	breast cancer resistance protein
Bcl-2	B-cell CLL/lymphoma 2
BRAF	v-raf murine sarcoma viral oncogene homolog B1
CCND1	cyclin D1
CDK4	cyclin-dependent kinase 4
CDKN2	cyclin-dependent kinase inhibitor 2A
COT/MAP3K8	mitogen-activated protein kinase kinase kinase 8
CRAF	v-raf-1 murine leukemia viral oncogene homolog 1
CTLA4	cytotoxic T-Lymphocyte Antigen 4
CTNNB1	β-catenin
4EBP-1	eukaryotic translation initiation factor 4E binding protein 1
EPHA2	EPH receptor A2
ERBB4	v-erb-a erythroblastic leukemia viral oncogene homolog 4
ERK	extracellular signal–regulated kinase
FAK	focal adhesion kinase
FISH	fluorescent in situ hybridization
HGF	hepatocyte growth factor
HIF-1α	hypoxia-inducible factor-1α
HRAS	v-Ha-ras Harvey rat sarcoma viral oncogene homolog
GF	growth factors
GNAQ	guanine nucleotide binding protein (G protein), q polypeptide
GRIN2A	glutamate receptor, ionotropic, N-methyl D-aspartate 2A
IC50	growth-adjusted inhibitory concentration of 50%
IGF1R	insulin-like growth factor 1 receptor
KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
MALDI-TOF	matrix-assisted laser desorption/ionization time-of-flight
MAPK	mitogen-activated protein kinase

MC1R	melanocortin-1 receptor
MEK	MAPK/ERK kinases
MET	met proto-oncogene
MITF	microphthalmia-associated transcription factor
MLPA	multiplex ligation-dependent probe amplification
MMP	matrix metalloproteinase
MRP4	multidrug resistance protein 4
mTOR	mammalian target of rapamycin
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NRAS	neuroblastoma RAS viral (v-ras) oncogene homolog
p70S6K	70-kDa ribosomal protein S6 kinase
PDGFRβ	platelet-derived growth factor receptor β
PI3K	phosphatidylinositol-3-kinase
PIK3CA	phosphoinositide-3-kinase, catalytic, alpha polypeptide
PIP2	phosphatidylinositol-4,5-biphosphate
PIP3	phosphatidylinositol-3,4,5-triphosphate
PTEN	phosphatase and tensin homolog
pTyr	phosphorylated tyrosine
RNAi	RNA interference
RTK	tyrosine kinase receptor
S6K	S6 kinase
SHC	Src homology 2 domain–containing transforming protein
siRNA	small interfering RNA
SRC	v-src sarcoma viral oncogene homolog
STAT3	signal transducer and activator of transcription 3
TGFβ	transforming growth factor, beta
TP53	tumor protein p53
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor

PART I

Abstract

The clinical activity of the BRAF inhibitor PLX4032 (vemurafenib) in patients with BRAFV600E mutant melanoma is limited primarily by the development of resistance leading to tumor progression. Strategies to overcome primary and acquired resistance are required. In a panel of 27 genetically characterized patient-derived melanoma cell lines the sensitivity to PLX4032 was dependent on BRAFV600E and independent from other gene alterations that commonly occur in melanoma, such as CDKN2A, and mutations of TP53, PTEN loss, and BRAF and MITF gene amplification. To investigate the molecular basis underlying acquired resistance to BRAF inhibitor, PLX4032-resistant cells were derived from a high sensitive BRAFV600E melanoma cell line, and used as a model. The resistant variant line showed increased AKT and ERK phosphorylation and enhanced IGF-1R/PI3K signaling. Combined treatment with PLX4032 plus PI3K inhibitors resulted in significant cell growth inhibition by decreasing pAKT and pERK signaling. To explore molecular mechanisms underlying primary resistance two melanoma cell lines lacking sensitivity to PLX4032 were used as models. Resistance to PLX4032 was maintained after CRAF down-regulation by siRNA, indicating that CRAF is not involved in the activation of ERK in the resistant cell lines. Treatment with the MEK inhibitor UO126 inhibited cell growth and decreased ERK phosphorylation indicating alternative activation of MEK-ERK signaling. Genetic characterization by MLPA and analysis of pTyr signaling by MALDI-TOF mass spectrometry revealed the activation of MET and SRC signaling, associated with the amplification of MET and of CTNNB1 and CCND1 genes, respectively. Testing of co-inhibition of the MET, SRC and MAPK signaling pathways by the combined treatment with the MET inhibitor, SU11274 or the SRC inhibitor, BMS-354825 plus PLX4032 resulted in a significant inhibitory effect on melanoma cell proliferation, survival, migration and invasive capacity.

These results support combinatorial approaches targeting MAPK pathway at different nodes and intercepting parallel signal transduction pathways as a strategy to override resistance to BRAF inhibitors.

State of the Art

Melanoma is an aggressive form of skin cancer and its incidence is on the rise worldwide. It is highly invasive and resistant to conventional therapy, making it the most lethal of human skin cancers. If melanoma is diagnosed early it can be cured by surgical resection, and about 80% of cases are dealt with in this way. Metastatic malignant melanoma is refractory to current therapies and has a very poor prognosis. The median survival rate for patients with metastatic disease is 8-9 months (Balch et al, 2009). Only recently, clinical trials of chemotherapy and of immunotherapy have shown significantly improved survival. Recent discoveries in the complex networks involved in melanoma proliferation, potential progression and survival have created many opportunities for targeted drugs and therapeutic approaches for this disease.

1. Melanoma subtypes

Cutaneous melanoma arises from the transformation of skin melanocytes, the melanin-producing cells located in the basal layer of the epidermis. By distributing pigment from melanosomes to keratinocytes melanocytes exert a protective role for the skin against UV radiation. Over 90% melanomas originate as cutaneous lesions, while 5% develop in mucosal surfaces (oral, gastrointestinal, ano-rectal, and vaginal) (Papaspyrou et al, 2011) and 5% in the uveal tract of the eye (Harbour et al, 2012). Cutaneous melanomas are classified into four major clinical-histologic subtypes (Clark et al, 1969; McGovern et al, 1973): 1- superficial spreading melanoma mostly occurring on areas of the body with intermittent sun exposure, such as the trunk and proximal extremities; 2- lentigo maligna melanoma associated with chronic sun-exposed areas of the body, such as the head; 3- acral melanoma mostly occurring on non sun-exposed regions, such as the palms, nail beds and soles of the head. This classification is however not predictive of disease progression, outcome and treatment response. The current melanoma staging system developed by American Joint Committee on Cancer staging system is based on primary tumor thickness, presence or absence of ulceration and of mitoses as well as on the extent of spread in regional lymph nodes and at distant body (Balch et al, 2009).

2. Mutations affecting key biological pathways in melanoma

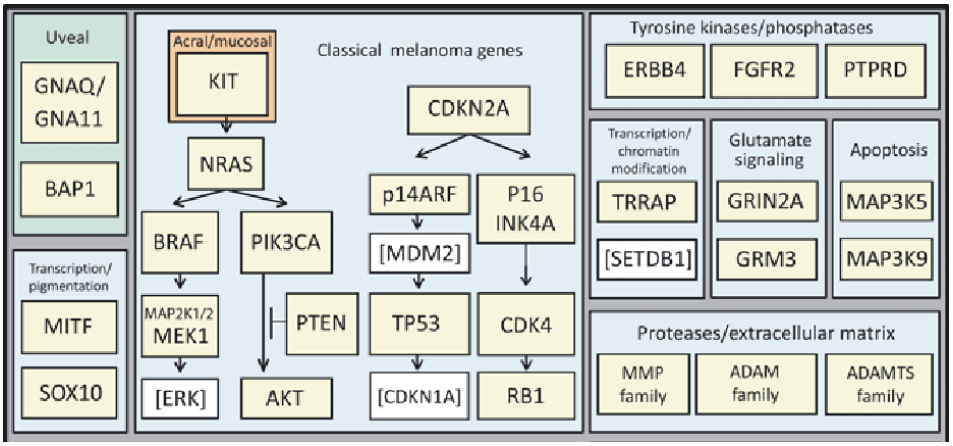

Figure 1. Summary of key pathways and genes frequently mutated in melanoma (modified from Dutton-Regester and Hayward, 2012).

Melanoma possesses a complex biological behavior resulting from a diverse range of genetic mutations. Somatic mutations are acquired during an individual's lifespan through environmental factors, primarily UV light exposure (Polsky and Cordon-Cardo, 2003). Genomic data support that UV light is the primary arbiter of high mutation rate observed in melanoma sequencing studies, as it induces point mutations causing nucleotide substitutions of thymine with cytosine (C>T) (Pleasance et al, 2010; Wei et al, 2011). Many somatic mutations have been identified in melanoma, not surprisingly a number of these occur in genes within the same biological pathways. The genes affected by mutations include oncogenes and tumor suppressor genes, namely CDKN2A, TP53, PTEN tumor suppressors and BRAF, NRAS, CKIT oncogenes which are defined 'classical melanoma genes'. (Dutton-Regester and Hayward, 2012). The advent of high-throughput technologies for sequencing have lead to significant progress in discovering and compiling mutation events in melanoma, adding a novel layer of complexity of analysis in discerning 'driver' mutation events responsible for the development of the tumor to those that are 'passenger' events (Walia et al, 2012). This is of particular significance in understanding the etiology of melanoma, with the observation of significantly higher rates of mutations in melanoma compared to other tumor types (Pleasance et al, 2010; Wei et al, 2011; Walia et al, 2012). A number of biological pathways and potential

targets to therapeutic intervention have been identified in these studies including ERBB4, matrix metalloproteinases (MMP) and GRIN2A genes, as shown in Figure 1. Somatic ERBB4 gene mutations resulting in hyperactivation of the ERBB4 receptor (Kurppa et al, 2009) have been identified in 19% of malignant melanoma (Prickett et al, 2009). The MMP have recently been shown to be frequently mutated in melanoma (Palavalli et al, 2009). A mutational analysis of 19 metalloproteinases-ADAM genes in human cutaneous metastatic melanoma identified eight to be somatically mutated, affecting 34% of the melanoma tumors analyzed (Wei et al, 2011). Functional analysis of the two frequently mutated ADAM genes, ADAM29 and ADAM7 demonstrated that the mutations affect adhesion of melanoma cells to specific extracellular matrix proteins and in some cases increase their migration ability, suggesting a role in melanoma progression. GRIN2A is mutated in 33% of melanoma samples (Wei et al, 2011). The mechanism by which mutations in GRIN2A have a tumorigenic effect is unclear; however, their frequency strongly suggests that they play a major role in melanoma and are worthy of future investigation.

3. Key signaling pathways implicated in melanoma

The implementation of novel high-throughput biotechnologies has led to the identification of a number of molecular pathways and genes involved in melanoma pathogenesis and progression. Melanoma develops through the disruption of the complex interconnected signaling pathways controlling cell proliferation, senescence and apoptosis. The most frequently altered biological pathways involved in melanoma progression are the RAS/RAF/MEK/ERK and PI3K/AKT pathways.

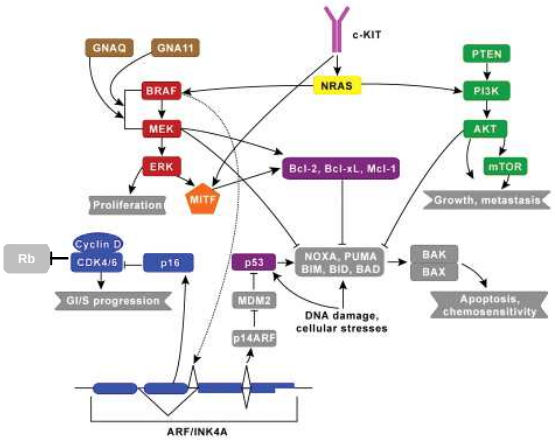

Figure 2. Signaling pathways implicated in melanoma (Modified from Vidwans et al, 2011).

3.1 RAS/RAF/MEK/ERK pathway

One of the most well known and characterized pathways commonly abrogated in melanoma is the mitogen activating protein kinase (MAPK) signaling cascade. The MAPK pathway is a phosphorylation-driven signal transduction cascade that couples intracellular responses to the binding of growth factors (GF) to cell surface receptors (Figure 2). In response to a variety of cellular stimuli, the G-protein RAS assume an activated state, leading to recruitment of RAF family (ARAF, BRAF and CRAF) from the cytosol to the cell membrane where they become activated. Activated RAF causes the phosphorylation and activation of MAP kinase extracellular signal regulated kinases 1 and 2 (MEK1/MEK2), which in turn phosphorylate and activate extracellular signal-regulated kinases 1 and 2 (ERK1/ERK2). Activated ERK translocate to the nucleus and phosphorylate several nuclear transcription factors (Elk-1, Myc, CREB, Fos and others) which bind promoters of many genes, including growth factor and cytokine genes that are important for stimulating cellular proliferation and survival, angiogenesis, invasiveness and metastasis. The MAPK pathway plays an important role in melanoma cell proliferation and survival, with ERK being constitutively activated in 90% of melanomas (Cohen et al, 2002). ERK hyperphosphorylation is due to mutations of NRAS (mutated in 10-20% of melanoma), and expecially of BRAF genes (approximately 50%), and less frequently of MAP2K1, MAP2K2 (approximately 8%) (Dutton-Regester and Hayward, 2012). Interestingly, NRAS and BRAF mutations are generally mutually exclusive events in melanoma, indicating that mutant BRAF or NRAS alone is able to activate the MEK/ERK pathway (Daniotti et al, 2004; Goel et al, 2006). Melanomas arising on non-chronically sun damaged skin are associated with frequent activating mutations in BRAF, a relatively high in frequency of NRAS mutations and no KIT mutations. Conversely, melanomas arising from mucosae, acral surfaces and skin with chronically sun damage show infrequent mutations in BRAF and NRAS and KIT gene amplification or mutations (Curtin et al, 2005; Curtin et al, 2006). KIT, located on chromosome 4q12, encodes a tyrosine kinase receptor for stem cell factor. Activation of KIT by ligand binding results in the stimulation of other signaling pathways producing proliferative and survival effects (Figure 2). Mutations result in constitutive activation of the KIT tyrosine kinase and

activation of multiple pro-survival signaling pathways including the MAPK and PI3K/AKT pathways. Genetic screens have shown that 46% of uveal melanomas exhibit mutations in GNAQ while are absent in cutaneous and mucosal melanomas (Onken et al, 2008; Van Raamsdonk et al, 2009). GNAQ gene, located on chromosome 9q21, encodes the q class of G-proteins α-subunits, involved in transmitting signals between G protein-coupled receptors and their downstream pathways, including activation of protein kinase C family members which are able to activate the MAPK pathway (Figure 2).
The microphtalmia-associated transcription factor (MITF), located on chromosome 3p14.1-p14.2, acts as a master regulator of melanocyte development, function and survival activated by MAPK signaling (Levy 2006). In melanocytes differentiation, it functions downstream of several pathways, including MC1R, MET and KIT (Figure 2). MAPK phosphorylates MITF leading to transcription of MITF-dependent genes, many of which are involved in pigmentation and survival, such as the antiapoptotic gene Bcl-2.

3.2 PI3K/AKT/mTOR pathway

The posphatidylinositol-3-kinase (PI3K) pathway is commonly altered in melanoma because of mutations in PTEN or activation of AKT resulting from tyrosine kinase receptors (RTK) activation (Figure 2).
The tumor suppressor gene PTEN, located on chromosome 10q23.3, encodes the lipid phosphatase PTEN, a negative regulator of PI3K/AKT pathway by dephosphorylating phosphatidylinositol-3,4,5-triphosphate (PIP3) in phosphatidylinositol-4,5-biphosphate (PIP2) (Robertson et al, 2005). Deletions or mutations in PTEN are found in about 40% of melanomas (Rodolfo et al, 2004), but low expression level of PTEN may be seen in 50% of melanomas, probably as a result of epigenetic silencing, altered subcellular localization, or ubiquitination (Zhou et al, 2000). In response of GF receptors the PI3K phosphorylates PIP2 to PIP3, leading to activation of the major downstream effector of the PI3K pathway, AKT/protein kinase B. Once activated, AKT phosphorylates its substrates including the serine/threonine kinase mammalian target of rapamycin (mTOR) which then phosphorylates S6 kinase (S6K) and inhibits 4E-BP, leading to increased protein translation as well as other

targets that regulate cell division and translation. mTOR is found to be activated in 73% human melanoma cell lines (Karbowniczek et al, 2008). AKT/protein kinase B is a serine protein kinase constitutively activated in about 60% of melanomas (Stahl et al, 2004). Recent studies have revealed deregulation of the PI3K signaling in a high proportion of melanomas. The three isoforms of AKT, namely, AKT1, AKT2 and AKT3, have different effects on cell proliferation. AKT3 is preferentially activated in melanoma in 43-60% of melanomas (Stahl et al, 2004). Mutations in PIK3CA locus, the gene encoding the catalytic subunit of PI3K, do not contribute to mechanisms of AKT deregulation, because are detected at very low frequencies (<5%) in melanoma (Omholt et al, 2006). Increased phospho-AKT (pAKT) expression in melanoma is associated with tumor progression and shorter survival (Fecher et al, 2007). Oncogenic RAS can also bind and activate PI3K, resulting in increased AKT activity (Sekulic et al, 2008). Inhibition of PI3K signaling can diminish cell proliferation and promote cell death. Consequently, inhibitors against multiple components of the PI3K/AKT pathway such as LY294002 and wortmannin, have been developed and are in various stages of clinical testing (Courtney et al, 2010).

4. BRAF as a therapeutic target

The BRAF gene, located on chromosome 7q34, encodes for a serine-threonine protein kinase and is the most commonly mutated gene in melanoma, observed in 50% of melanoma (Davies et al, 2002). Ninetyfive % mutations of BRAF occur in exon 15, which encodes the catalytic domain of the BRAF protein, and determine the substitution at the valine with glutamic acid at position 600 (V600E). Alternative point mutations at the same position (V600K, V600D, V600R) contribute for 5-6% of the total (Long et al, 2010). The V600E mutation introduces a conformational change in the kinase domain that increase the activity of the kinase resulting in >10-fold more active than the wild-type kinase. BRAFV600E mutation leads to constitutive activation and phosphorylation of MEK/ERK pathway by phosphorylating MEK, stimulating cancer cell proliferation and survival (Davies et al, 2002).
Mutant BRAF transmits survival signals through a variety of cytoplasmic and cytoskeletal targets and initiates nuclear transcription, resulting in expression of several cancer-associated genes, including those for

cyclin D, hypoxia-inducible factor-1α (HIF-1α), vascular endothelial growth factor (VEGF), MMP, urokinase and integrins regulating cellular proliferation, angiogenesis, tissue invasion and metastases (Kohno et al, 2006; Kumar et al, 2007; DeLuca et al, 2008).
BRAFV600E mutation is an early mutational event since it is detected also in nevi suggesting that BRAFV600E alone is not sufficient for malignant transformation and additional alterations are needed for melanocyte transformation (Pollock et al, 2003; Kumar et al, 2004). The association of BRAF mutation with PTEN (Dankort et al, 2009), p16 (Dhomen et al, 2009), p53 (Patton et al, 2005) genes has been described. It has been reported that melanocortin-1 receptor (MC1R) variants increase the risk of melanoma with BRAF mutations leading to the hypothesis that BRAF activation may be indirectly induced by UV radiation (Landi et al, 2006; Fargnoli et al, 2008; Scherer et al, 2010).
The frequency and specificity of BRAFV600E mutation, together with the strict dependence of melanoma cell growth and survival on BRAFV600E activity, a phenomenon called oncogene addiction, have pointed to BRAFV600E as a promising therapeutic target. BRAF depletion by siRNA inhibited the MAPK cascade, induced growth arrest, and promoted apoptosis (Hingorani et al, 2003; Karasarides et al, 2004).
Several authors have reported a BRAF mutation-associated gene expression signature in melanoma cells by microarray analysis (Pavey et al, 2004; Bloethner et al, 2005; Johansson et al, 2007). In particular, genes that encode proteins involved in RAS/RAF/MEK/ERK signaling were identified among the genes differentially expressed between melanoma cell lines with or without BRAF mutation (Bloethner et al, 2005) that potentially represent novel therapeutic targets.

A series of small molecules which inhibit BRAF activity have been developed. The first BRAF inhibitor to be investigated in melanoma was the multikinase inhibitor sorafenib (BAY43-9006), which targets BRAF as well as CRAF, platelet-derived growth factor receptor β (PDGFRβ), and vascular endothelial growth factor receptor (VEGFR) (Wilhelm et al., 2004). Sorafenib was tested in melanoma patients as monotherapy and combined with cytotoxic chemotherapy-dacarbazine (McDermott et al, 2008), temozolomide (Amaravadi et al, 2009), and carboplatin plus paclitaxel (Hauschild et al, 2009) with negative results. Then, a new generation of highly specific and potent BRAF inhibitors has been

developed. Two agents have recently demonstrated significant clinical benefit in melanoma, vemurafenib and dabrafenib. Vemurafenib (PLX4032/RG7204/RO5185426, zelboraf) is an highly specific ATP competitive inhibitor of BRAFV600E. PLX4032 is also effective against V600K mutation (Rubinstein et al, 2010). PLX4032 suppresses the activated oncogenic pathway by inhibiting the ERK kinase cascade, as evidenced by the inhibition of phosphorylated ERK (pERK). Phase I/II trial resulted in an 80% response rate, as determined by complete or partial tumor regression, in patients with BRAFV600E tumors whereas patients without the mutation did not respond (Flaherty et al, 2010; Chapman et al, 2011; Sosman et al, 2012). This is the highest response rate to date for a melanoma drug. However, the responses are temporaneous and resistance to BRAF inhibitors emerges within few months. Side effects include skin rash, photosensitivity, hair loss, fatigue, joint pain and skin tumors, mostly squamous-cell carcinomas and keratoacanthomas, in 15-30% of patients (Robert et al, 2011). Another specific inhibitor is dabrafenib (GSK2118436), an ATP competitive inhibitor of the mutant BRAF V600E/D/K. In a phase I/II study, 60% melanoma patients with mutant BRAF had more than 20% tumor reductions (Kefford et al, 2010); notably, reduction of brain metastases was observed (Long et al, 2010). GSK2118436 is under evaluation in a phase II study for the treatment of patients with brain metastases, and a phase I study has commenced of GSK2118436 in combination with the MEK inhibitor GSK1120212. Side-effects were very similar to what has been observed with PLX4032. Both PLX4032 and GSK2118436 were reported to have a stimulatory effect on MAPK pathway in cell lines with wild type BRAF that harbor upstream pathway activation such as oncogenic RAS or upregulated receptor tyrosine kinases (Poulikakos et al, 2010; Hatzivassiliou et al, 2010). This effect is probably driven by the formation of RAF dimers that lead to signaling through CRAF and consequent MAPK pathway hyperactivation (Heidorn et al, 2010). This phenomenon may explain the appearance of secondary skin tumors which are characterized by frequently mutations in HRAS (Su et al, 2012).

MEK and ERK kinases lie downstream of BRAF in the signaling pathway and are other potential targets of new drugs. Several MEK inhibitors are under evaluation for the treatment of metastatic melanoma, such as AZD6244 and GSK1120212 which demonstrated an objective response

rate of 44% with a median progression free-survival of 7 months (Infante et al, 2010).

4.1. Resistance mechanisms to BRAF inhibition

Resistance to BRAF inhibition includes primary resistance or insensitivity to treatment and secondary resistance, acquired after chronic treatment. Primary resistance defines those tumors that progress early in the course of therapy, whereas secondary resistance connotes progression after an initial response. Primary resistance to BRAF inhibition is observed in 20% of patients with BRAF mutant melanoma treated with PLX4032 (Flaherty et al, 2010). Preclinical studies have identified elevated levels of CRAF are associated to primary resistance to BRAF inhibition (Montagut et al, 2008) in addition to CCND1 gene amplification, overexpression of cyclin D1 and of CDK4 (Smalley et al, 2008). The majority of patients responded to PLX4032 and GSK2118436 treatment but relapsed within 2-18 months and developed resistance to the treatment (Flaherty et al, 2010; Arkenau et al, 2011).
To date, preclinical studies have identified multiple mechanisms of acquired resistance to BRAF inhibitors, some of which were shown to occur in metastatic lesions from treated patients. Reactivation of the MAPK pathway seems to be involved in the majority of the cases of acquired resistance but signaling transduction by parallel pathways was also identified (Johannessen et al, 2010; Nazarian et al, 2010; Wagle et al, 2011). In general, acquired resistance mechanisms can be classified into ERK-Dependent and ERK-Independent mechanisms (Figure 3 and Table 1).

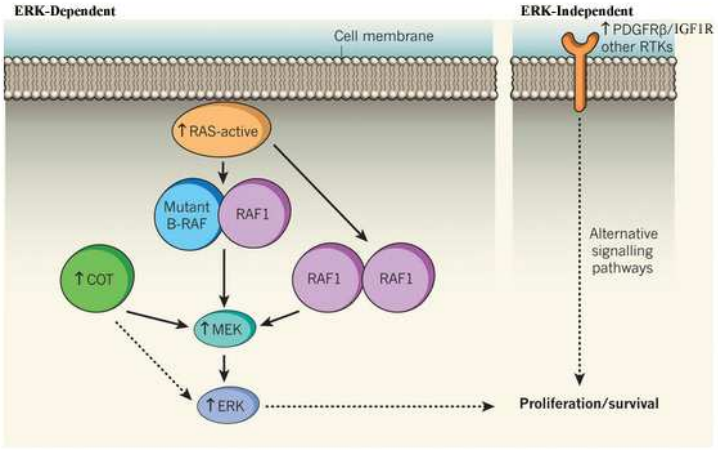

Figure 3. Mechanisms of acquired resistance to BRAF inhibitors (Modified from Solit et al, 2010).

4.2.1 ERK-Dependent mechanisms

The vast majority of acquired resistance mechanisms to BRAF inhibitors display reactivation of ERK signaling despite the presence of inhibitor.
Elevated CRAF activity was identified as a mechanism of resistance to the BRAF inhibitor AZ628 in preclinical studies (Montagut et al, 2008). In AZ628 resistant clones generated in vitro from a BRAF mutant melanoma cell line, higher CRAF protein levels were detected, compared to drug sensitive parental cells, with tumor cells appearing to have switched signaling from BRAF to CRAF. However, no CRAF gene amplification was detected.
While secondary mutations in the kinase domain of BRAF have not yet been identified as a cause of BRAF inhibitor resistance, Corcoran et al have identified amplification of the mutant BRAF allele in two independent BRAF mutant colorectal cancer cell lines resistant to the MEK inhibitor AZD6244 (Corcoran et al, 2010).
Furthermore, BRAF gene amplification was reported to occur in four out of twenty patients with acquired resistance to BRAF inhibitor PLX4032 providing evidence for alterations in the drug target causing clinical relapse (Shi et al, 2012).
The first resistance mechanism that involves a structural change in BRAF was reported by Poulikakos et al showing dimerization of aberrantly BRAFV600E splicing variants, lacking the RAS-binding domain, as a mechanism of acquired resistance to PLX4032 (Poulikakos et al, 2011). Acquired resistance mediated by BRAFV600E splicing variants is due to insensitivity of the enzyme to RAF inhibitors. The identification of BRAF variants in tumors from six of nineteen patients with acquired resistance to PLX4032, indicates the clinically importance of this mechanism. BRAF splicing variants were not detected in two samples derived from patients with intrinsic resistance, or in melanoma cell lines and tumor biopsies not exposed to BRAF treatment with inhibitors.
NRAS mutations were identified as a mechanism of acquired resistance to the BRAF inhibitor PLX4032 (Nazarian et al, 2010). Cell lines resistant to PLX4032 were derived from three melanoma cell lines with BRAFV600E. In one of these cell lines an activating NRAS mutation (Q61K) was identified which was not detected in the original cell line. This mutation was also present in an isolated nodal metastasis from a

patient with BRAF mutant melanoma, which progressed after an initial response to treatment with PLX4032. A second NRAS mutation, Q61R, was identified in a second progression site in the same patient. Mutated NRAS was known to induce a switch from BRAF to CRAF, resulting in persistent MAPK signaling (Dumaz et al, 2006).

COT/MAP3K8, a MAPK downstream of BRAF, was identified as potential mediator of BRAF inhibitor resistance by employing cDNA expression for screening kinases able to confer resistance to PLX4720, a compound closely related to PLX4032 (Tsai et al, 2008; Johannessen et al, 2010). COT levels were observed to increase in cell lines treated with BRAF inhibitors, suggesting that it may be involved with feedback regulation of MEK activity. Moreover, COT expression was associated with intrinsic resistance in BRAFV600E cultured cell lines and sustained ERK phosphorylation in the presence of BRAF inhibitor. Biopsy samples from three patients with metastatic melanoma undergoing treatment with PLX4032 showed higher COT expression during treatment than before treatment, and high levels were detected in a relapse specimen. COT-expressing cell lines were shown refractory not only to BRAF inhibition but also to MEK1/2 inhibitors. In the context of MEK inhibition, these cell lines showed sustained ERK phosphorylation, raising the possibility that COT may activate ERK through a MEK-independent mechanism as well as a MEK-dependent mechanism. ERK inhibition or direct COT inhibition may be needed to bypass this mechanism.

Activating MEK1 point mutations (P124L, Q56P, C121S) were identified in resistant melanoma cells (Emery et al, 2009; Wagle et al, 2011). These mutations were characterized biochemically to activate ERK and could account for reactivation of MAPK signaling pathway. P124L MEK1 mutation, was identified in a resistant metastatic focus that emerged in a melanoma patient treated with the MEK inhibitor, AZD6244. Both P124L and Q56P MEK1 mutants conferred cross-resistance to PLX4720, a selective B-RAF inhibitor (Emery et al, 2009). C121S MEK1 mutation was found in one tumor sample of a patient after disease progression on PLX4032 treatment, and in a second sample from a tumor excised before treatment (Wagle et al, 2011).

4.2.2 ERK-Independent mechanisms

Several examples of resistance mechanisms have been reported that do not rely on sustained ERK signaling, indicating that ERK-independent pathways can maintain the tumorigenicity of BRAFV600E melanoma in the absence of ERK activation. The activation of signaling of RTK can bypass BRAF inhibition by activating parallel signaling pathways that redundantly regulate downstream mediators of cellular proliferation and survival, such as cyclin D1, BAD and 4E-BP1 (She et al, 2010). Activation of PDGFRβ was observed in three BRAF mutant melanoma cell lines made resistant to PLX4032 by chronic exposure compared to sensitive cell lines. Consistent with an ERK-independent resistance mechanism, the growth of PDGFRβ-overexpressing resistant cells was insensitive to MEK inhibitors. Four of eleven clinical post-relapse biopsies from melanoma patients treated with PLX4032 showed increased PDGFRβ expression compared to pre-treatment biopsies (Nazarian et al, 2010). RNAi-mediated knockdown of PDGFRβ led to growth inhibition in the presence of PLX4032, validating PDGFRβ as the cause of acquired resistance. However, the combination of PDGFRβ inhibitor and PLX4032 did not restore sensitivity to resistant cell lines indicating that additional mechanisms may contribute to resistance, possibly involving other RTK.
The insulin-like growth factor 1 (IGF1R) signaling pathway was implicated in resistance to BRAF inhibitor SB-590885. IGF1R, as well as other RTK, is known to activate PI3K/AKT signaling. Resistant cells displayed elevated levels of phoshorylated IGF1R (pIGF1R) and AKT. Pharmacologic inhibition of IGF1R decreased proliferation and reduced pAKT levels in resistant cells. Combined inhibition of IGF1R and MEK induced apoptosis, suggesting that ERK-independent survival signaling was mediated by IGF1R in resistant cells (Villanueva et al, 2010). In addition, immunohistochemical analysis of five paired tissue sets from relapsed melanoma patients treated with PLX4032 showed increased expression of IGF1R in two patients, one of which also had increased levels of pAKT. In the post relapse sample of one patient without a concomitant IGF1R increase a homozygous loss of PTEN and increased levels of pAKT were found indicating that PTEN loss could be linked to resistance to BRAF inhibitors.

	Mechanism	Clinicalrelevance of in vitro acquired resistance studies	Reference
ERK-Dependent	BRAF amplification	Not tested	Corcoran et al, 2010
		4/20 patients	Shi et al, 2012
	BRAF splicing variants	6/19 patients	Poulikakos et al, 2011
	MEK1 mutation	1 patient	Emery et al, 2009; Wagle et al, 2011
	NRAS mutation	2/16 biopsies from 1 patient	Nazarian et al, 2010
	CRAF upregulation	Not tested	Montagut et al, 2008
	COT upregulation	3 patients	Johannessen et al, 2010
ERK-Independent	PDGFRβ upregulation	4/11 patients	Nazarian et al, 2010
	IGF1R upregulation	2/5 patients	Villanueva et al, 2010

Table 1. Molecular mechanisms of acquired resistance to BRAF inhibitors

Understanding the biology and deciphering the genetics of melanoma has been crucial for the development of new therapies. Due to the variety of the resistance mechanisms described so far, it appears that there is not a single strategy to bypass resistance to BRAF targeted drugs that will fit all patients. Compounds blocking multiple levels of the signaling pathways are being actively researched. The V600EBRAF inhibitor PLX4032 is now an approved agent for the treatment of advanced melanoma. However, this drug is suited only for 50% of the patients who carry metastatic melanoma with the BRAF mutation, and even in these patients the responses are transitory. For those not presenting the mutation, finding other targets is urgent. The combination of kinase inhibition with conventional cytotoxic chemotherapy, immunotherapy or multiple kinase inhibition guided by the tumor molecular profile are currently evaluated as new strategies for personalized melanoma treatment.

Aim of the Project

Blocking oncogenic signaling induced by the BRAFV600E mutation is a promising approach for melanoma treatment. The specific BRAFV600E kinase inhibitor **PLX4032** showed a remarkable clinical activity in patients with BRAF mutant melanoma. However, resistance to PLX4032 develops after treatment and strategies to prevent and overcome resistance are required.
To overcome PLX4032-resistance the following specific objectives were identified:

- to evaluate PLX4032 activity on cell proliferation, apoptosis, cell cycle and downstream signaling pathways in a panel of BRAFV600E melanoma cell lines with defined genetic alterations

- to characterize at the genetic and molecular levels melanoma cell lines showing poor sensitivity to PLX4032

- to generate resistant cell line variants by chronic exposure to PLX4032

- to identify and validate new potential targets for pharmacological intervention associated with the lack of sensitivity to PLX4032 by molecular studies

- to evaluate the effects of the combination of PLX4032 with other kinase inhibitors to bypass resistance to BRAF inhibition.

Results and Discussion

1. Effects of PLX4032 on growth and survival in BRAFV600E mutated melanoma cells

The antiproliferative effects of PLX4032 was tested in 27 genetically characterized short term melanoma cell lines, including 20 lines heterozygous for the BRAFV600E mutation and 7 lines carrying wild type BRAF gene. Eighteen out of 20 BRAFV600E mutated melanoma cell lines were highly sensitive to growth inhibition by PLX4032 when evaluated by MTT proliferation assays, with IC50 in the µM range (0.01-1), while two lines showed IC50 about 10 µM indicating primary resistance to the compound. The different IC50 values observed in the sensitive cell lines were not associated with alterations in CDKN2A, TP53, PTEN genes, amplification of BRAF or MITF genes, or to the expression of KIT protein. No effect on proliferation was detected in melanomas carrying wild type BRAF gene, indicating the specificity of PLX4032 for BRAFV600 mutated melanomas (Figure 1A).
Studies in other cancer types suggest that activation of the PI3K pathway is one mechanism by which tumor cells may bypass apoptotic signals initiated through anti-tumor agents (Halilovic et al, 2010; Smalley and Sondak, 2010). PTEN loss in conjunction with BRAF mutation has been shown to enable melanoma cell proliferation and survival through activation of PI3K/AKT signaling pathway, possibly decreasing dependency of ERK signaling (Dankort et al, 2009). To evaluate the role of PTEN in the effects of PLX4032 it has been tested whether PLX4032 exerted different effects in melanoma cell lines carrying or lacking intact PTEN. When response of melanoma cells to PLX4032 concentrations inhibiting cell growth was examined, an accumulation of cells in the G1 phase in the majority of sensitive but not in resistant lines was found, indicating proliferative block. Independently of PTEN expression evaluation of apoptosis, detected by adenylate kinase (AK) release or by activated caspase-3 detection, showed higher levels of apoptotic cells in PTEN-positive samples, indicating a role for PTEN in the induction of cell death in response to PLX4032 (Figure 1B). Together, these data confirmed the high specificity of PLX4032 for BRAFV600E mutated cell lines independently of other common alterations occurring in melanoma. In addition, two cell lines carrying BRAFV600E mutation, LM20 and

LM38, and demonstrating resistance to the cytotoxic effects of PLX4032 were identified.

2. Modulation of MAPK and PI3K/AKT signaling pathways by PLX4032 in sensitive and resistant cell lines

In an attempt to evaluate the signaling response associated to susceptibility to PLX4032, the effect of treatment on downstream pathways regulating cell growth and proliferation in BRAFV600E mutated melanomas was examined. Exposure to PLX4032 resulted in a marked decrease in pERK, pAKT and p-p70S6K, indicating that treatment with PLX4032 blocked the downstream signaling in most drug-sensitive cell lines, independently of PTEN status (Figure 2). In addition, cyclinD1 expression was downregulated in all drug-sensitive cells, in agreement with a G1 accumation in the cell cycle. In contrast, treatment did not inhibit pERK, pAKT and p-p70S6K signaling in the resistant cell lines LM20 and LM38. These data suggest that these cell lines can survive in the absence of MAPK abrogation due to other oncogenic events beyond BRAF.

3. Chronic BRAF inhibition leads to acquired PLX4032 resistance

To investigate if chronic BRAF inhibition could lead to acquired drug resistance, four PLX4032-sensitive V600E melanoma cell lines (LM17, LM36, LM16, LM25) were chronically treated with the drug. The resistant variants (LM17R, LM36R, LM16R, LM25R) were obtained after treatment with PLX4032 (3.2 µM) for 96 hours, allowing the few surviving cells to regrow, and repeating treatment for 11 times (Figure 3). The LM17 and LM17R cell lines were used as a model system to investigate the molecular basis underlying acquired resistance to BRAF inhibitor. Whereas LM17 cells were highly sensitive to BRAF inhibition by PLX4032, LM17R cells showed reduced sensitivity to the antiproliferative effect of the drug and diminished cell cycle arrest, AK release and caspase 3 activation (Figure 4A). Western blot analysis showed that pERK, pAKT and cyclinD1 signaling was maintained after exposure to PLX4032 in LM17R in contrast with a strong signaling down regulation in LM17 cells (Figure 4B). Increase in pAKT level was noted in LM17R compared to LM17. These findings suggest that acquired resistance to

BRAFV600E inhibition could occur through reactivation of MAPK signaling and activation of alternative pathways as PI3K/ AKT.

To investigate pathways activated in response to chronic treatment to PLX4032 a phospho-RTK array was used to assess phosphorylation of RTK in resistant compared to parental cells. pIGF1R levels resulted clearly upregulated among the tested RTK. Western blot analysis of pIGF1R confirmed the upregulation in the resistant line (Figure 4C). IGF1R can activate both the MAPK and PI3K signaling pathways, thus explaining the increase in pAKT levels observed in LM17R cells (Neudauer et al, 2003). Consistently with an increased dependency from AKT mediated signaling for proliferation, the effect of PI3K inhibitors, LY294002 and wortmannin, in LM17R versus LM17 was examined. Both LY294002 and wortmannin had antiproliferative effect as a single agent on both cell lines. In LM17R, the combination of LY294002 plus PLX4032 or wortmannin plus PLX4032 improved the response to either drug alone compared to the parental cell line (Figure 5A). These data suggest a link between BRAF inhibition and enhanced IGF1R-mediated PI3K signaling. The efficacy of the combined PI3K and MAPK pathway inhibitors was verified by western blot analysis for pAKT and pERK. Exposure of LM17R cells to LY294002 plus PLX4032 showed a clear reduction of pERK and a moderate decrease of pAKT compared with either agent alone (Figure 5B). In contrast, in the intrinsically resistant LM20 cell line, the combination of LY294002 or wortmannin plus PLX4032 did not increase sensitivity to PLX4032 confirmed by minimal reduction in growth inhibition and unresponsiveness of pERK and pAKT (Figure 5C) indicating that additional signaling pathways may be involved in growth and survival of BRAFV600E cell lines showing primary resistance to PLX4032.

4. Resistance to PLX4032 is independent from CRAF or MEK activity

Several different mechanisms involved in resistance to BRAF inhibition have been described. Among them increased CRAF protein levels and switching from BRAF to CRAF dependency has been associated with the in vitro acquired resistance by BRAF inhibition to AZ628 (Montagut et al, 2008). To explore the role of CRAF expression in the persistent

MAPK signaling observed in the resistant lines, a RNA interference (RNAi) approach was used. LM38 and LM17R cells were transfected with CRAF or control siRNA for 24 hours before being treated for 24 hours with PLX4032. Immunoblotting demonstrated specific and effective down-regulation of CRAF protein levels in both PLX4032-resistant cell lines, LM38 and LM17R after transfection with CRAF specific siRNA. However, CRAF down-regulation did not affect pERK levels and sensitivity to PLX4032 indicating that CRAF is not involved in the activation of ERK in the resistant cell lines (Figure 6AB). To confirm that PLX4032-resistant lines remain dependent on MAPK activation for proliferation, the effect of MEK inhibition using the MEK inhibitor UO126 was examined. Treatment with UO126 inhibited ERK phosphorylation and decreased proliferation in resistant cell lines indicating that other pathways may promote survival of these cells (Figure 6CD).

5. Characterization of melanomas showing resistance to PLX4032

Resistant cell lines showing a low sensitivity to PLX4032 may be less dependent on the BRAFV600E oncogenic signaling, relying on the co-activation of other signaling pathways acting in parallel or upstream BRAF. In the search for new potential markers associated with the lack of sensitivity to PLX4032 and to identify candidate genes, Multiplex Ligation-dependent Probe Amplification (MLPA) analysis measuring the copy number of genes that are often deleted or amplified in tumors was used to genetically characterize the resistant melanomas. MLPA analysis showed no difference in the pattern of alterations between LM17 and LM17R, indicating that the acquisition of PLX4032 resistance is not associated to gain or loss of the tested genes (Part II, figure W2). In contrast amplification of CCND1 at 11q13 and of CTNNB1 at 3p21 were detected in LM20 cells, while LM38 line showed a different pattern of alterations, which included MET amplification at 7q31. MET and CCND1 gene amplification in LM38 and in LM20 were confirmed by FISH analysis (Figure 7A) and gene copy number by qPCR (Part II, table W1).

To further explore the mechanisms of drug resistance in an attempt to identify potential target molecules in BRAFV600E-mutated melanoma showing poor sensitivity to PLX4032, a proteomic-multiplexed analysis of the phosphotyrosine (pTyr) signaling and antibody validation was used

to screen pTyr proteins modulated by treatment, comparing sensitive and resistant melanomas. A significant degree of heterogeneity of profiles before and after PLX4032-treatment was observed (Part II, figure W3). MALDI-TOF mass spectrometry analysis was used to identify the most abundant phosphorylated proteins in untreated LM20 and LM38 cell lines. The identified proteins indicated that in LM20 cells pTyr-based cell signaling was based on SRC/FAK axis, while MET axis was prevalently activated in LM38 cells (Figure 7C). These data appeared consistent with the genetic data showing MET gene amplification in LM38 cells and CTNNB1 in LM20 cells. In agreement with proteomic results, immunoblotting analysis revealed the phosphorylated MET (pMET) receptor in LM38 cells, and the phosphorylated form of STAT-3 (pSTAT3), activated downstream SRC, in LM20 cells (Figure 7B).

6. Combined targeting of MET and SRC signaling pathways to overcome PLX4032 resistance

On the basis of the results of molecular profiling, MET and SRC represented new potential targets activated in LM38 and LM20 cell lines, intrinsically resistant to PLX4032. To see if targeting the MAPK signaling pathways at parallel nodes is effective in PLX4032- resistant cell lines the effect of combining PLX4032 with the MET inhibitor SU11274 or the SRC inhibitor BMS-354825 was tested.
SU11274, when used alone, inhibited cell growth in most of melanoma cell lines including PLX4032-resistant cells. The combined treatment with SU11274 and PLX4032 resulted in a synergistic reduction of proliferation in the PLX4032-resistant cell line, LM38 (interaction index of 2.5). Combination of SU11274 with PLX4032 enhanced G1 cell cycle arrest and AK release in the absence of caspase 3 activation (Figure 8A). A similar antiproliferative effect was obtained after treating LM38 cells with PLX4032 plus other MET inhibitors (JNJ, SGX523 and PHA) (Figure 8B). The combined blockade of both pathways was also verified by western blot analysis resulting in down-regulation of MET signaling through pFAK and pSHC while pERK and pAKT were not affected (Figure 8C). To determine whether MET and BRAF signaling pathways cooperate, LM38 cells were transfected with siRNA targeted to MET and MET silencing checked by testing MET expression by western blot analysis. The treatment with MET-siRNA plus PLX4032 showed a

significant inhibitory effect on cell proliferation compared to single agents alone, indicating an interaction between the two signaling pathways (Figure 8D). After the treatment with MET-siRNA, cells displayed down-regulation of pSHC levels but not of pERK and pAKT (Figure 8E). MET inhibition with drugs or by specific siRNA confirmed the role of MET signaling pathway in LM38 cells resistant to PLX4032. HGF/MET signaling promotes multiple biological activities, including motility and invasion. The effect of the combined treatment with PLX4032 and SU11274 on the ability of the cells to invade matrigel and migrate in vitro was evaluated. Indeed, blocking MET signaling by treatment with SU11274 alone or in combination with PLX4032 strongly inhibited matrigel invasion, confirming the role of MET signaling in mediating the invasive capacity in these cells (Figure 9A). Notably, a moderate effect was observed after treatment with PLX4032, indicating that BRAF inhibition, although not affecting cell growth, may alter the invasive activity of melanoma cells, even in the presence of exogenous HGF. Scratch wound assays showed that the combination of PLX4032 with SU11274 prevented wound closure, whereas the single drugs impaired wound healing to a limited extent, confirming the effect of the combination on cell migration (Figure 9C). In addition, the combined drugs downregulated the expression of β1-integrin, the receptor for the extracellular matrix protein laminin, involved in adhesive and invasive cellular processes (Figure 9B).

BMS-354825 was reported to downregulate activated SRC, FAK, and EphA2 in melanoma cells and to inhibit proliferation in some melanoma cell lines (Eustace et al, 2008; Jilaveanu et al, 2011). Although BMS-354825 by itself did not affect the viability of LM20 cells, the combined treatment BMS-354825 plus PLX4032 significantly reduced growth in a synergistic manner (interaction index value of 2.1), increased AK release and the number of caspase 3-positive cells. A similar antiproliferative effect was obtained after treating LM20 cells with PLX4032 plus another SRC inhibitor, E804 (Figure 10A). BMS-354825 treatment downregulated pSRC levels and the downstream targets paxillin and p130CAS; in addition, BMS-354825 reduced pFAK levels. In contrast, no effect was detectable on pERK and pAKT levels, suggesting that it is not a necessary requirement to impair cell proliferation (Figure 10B). The combined treatment with PLX4032 and BMS-354825 decreased MMP-2 production by LM20 melanoma cells, which was measured using gelatin-

gel zymography (Figure 10C), and reduced the expression of β1-integrin (Figure 10D).

SRC and MET have been implicated in the development and progression of several tumor types as a result of interaction with receptor tyrosine kinases and their downstream effectors leading to proliferation, cell growth, survival, motility, migration and angiogenesis (Stella et al, 2010; Sen et al, 2011). Aberrant MET activation due to overexpression, mutations or gene amplifications, has been associated with poor clinical outcome and drug resistance in lung, hepatic, renal, and colorectal carcinoma (Stella et al, 2010). Genomic amplification of MET has been found in 47% of metastatic melanomas (Moore et al, 2008). It has been shown that targeted inhibition of MET either via SU11274 or specific small interfering RNA (siRNA) led to decreased cell growth and viability of melanoma cells. SU11274 inhibition of MET abrogated tyrosine phosphorylation of cellular proteins, including MET itself, as well as its downstream signaling proteins (Puri et al, 2007; Kenessey et al, 2010). These findings implicate the MET pathway in melanoma progression and suggest that MET inhibition might provide an effective therapeutic approach. The nonreceptor protein tyrosine kinase SRC plays a crucial role in the signal transduction pathways involved in cell division, motility, adhesion, and survival in both normal and cancer cells. Aberrant expression and activation of SRC occur in breast, prostate, lung, and colorectal carcinomas, in association with poor clinical outcome (Sen et al, 2011). Simultaneous inhibition of BRAF and MET/SRC signaling pathways showed antiproliferative and proapoptotic effects in PLX4032-resistant cells. In addition, these results demonstrated that the combination of SU11274 or BMS-354825 plus PLX4032 reduced invasive and migratory capacities of PLX4032-resistant cell lines, suggesting that they may represent effective drug combinations to inhibit melanoma growth and dissemination.

Conclusions and Future Prospects

The results collected in this thesis confirmed the high in vitro specificity of the BRAF inhibitor, PLX4032 for a subset of melanoma cell lines bearing mutant BRAFV600E. The antiproliferative effect of PLX4032 was accompanied by cell cycle arrest, increase in cell death and inhibition of ERK phosphorylation, indicating that PLX4032 blocks the downstream signaling resulting from constitutively active BRAFV600E. A large panel of melanoma cell lines showed differences in the sensitivity to PLX4032, and two BRAFV600E melanoma cell lines showing intrinsic resistance to the cytotoxic effects of the drug were identified. Data reported here showed that differential response to PLX4032 in BRAFV600E mutant melanoma cell lines may be explained by different resistance mechanisms. Sensitive cell lines may have a preferential MAPK pathway-addiction, and cells with low sensitivity may be less dependent on the BRAFV600E oncogenic signaling, relying on the co-activation of other signaling pathways. The genetic and molecular characterization of the PLX4032-resistant lines showed constitutive activation of MET and SRC kinases, representing potential new candidate targets involved in primary resistance. Inhibition of MET and SRC signaling by specific inhibitors in combination with PLX4032 restrain growth, survival and invasive capacity of melanoma cells bypassing resistance to BRAF inhibition. Taken together, the results reported here highlight the complexity and functional redundancy within the melanoma signaling network and support evidence that combinatorial approaches targeting MAPK pathway at different nodes and/or parallel signal transduction pathways may represent a strategy to override resistance to BRAF inhibitors.

An issue that deserves consideration in the context of BRAF targeting drugs is the immune response at tumor site. Significant clinical results have been obtained with Ipilimumab, a human monoclonal antibody that blocks cytotoxic T lymphocyte-associated antigen 4 (CTLA4) improving survival in patients with metastatic melanoma (Hodi et al, 2010). Oncogenic BRAF signaling has been shown to contribute to melanoma immune escape by inducing the expression of immunosuppressive cytokines such as IL8, IL6, IL10, TGFβ and VEGF (Zou et al, 2005). Cytokine genes including IL8 were identified as transcriptional targets of

BRAF signaling by gene expression profiling (Packer et al, 2009). Thus, BRAF represents a potential molecular target not only for inhibiting cellular proliferation but also for melanoma induced suppression of immune surveillance. It has been reported that MEK inhibitor UO126 or RNAi for BRAFV600E decreased production of IL10, VEGF and IL6 from melanoma cells (Sumimoto et al, 2006). In vivo, BRAF inhibitors were showed to induce marked T cell infiltration into metastatic melanoma lesions (Wilmott et al, 2012). These findings corroborate the results of in vitro studies performed on mutant BRAF melanoma cell lines suggesting that inhibition of the MAPK pathway with a BRAFV600E inhibitor resulted in increased expression of melanocyte differentiation antigens, and improved tumor cells recognition by antigen-specific T-cell (Kono et al, 2006; Boni et al, 2010). T-cell viability and function was not compromised when tested in vitro after treatment with BRAFV600E inihibitor at therapeutic concentrations (Comin-Anduix et al, 2010). These data indicate that BRAF inhibitors render melanoma cells more susceptible to immune attack, and combination therapies including BRAF inhibitors and immunotherapy may be developed to extend the duration of treatment response. I have recently begun to study the effects of BRAF and MEK inhibitors on the modulation of melanoma differentiation antigens and production of cytokines in a panel of melanoma cell lines with intrinsic or acquired resistance to PLX4032. The cell lines are going characterization for GF and cytokines network profiles associated to resistance to BRAF inhibition in order to identify cytokines signaling representing potential new targets for combined treatment.

Figures

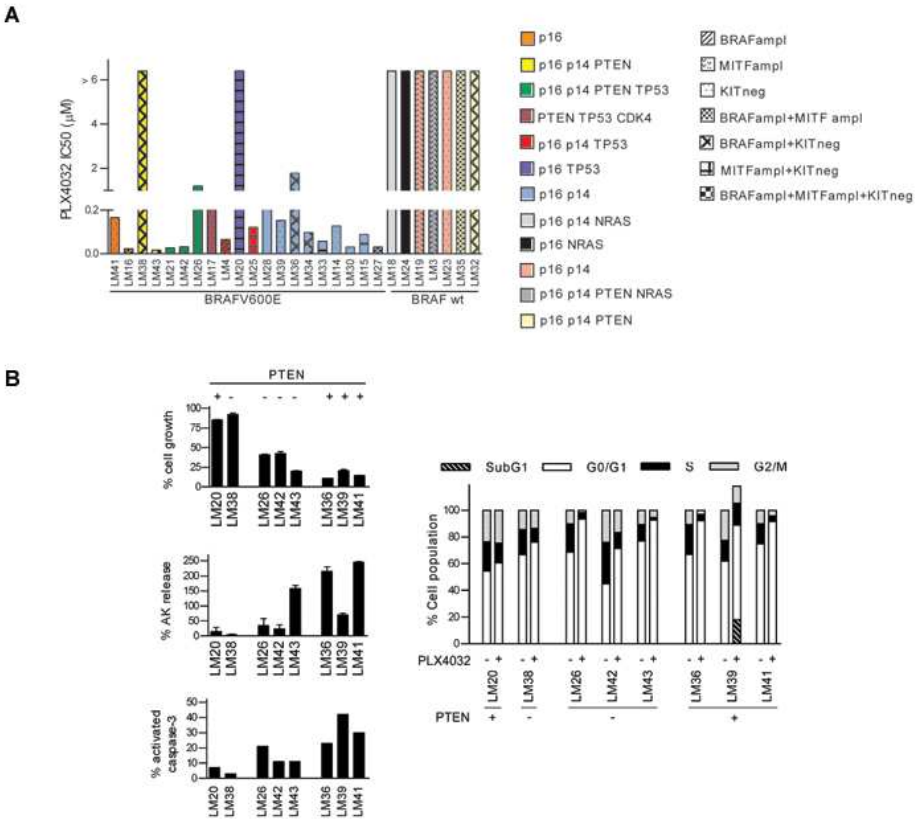

Figure 1. Effects of PLX4032 on BRAFV600E-mutated melanoma cells expressing or lacking PTEN. (A) Inhibitory effect of PLX4032 on cell growth in a panel of 27 genetically characterized melanoma cell lines after 72 hours of drug exposure. The different mutation profiles are indicated. Ampl, amplification; neg, negative. (B) Inhibition of cell growth (72 hours), AK release (72 hours), activated caspase 3 (48 hours), percentages of cells in sub G1, G1, S and G2/M phases of cell cycle (24 hours) after treatment with PLX4032 (3.2 μM). The percentage of growth was calculated by MTT assays as: (OD570 of wells that contained the drug/OD570 of the drug-free wells) × 100. Error bars, SD.

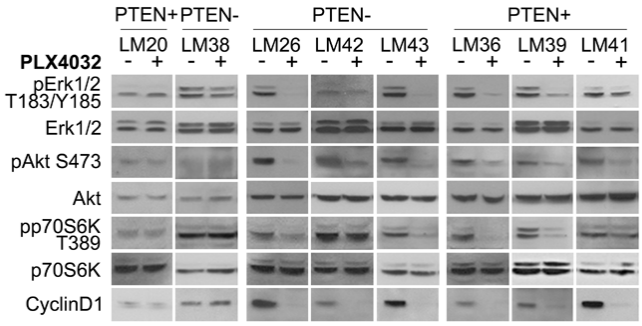

Figure 2. Modulation of phospho-signaling pathways after 24 hours PLX4032 treatment (3.2 μM).

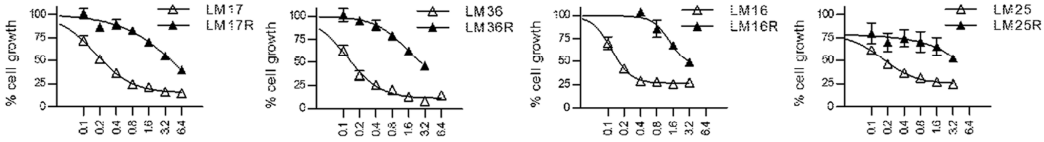

Figure 3. PLX4032-resistant variants selected by longterm exposure. Calculated IC50 values were 0.2 and 4 μM for LM17 and LM17R, 0.07 and 7.1 μM for LM36 and LM36R, 0.09 and 8.5 μM for LM16 and LM16R, 0.1 and >10 μM for LM25 and LM25R.

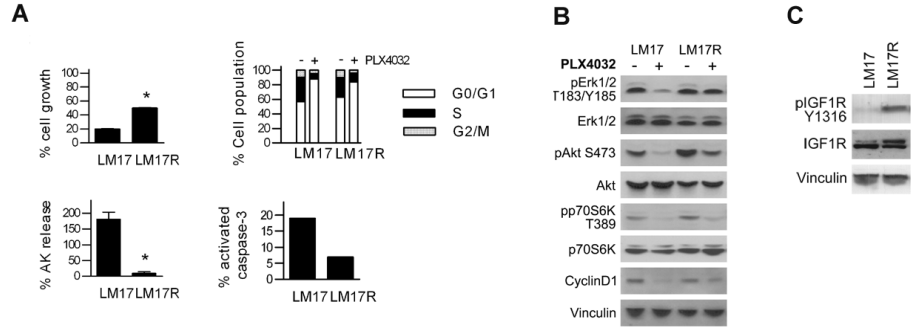

Figure 4. LM17R variants showed decreased sensitivity to inhibition of cell growth, induction of cell death and cell cycle arrest after PLX4032 treatment. (A) Cell growth, cell death, caspase 3 staining, and cell cycle analysis were tested at 72, 48, and 24 hours of treatment with PLX4032 (3.2 μM). ∗: $p < 0.0001$ by Student's t test. (B-C) Enhanced ERK, AKT and IGF1R signaling in the resistant variant LM17R. Expression levels as evaluated by western blot analysis in parental and resistant cells treated or not with PLX4032 (3.2 μM for 24 hours).

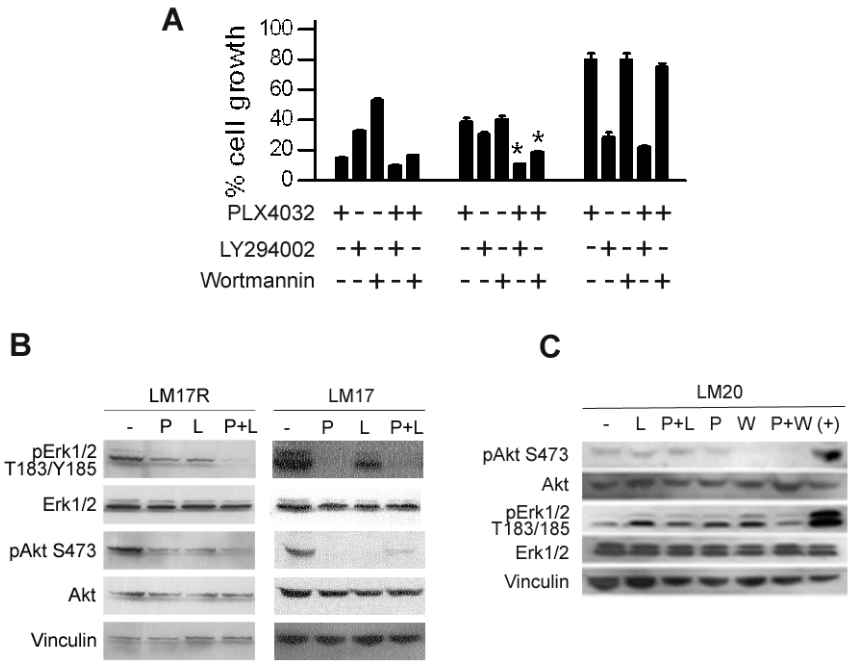

Figure 5. Cotreatment with PI3K inhibitors and PLX4032 inhibits growth and signaling in LM17R melanoma. (A) Growth inhibition (72 hours), in LM17, LM17R and LM20 cells treated with PLX4032 and/or LY294002 (60 μM)/wortmannin (10 μM). ∗: $p < 0.0001$ by one-way ANOVA followed by the Bonferroni correction. (B-C) Western blot analysis showing the regulation of downstream PI3K targets in LM17R compared to LM17 cells and in the resistant line LM20 after 24 hours of treatment with PLX4032 and/or LY294002/wortmannin.

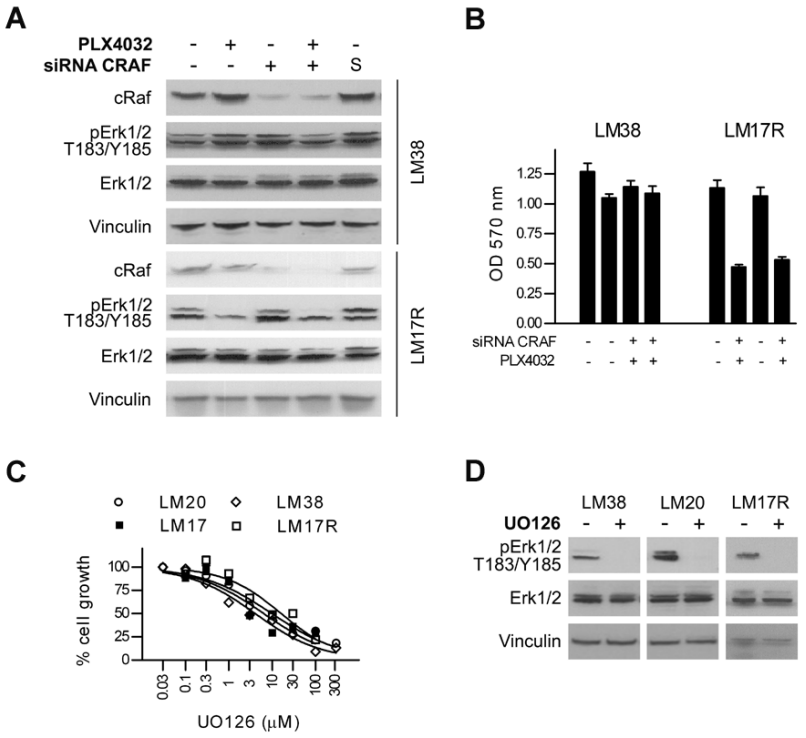

Figure 6. Resistance to PLX4032 is independent of CRAF and MEK. (A) Western blot analysis of LM38 and LM17R cells that were transfected with CRAF or control siRNA for 24 hours before being treated for 24 hours with PLX4032 (3.2 μM), showing CRAF protein levels downregulated to 14% and to 20%, respectively, as determined by quantification of the signal by Image Quant v5.2 software. (B) LM38 and LM17R cells were treated for 24 hours with CRAF or control siRNA before 72 hours of treatment with PLX4032 (3.2 μM). Absorbance at 570 nm after MTT staining is shown, indicating that CRAF inhibition does not increase sensitivity to PLX4032. (C) The proliferation of melanoma LM20, LM38, LM17R, and LM17 cells was similarly inhibited by 72 hours of treatment with UO126. (D) Levels of pERK are downregulated after 24 hours of incubation with UO126 (25 μM).

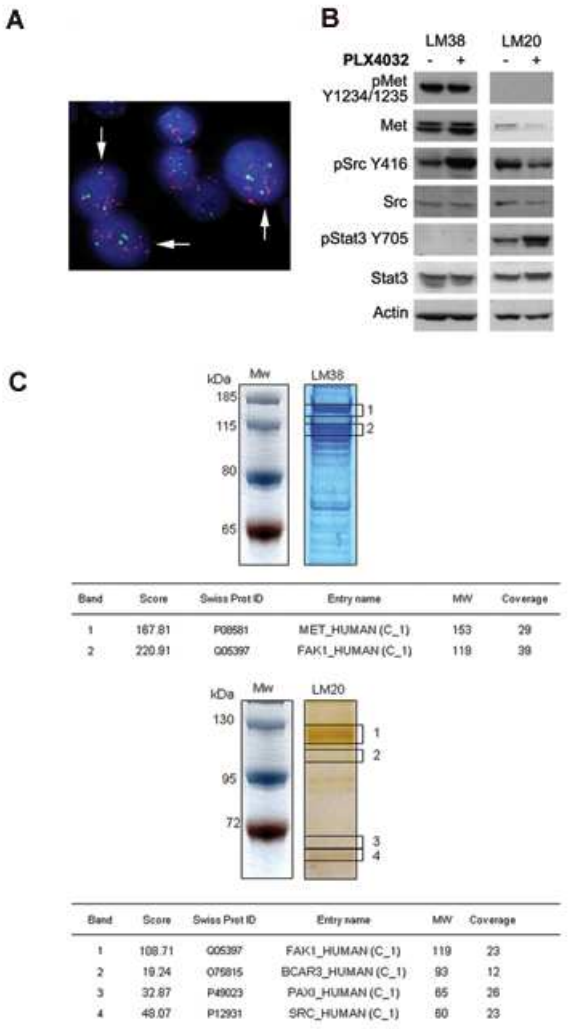

Figure 7. Molecular characteristics of the PLX4032-resistant LM20 and LM38 cell lines. (A) FISH analysis with orange labeled probe targeting MET gene and green control probe in LM38 cells. Four green and more than eight orange signals are shown in cells indicated by the arrows. (B) Detection of pMET, pSRC, and pSTAT3 signals after 24 hours of treatment with PLX4032 (3.2 μM) in LM38 and LM20 cells by Western blot analysis. (C) Identification of some relevant immunoaffinity-purified proteins from LM38 and LM20 cell lines. Top: Coomassie blue staining of anti-pTyr affinity-purified proteins from LM38 cells. Bottom: Silver staining of anti-pTyr affinity-purified proteins from LM20 cells. Protein extracts were incubated with antipTyr agarose-conjugated antibody. Bound proteins were washed, eluted, and resolved by 4% to 12% SDS-PAGE. Mw indicates molecular weight markers. Swiss Prot ID indicates accession number.

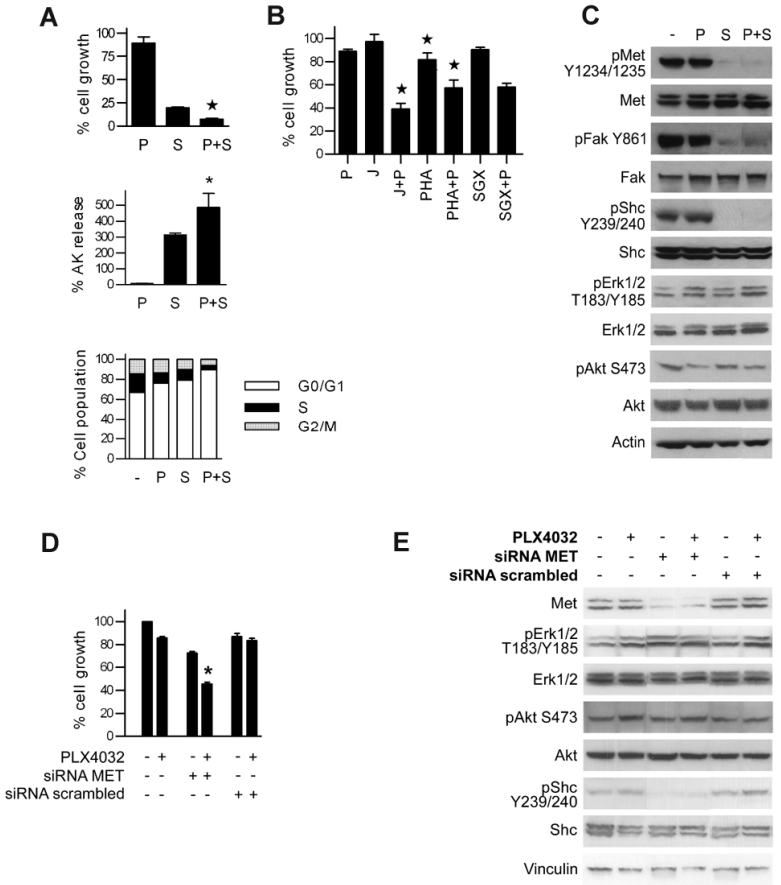

Figure 8. Cotreatment with MET inhibitor and PLX4032 inhibits growth and increased cell death in LM38-resistant melanoma. (A) Growth inhibition (72 hours), AK release (72 hours), and cell cycle (24 hours) in LM38 cells treated with PLX4032 and/or SU11274. ∗:$p < 0.0001$ by one-way ANOVA followed by the Bonferroni correction. ★: interaction index = 2.5. Interaction index values was used to evaluate drug interaction with values greater than 1 indicating synergism. (B) The inhibitory effect of PLX4032 combined with JNJ-38877605 (J), PHA-665752 (PHA), and SGX-523 (SGX) on proliferation is shown. ★: interaction index = 2.2, 1.22, and 1.33, respectively. ∗: $p < 0.0001$ by one-way ANOVA followed by the Bonferroni correction. (C) Western blot analysis showing the regulation of downstream MET targets in LM38 cells after 24 hours of treatment with PLX4032 and/or SU11274. (D) LM38 cells were treated for 96 hours with MET or control siRNA and with PLX4032 (3.2 μM). After MTT staining, the percentage of cell growth was calculated compared with the untreated control. ∗: $p < 0.0001$ by one-way ANOVA followed by the Bonferroni correction. (E) Western blot analyses of LM38 cells showing modulation of MET signaling after 96 hours of the indicated treatments. MET protein levels were downregulated to 20%, as determined by quantification of the signal by Image Quant v5.2 software.

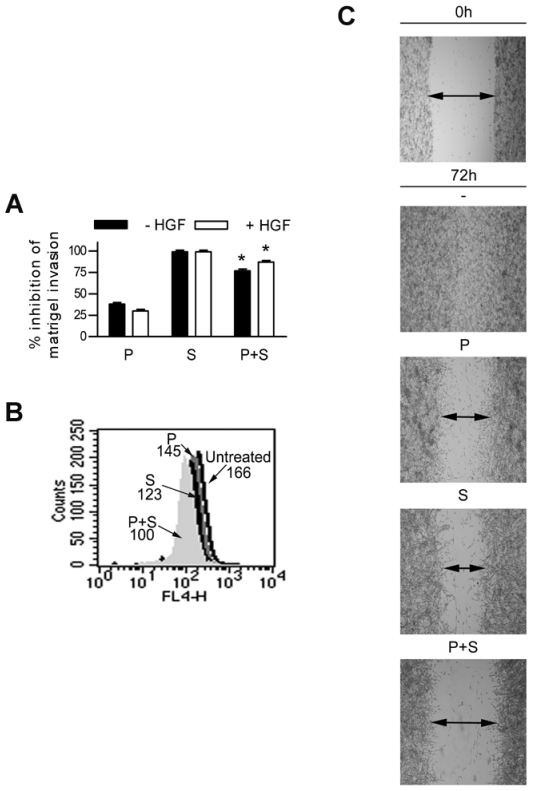

Figure 9. Cotreatment with MET inhibitor and PLX4032 inhibits invasion, and migration of LM38-resistant melanoma. (A) The Matrigel cell invasion assay showing the effect of exposure to PLX4032, SU11274 or both in LM38 cells. The percent inhibition of migration at 24 hours with or without HGF compared with that of untreated cells is shown. ∗: $p < 0.0001$ compared with treatment with PLX4032 by Student's t test. (B) FACS analysis of β1-integrin expression after 24 hours of exposure to PLX4032 and/or SU11274 in LM38 cells. Mean fluorescence intensity after treatment is indicated. (C) Scratch wound assay showing closure of a scratch wound in cultured LM38 cells under control conditions or in the presence of PLX4032, SU11274, or both for 72 hours. Medium was replaced every day to remove detached dead cells. Magnification, x2.5. − indicates untreated control; P, PLX4032 (3.2 μM); S, SU11274 (10 μM).

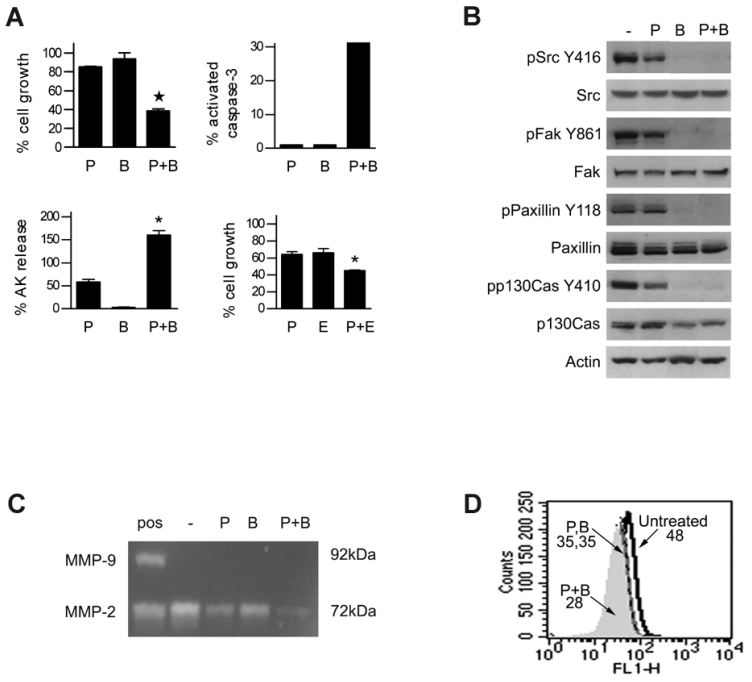

Figure 10. Cotreatment with SRC inhibitors and PLX4032 inhibits LM20 melanoma cell growth and downregulates MMP-2 and β1-integrin. (A) Growth inhibition, AK release (72 hours), and activated caspase 3 (48 hours) in LM20 cells that were treated with PLX4032 and/or BMS-354825. ∗: $p < 0.0001$ by one-way ANOVA followed by the Bonferroni correction. ★: interaction index = 2.1. Interaction index values was used to evaluate drug interaction with values greater than 1 indicating synergism. Bottom right, growth inhibitory effect of PLX4032 combined with the SRC inhibitor E804. ∗: $p < 0.0001$ compared with single treatments by Student's t test. (B) Western blot analysis showing regulation of downstream SRC targets in LM20 cells after 24 hours of treatment with PLX4032 and/or BMS-354825. (C) Gelatin zymography detecting MMP-2 in supernatants from LM20 cells collected after 24 hours of exposure to PLX4032, BMS-354825, or their combination. MMP-2 band was detectable at 72 kDa. (D) FACS analysis of β1-integrin expression after 24 hours of exposure to PLX4032 and/or BMS-354825 in LM20 cells. Mean fluorescence intensity after treatment is indicated. − indicates untreated control; B, BMS-354825 (100 nM); E, E804; P, PLX4032 (3.2 μM); pos, positive control.

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Acknowledgemens

It is a pleasure to thank the many people who made this thesis possible.

I would like to express my sincere gratitude to my tutor Dr Monica Rodolfo for her patience, constructive comments, and for her important support during my PhD project.

I am deeply grateful to all my colleagues for their valuable advice, friendly help and for creating a pleasant working atmosphere. Many thanks go in particular to Viviana Vallacchi, Paola Deho and Simona Frigerio for their precious help in the numerous assays in my study.

Thanks to Chiara Camisaschi for the time we spent together during these three years of PhD school.

I wish to thank Patricia Hamilton who patiently tried to improve my English.

Lastly, and most importantly, I wish to thank my parents for their loving encouragement and my boyfriend Maurizio whose kind support during the final stages of this thesis has been so appreciated. To them I dedicate this thesis.

PART II

NEOPLASIA
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Volume 13 Number 12 December 2011 pp. 1132–1142

Identification of MET and SRC Activation in Melanoma Cell Lines Showing Primary Resistance to PLX4032[1,2]

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Abstract

PLX4032/vemurafenib is a first-in-class small-molecule $BRAF^{V600E}$ inhibitor with clinical activity in patients with BRAF mutant melanoma. Nevertheless, drug resistance develops in treated patients, and strategies to overcome primary and acquired resistance are required. To explore the molecular mechanisms involved in primary resistance to PLX4032, we investigated its effects on cell proliferation and signaling in a panel of 27 genetically characterized patient-derived melanoma cell lines. Cell sensitivity to PLX4032 was dependent on $BRAF^{V600E}$ and independent from other gene alterations that commonly occur in melanoma such as *PTEN* loss, *BRAF*, and *MITF* gene amplification. Two cell lines lacking sensitivity to PLX4032 and harboring a different set of genetic alterations were studied as models of primary resistance. Treatment with the MEK inhibitor UO126 but not with PLX4032 inhibited cell growth and ERK activation. Resistance to PLX4032 was maintained after CRAF down-regulation by siRNA indicating alternative activation of MEK-ERK signaling. Genetic characterization by multiplex ligation-dependent probe amplification and analysis of phosphotyrosine signaling by MALDI-TOF mass spectrometry analysis revealed the activation of MET and SRC signaling, associated with the amplification of *MET* and of *CTNNB1* and *CCND1* genes, respectively. The combination of PLX4032 with drugs or siRNA targeting MET was effective in inhibiting cell growth and reducing cell invasion and migration in melanoma cells with *MET* amplification; similar effects were observed after targeting SRC in the other cell line, indicating a role for MET and SRC signaling in primary resistance to PLX4032. Our results support the development of classification of melanoma in molecular subtypes for more effective therapies.

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Abbreviations: AK, adenylate kinase; AKT, v-akt murine thymoma viral oncogene homolog; ANOVA, analysis of variance; BCRP, breast cancer resistance protein; BRAF, v-raf murine sarcoma viral oncogene homolog B1; CCND1, cyclin D1; CRAF, v-raf-1 murine leukemia viral oncogene homolog 1; CTNNB1, β-catenin; ERK, extracellular signal–regulated kinase; FAK, focal adhesion kinase; FISH, fluorescent *in situ* hybridization; HGF, hepatocyte growth factor; IC50, growth-adjusted inhibitory concentration of 50%; IGF1R, insulin-like growth factor 1 receptor; KIT, v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinases; MET, met proto-oncogene; MITF, microphthalmia-associated transcription factor; MLPA, multiplex ligation-dependent probe amplification; MMP-2, matrix metalloproteinase 2; MRP4, multidrug resistance protein 4; NRAS, neuroblastoma RAS viral (v-*ras*) oncogene homolog; $p70^{S6K}$, 70-kDa ribosomal protein S6 kinase; PTEN, phosphatase and tensin homolog; pTyr, phosphorylated tyrosine; SHC, Src homology 2 domain–containing transforming protein; SRC, v-*src* sarcoma viral oncogene homolog; STAT3, signal transducer and activator of transcription 3; TP53, tumor protein p53

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[1]This work was supported by EU grant CHEMORES (no. 037665) and by Associazione Italiana Ricerca sul Cancro. E.V. and V.V. were supported by the Italian Health Ministry (RFPS project 2006 no. 4-341458). The authors declare no conflict of interest.
[2]This article refers to supplementary materials, which are designated by Table W1 and Figures W1 to W4 and are available online at www.neoplasia.com.
Received 4 August 2011; Revised 4 November 2011; Accepted 8 November 2011

DOI 10.1593/neo.111102

Introduction

Among the common gene alterations occurring in melanoma pathogenesis, the most frequent is the T1799A transversion in the v-raf murine sarcoma viral oncogene homolog B1 (*BRAF*) gene that causes a glutamic acid substitution for valine at position 600 in the encoded kinase, which is detectable in approximately 50% of tumor lesions. BRAF is a serine/threonine–specific protein kinase that is activated by RAS G protein, which is activated downstream of growth factor receptors, cytokines, and hormones in the RAS/MEK/extracellular signal–regulated kinase (ERK) signaling cascade. The V600E change activates the RAF kinase function to constitutively activate the mitogen-activated protein kinase (MAPK) pathway through the hyperactivation of ERK, which promotes cell survival, proliferation, invasion, and angiogenesis. BRAF mutation acts as a driver determining a state of "oncogene addiction," unresponsive to inhibition by MAPK/ERK kinase (MEK)–dependent feedback but displaying increased sensitivity to the direct inhibition of BRAF and MEK [1]. MAPK signaling determines the cascade activation of other pathways that interact at different levels. This network signals also to the phosphoinositide 3-kinase/v-akt murine thymoma viral oncogene homolog (AKT)/mammalian target of rapamycin pathway, which is constitutively activated in melanoma and may offer compensatory routes to promote cell proliferation and survival [2].

In view of the relevance of RAS/BRAF/MAPK–activated signaling in melanoma, several inhibitors have been produced targeting the RAF kinases, some showing selectivity for mutant BRAF, or targeting the downstream kinase MEK. Several of these inhibitors are currently being evaluated in clinical trials [3]. PLX4032 is an azaindole derivative ATP-competitive inhibitor specific for V600E mutant BRAF which displayed promising efficacy in preclinical studies [4–7]. Phase 1 to 2 clinical trials have shown response rates of more than 50% in patients with melanoma carrying the $BRAF^{V600E}$ mutation, a result confirmed in a phase 3 trial reporting improved rates of overall and progression-free survival [8–10]. Despite this encouraging evidence, the clinical results pointed at secondary resistance as a common feature of kinase-targeted drugs and a major issue for investigations. Studies investigating the mechanisms associated to the acquisition of resistance have reported different genetic and epigenetic alterations, which promote ERK activation by MEK-dependent mechanisms bypassing BRAF inhibition, detectable in tumor biopsies from patients who developed resistance to PLX4032 treatment after clinical response. These alterations included *de novo* somatic mutations in *MEK1*, neuroblastoma RAS viral (v-*ras*) oncogene homolog (*NRAS*), or phosphatase and tensin homolog (*PTEN*) genes, but not in the targeted *BRAF* gene, as well as hyperactivation of platelet-derived growth factor receptor β, insulin-like growth factor 1 receptor (IGF1R), and MAP3K8 kinases [11–14].

In the current report, we focused on melanoma showing primary resistance that were identified by screening a panel of patient-derived genetically characterized $BRAF^{V600E}$-mutated melanoma cell lines to identify alterations that are associated with the cellular response to PLX4032. We investigated at the genetic and molecular levels two melanoma cell lines that displayed poor sensitivity to PLX4032 as models of primary resistance. By genetic characterization and by using a phosphoproteomic approach, we identified and validated further targets for pharmacological intervention and examined the effects of the combination of PLX4032 with other kinase inhibitors as an approach to overcome resistance.

Materials and Methods

Cells and Cellular Assays

The short-term melanoma cell lines LM4-LM41 have previously been described [15]; LM42 and LM43 were derived from visceral metastases and were similarly generated and characterized. The cell line LM17R was generated by treating the parental cell line LM17 with PLX4032 (3.2 μM) for 96 hours, allowing the few surviving cells to regrow, and repeating treatment for 11 times. MTT assays were used to evaluate the inhibition of cell growth at 72 hours, adding drugs 24 hours after cell plating. The bioluminescent ToxiLight bioassay kit (Lonza, Valais, Switzerland) was used to measure the release of adenylate kinase (AK) from dying cells. Caspase 3 activation was measured using the Active Caspase 3 Apoptosis Kit (Becton Dickinson, Franklin Lakers, NJ). The analysis of the cell cycle was performed by determining the DNA content distribution after propidium iodide staining using a FACSCalibur and ModFit LT v3.1 software. Silencing of v-raf-1 murine leukemia viral oncogene homolog 1 (CRAF) and met proto-oncogene (MET) was obtained using SMART pool small interfering RNA (siRNA; L-003601 and L-003156; Dharmacon, Lafayette, CO) and Lipofectamine 2000 (Gibco, Grand Island, NY). A scrambled control was used (D-001810-10). Invasion assays were performed as previously described [16] on cells exposed for 24 hours to the inhibitors. Scratch wound assays were set on confluent cell monolayer in six-well plates. The monolayer was scratched using a sterile pipette tip, rinsed to remove detached cells, and treated with inhibitors for 72 hours. Matrix metalloproteinase 2 and 9 (MMP-2/-9) activity was assessed using 10% SDS-PAGE gelatin substrate zymography (Invitrogen, Carlsbad, CA) in serum-free conditioned medium after concentration with Amicon Ultra 10K (Millipore, Billerica, MA). Anti–human β_1-integrin antibody (552828; Becton Dickinson) was used with APC-conjugated anti-rat immunoglobulin G (Jackson ImmunoResearch, Plymouth, PA) and analyzing staining by FACS analysis. Fluorescent *in situ* hybridization (FISH) analysis was performed using the probe kit D7S522/CEP7 according to the manufacturer's protocol (Abbott Vysis, Abbott Park, IL).

Genetic Analysis

Copy numbers of *BRAF*, *microphthalmia-associated transcription factor* (*MITF*), *MET*, *cyclin D1* (*CCND1*), and *β-catenin* (*CTNNB1*) genes in melanoma samples were determined by quantitative real-time polymerase chain reaction (PCR) analysis using TaqMan Copy Number Assays from Applied Biosystems (Branchburg, NJ). In particular, the copy number of *BRAF* gene was evaluated by targeting intron 13 (Hs04958893_cn) and intron 16 (Hs05004157_cn), whereas a single assay was used for *MITF* (Hs02258756_cn), *MET* (Hs00305306_cn), *CCND1* (Hs01425024_cn), and *CTNNB1* (Hs02393264_cn). TaqMan copy number reference assay RNase P was used as endogenous reference gene. DNA isolated from blood samples of healthy donors was used as control. PCRs were performed in quadruplicate and run on the ABI Prism 7900HT machine. Results were analyzed using the Copy Caller software version 1.1 and copy numbers 4 or higher were considered gene amplifications. The methylation status of the *PTEN* promoter was determined after bisulfite conversion using the EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA) by performing PCR analysis using previously reported primers and protocols with minor modifications [17]. Multiplex ligation-dependent probe amplification (MLPA) SALSA kits P005, P006, and P007 were used to profile changes in chromosomal regions as detailed by the manufacturer

(MRC-Holland, Amsterdam, the Netherlands). Results were analyzed by Coffalyser v 9.4 software by normalizing to three samples of normal DNA. The resulting values were categorized as homozygous loss (≤0.3), loss of heterozygosity (≤0.6), gain (≥1.3), and amplification (≥2).

Materials

The following antibodies were used: anti-pERK1/2 (M8159), anti-ERK (M5670), and anti-vinculin (V9131) from Sigma (St. Louis, MO); anti-AKT (610861) from Becton Dickinson; anti-pAKT (4051), anti-pSRC (2105), anti-pMET (3077), anti–phosphorylated signal transducer and activator of transcription 3 (STAT3; 9131), anti-pPaxillin (2541), and anti-pp130CAS (4011S) from Cell Signaling Technology (Danvers, MA); anti-Src (05-184), anti-p70 S6 kinase ($p70^{S6K}$; 05-781), anti-pp70 S6 kinase (04-392), and anti-Src homology 2 domain–containing transforming protein (SHC; 06-203) from Upstate Biotechnology (Lake Placid, NY); anti-CCND1 (M7155) from Dako (Glostrup, Denmark); anti-MET (sc-10), anti-STAT3 (sc-483), anti-CRAF (sc-133), anti-phosphorylated focal adhesion kinase (pFAK; sc-101679), anti-FAK (sc-932), anti-pSHC (sc-18074-R), and anti-actin (sc-166) from Santa Cruz Biotechnology (Santa Cruz, CA); anti-paxillin (P13520) from Transduction Laboratories (Lexington, KY); anti-p130CAS (ab33539) from Abcam (Cambridge, UK); anti–breast cancer resistance protein (BCRP; MON9041) and anti–multidrug resistance protein 4 (MRP4; MON9069) from Monosan (Valter Occhiena, Torino, Italy); anti-KIT (v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog; 566) from MBL (Woburn, MA); and peroxidase-conjugated secondary antibodies anti-mouse immunoglobulin (Becton Dickinson) and anti-rabbit immunoglobulin G (Amersham, Piscataway, NJ) were used. For anti–phosphorylated tyrosine (pTyr) immunoprecipitation and MALDI-TOF mass spectrometry analysis, samples were processed as previously described [18,19]. Only proteins identified in at least three separate experiments were considered.

PLX4032 was obtained by agreement with Plexxikon, Inc (Berkeley, CA). SU11274/Sugen, UO126, PHA-665752 (Sigma), BMS-354825/Dasatinib (LC Laboratories, Woburn, MA), JNJ-38877605, SGX-523 (Selleck, Houston, TX), and E804/Indirubin (Calbiochem, Gibbstown, NJ) were purchased. After dose-response tests, the drugs were used at the concentrations indicated.

Data Analysis

Fitted lines were generated using the four-parameter nonlinear regression with a sigmoidal dose response (variable slope), and the IC_{50} values (growth-adjusted inhibitory concentration of 50%) for inhibition of cell growth at 72 hours of PLX4032 treatment were calculated using Prism v 5.0 software. Student's *t* test and one-way analysis of variance (ANOVA) followed by the Bonferroni correction were used to evaluate statistical significance. Drug interaction was evaluated as described elsewhere [20] with interaction index values greater than 1 indicating synergism. The reported data are representative of three independent experiments.

Results

PLX4032 Growth Inhibitory Effects in $BRAF^{V600E}$-Mutated Melanoma Cells Are Not Associated with Other Common Melanoma Gene Alterations Including PTEN Loss

The growth inhibitory effect of PLX4032 was tested in a panel of 27 genetically characterized melanoma cell lines, including 20 lines that were heterozygous for the V600E BRAF mutation and 7 lines carrying wild-type *BRAF* gene. The effect of other genetic alterations, including mutations in *CDKN2A*, *PTEN*, and tumor protein p53 (*TP53*) and amplification of *BRAF* and *MITF*, on melanoma cell sensitivity to PLX4032 was considered. We found that PLX4032 inhibition of cell growth was strictly dependent on the presence of $BRAF^{V600E}$ and independent of other gene alterations. In fact, 18 of 20 $BRAF^{V600E}$-mutated melanoma cell lines were sensitive to the compound, with IC_{50} values ranging between 0.01 and 1 μM, whereas 2 cell lines displayed a poor sensitivity and showed IC_{50} values that were approximately 10 μM. The different IC_{50} values were not associated with the mutational profiles of the cell lines, including the amplification of the *BRAF* or *MITF* genes, or to the expression of KIT protein (Table 1).

Melanoma cell lines LM20 and LM38 showed primary resistance to PLX4032 lacked p16 and KIT protein expression but showed different gene alterations because LM20 cells harbored *MITF* amplification and mutated *TP53*, whereas LM38 lacked *p14/ARF* gene and PTEN expression because of gene methylation. PTEN deficiency has been hypothesized to promote melanoma cell proliferation and survival through AKT activation, which may decrease the dependency on ERK signaling. Moreover, PTEN loss has been detected in a melanoma tissue biopsy obtained from a patient relapsing on treatment with PLX4032 [13]. When response of melanoma cell lines to PLX4032 concentrations inhibiting cell growth was examined, we found that the drug produced an accumulation in the G_1 phase of cell cycle regardless of PTEN status (Figures 1 and W1). Growth inhibition was associated with apoptotic cell death, as documented by AK release and activation of caspase 3, at higher levels in PTEN-positive samples, indicating a role for PTEN in the induction of cell death in response to PLX4032 (Figure 1, *A* and *B*).

Modulation of MAPK and AKT Signaling by PLX4032 Treatment

To define the cellular response that was associated with PLX4032 sensitivity, we examined the effect of treatment on downstream signaling pathways that regulate cell growth and survival. PLX4032 treatment strongly reduced the levels of pERK and pAKT in most drug-sensitive cell lines, independently of PTEN status. In addition, down-regulation of $p70^{S6K}$, which is activated downstream of the mammalian target of rapamycin signaling, was detectable in most lines, and CCND1 expression was downregulated in all drug-sensitive cell lines, consistently with an accumulation in the G_1 phase of the cell cycle. In contrast, pAKT, pERK, $pp70^{S6K}$, and cyclin D1 levels were not affected by the treatment in the resistant LM20 and LM38 cells, in keeping with the poor antiproliferative and cytotoxic effects (Figure 1*C*).

A resistant cell line (LM17R) was generated by repeated drug exposure from the cell line LM17, which showed extensive cell death after PLX4032 treatment. LM17R showed reduced sensitivity to the antiproliferative effect of PLX4032, diminished AK release, caspase 3 activation, and G_1 block of the cell cycle, as well as unresponsiveness of pERK, pAKT, and CCND1 (Figure 2). Sequence analysis confirmed the presence of the heterozygous V600E BRAF mutation and excluded the presence of secondary mutations in exons 11 and 15 and in *RAS* gene; in addition, the same number of copies of the *BRAF* gene as the parental LM17 cells was detected.

To assess whether the MAPK pathway can be modulated downstream of mutated BRAF in resistant cells, we tested whether MEK

Table 1. Genetic Characterization and PLX4032 Sensitivity of the Melanoma Cell Lines.

Melanoma*	IC_{50} PLX4032 (μM)†	BRAF‡	nBRAF 7q34§	nMITF 3p14§	NRAS‡	PTEN	TP53	p16	p14/ARF	cKIT¶
LM4	0.065	V600E	4	2	wt	wt/+	Y236H	del	del	+
LM14	0.128	V600E	2	2	wt	wt/+	wt	del	del	–
LM15	0.088	V600E	3	4	wt	wt/+	wt	del	del	–
LM16	0.023	V600E	4	10	wt	wt/+	wt	L65P	wt/+	*L862L*/+
LM17	0.238	V600E	3	3	wt	P38S/+	S127F	*A148Tl*+	wt/+	–
LM17R	**4.044**	**V600E**	**3**	**3**	**wt**	+				
LM20	**8.907**	**V600E**	**2**	4	**wt**	**wt/+**	**Y234C**	**wt/–**	**wt/+**	**–**
LM21	0.027	V600E	3	3	wt	P246S/+	S127F	del	del	+
LM25	0.122	V600E	2	6	wt	wt/+	E258K	R80stop/–	P94L/–	–
LM26	1.195	V600E	3	3	wt	C105*fs*X112/–	R175H	del	del	–
LM27	0.031	V600E	7	4	wt	wt/+	wt	IVS2-2A>G/–	wt/–	+
LM28	0.398	V600E	3	3	wt	wt/+	wt	del	del	+
LM30	0.031	V600E	2	2	wt	wt/+	wt	del	del	–
LM33	0.057	V600E	2	5	wt	wt/+	wt	del	del	–
LM34	0.097	V600E	5	3	wt	wt/+	wt	*A148Tl*–	wt/–	–
LM36	1.778	V600E	4	2	wt	wt/+	wt	del	del	I798I/–
LM38	**8.871**	**V600E**	**11**	3	**wt**	**M/–**	**wt**	**del**	**del**	***L862L*/–**
LM39	0.153	V600E	2	2	wt	wt/+	wt	del	del	–
LM41	0.166	V600E	3	3	wt	wt/+	wt	wt/–	wt/+	–
LM42	0.032	V600E	2	3	wt	M/–	c993+1 G>A	del	del	–
LM43	0.017	V600E	8	4	wt	M/–	wt	del	del	*L862L*/–
LM3	>6.400	wt	3	4	Q61R	wt/–	wt	wt/–	wt/–	+
LM18	>6.400	wt	2	3	Q61R	wt/+	wt	del	del	+
LM19	>6.400	wt	3	9	wt	wt/+	wt	del	del	+
LM23	>6.400	wt	2	3	wt	wt/+	wt	del	del	–
LM24	>6.400	wt	2	3	Q61R	wt/+	wt	del	wt/+	+
LM32	>6.400	wt	8	3	wt	wt/–	wt	del	del	–
LM35	>6.400	wt	4	6	wt	M/–	wt	del	del	+

+, – indicates presence or absence of protein expression; del, homozygous deletion; M, gene methylation; wt, wild-type gene.

Polymorphisms are shown in italics. PLX4032-resistant cell lines are shown in bold.

*Cell lines are numbered according to the list reported in Daniotti et al. [15]. All lines are shown wild-type for *PI3KCA* exons 9 and 20, *GNAQ* exon 5, and *CDK4* exon 2, except LM17 cells that carry a K22R mutation in *CDK4* exon 2.

†IC_{50} values were calculated by modeling results of growth inhibition assays using a nonlinear regression curve fit with a sigmoidal dose-response (variable slope).

‡Heterozygous mutations; exons 11 and 15 for *BRAF* and exons 1 and 2 for *NRAS* gene were sequenced.

§Gene copy number as evaluated by quantitative PCR as detailed in Materials and Methods.

¶*KIT* gene exons 9, 11, 13, 14, 17, and 18 were sequenced. Polymorphism *L862L* (rs3733542) in exon 18 and the silent mutation I798I in exon 17 were detected. KIT protein expression was evaluated by Western blot.

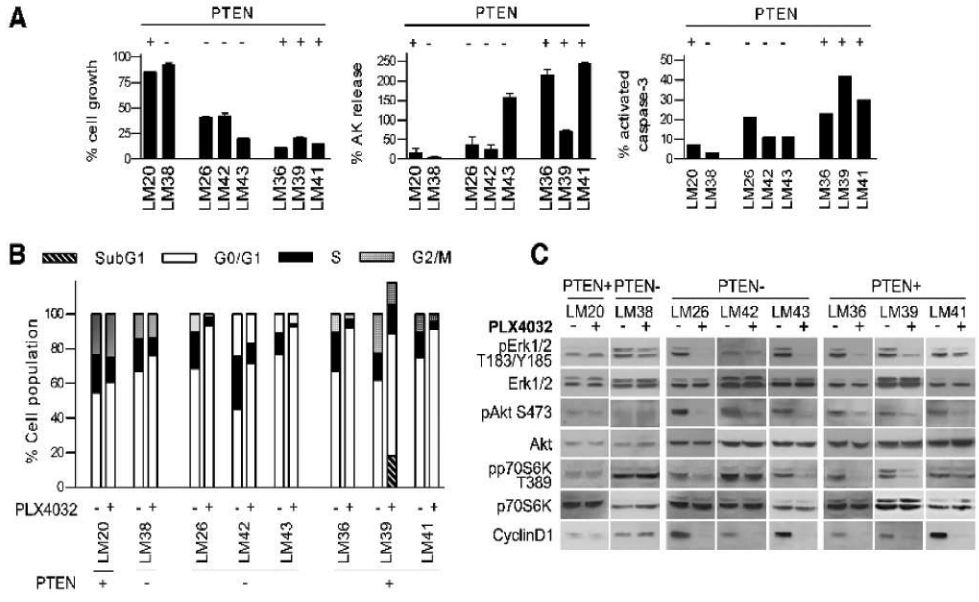


Figure 1. Effects of PLX4032 on $BRAF^{V600E}$-mutated melanoma cells expressing or lacking PTEN. (A) Inhibition of cell growth (72 hours), AK release (72 hours), and activated caspase 3 (48 hours) after treatment with PLX4032 (3.2 μM). The percentage of growth was calculated as: (OD_{570} of wells that contained the drug/OD_{570} of the drug-free wells) × 100. Error bars, SD. (B) Percentages of cells in sub G_1, G_1, S, and G_2/M phases of cell cycle after 24 hours of treatment with PLX4032 (3.2 μM). (C) Modulation of phospho-signaling after 24 hours of PLX4032 treatment (3.2 μM).

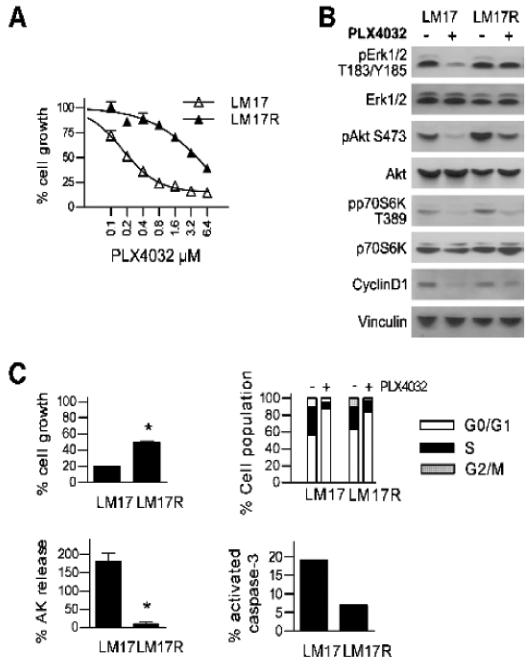


Figure 2. Selection of PLX4032-resistant variant LM17R by long-term exposure of LM17 cells to PLX4032. (A) Growth curves showing decreased sensitivity to PLX4032 in LM17R cells. Calculated IC_{50} values were 0.2 and 4 μM for LM17 and LM17R, respectively. (B) Enhanced ERK and AKT signaling in the resistant variant LM17R. Expression levels as evaluated by Western blot analysis in parental and resistant cells treated with PLX4032 (3.2 μM for 24 hours). (C) Cell growth, cell death, caspase 3 staining, and cell cycle analysis were tested at 72, 48, and 24 hours of treatment with PLX4032 (3.2 μM). *$P < .0001$ by Student's t test.

inhibition affected pERK levels and cell proliferation. Treatment with the MEK1/2 inhibitor UO126 reduced pERK signal and inhibited proliferation in LM20 and LM38 as well as in LM17R cells compared with that in LM17 (Figure 3, *A* and *B*), indicating that these cell lines retained the susceptibility to MEK inhibition.

A shift in signaling from BRAF to CRAF after BRAF inhibition has been described in melanoma cells, with CRAF mediating ERK activation [21]. Therefore, we silenced CRAF in LM38 cells using specific siRNA to test whether the sensitivity to PLX4032 increased by reducing CRAF levels. The CRAF siRNA downregulated CRAF protein levels without affecting pERK levels and cell sensitivity to PLX4032. Similar results were obtained also in LM17R cells (Figure 3, *C* and *D*).

Molecular Characterization of Melanoma Cell Lines Showing Resistance to PLX4032

To identify new potential markers that are associated with PLX4032 resistance and candidate genes, the MLPA analysis was used to genetically characterize the resistant melanoma cell lines. Several probes showed values indicating gene gain or loss (Figure W2). Amplification of *CCND1* at 11q13 and of *CTNNB1* at 3p21 was detected in LM20 cells, whereas the LM38 line showed a different pattern of alterations, including *MET* amplification at 7q31. *MET*, *CCND1*, and *CTNNB1* gene amplifications in LM38 and in LM20 were confirmed by FISH analysis (Figure 4*A* and data not shown) and by using quantitative PCR assessing gene copy number (Table W1). MLPA analysis showed no difference in the pattern of alterations between LM17R and LM17, indicating that the acquisition of resistance to PLX4032 was not associated to gain or loss of the tested genes.

To further explore the mechanisms of PLX4032 resistance, a proteomic-multiplexed analysis of pTyr signaling and antibody validation was used to screen pTyr proteins that were modulated by treatment in PLX4032-sensitive and -resistant melanoma cells. We observed a high degree of heterogeneity in the pTyr profiles in the different cell lines (Figure W3). To identify the most abundant phosphorylated proteins in LM20 and LM38 cell lines, protein bands from anti-pTyr immunoprecipitates of cell lysates were resolved in SDS-PAGE, excised from preparative silver-stained gel, and processed for MALDI-TOF mass spectrometry analysis. The identified proteins indicated that pTyr-based cell signaling was activated in the v-*src* sarcoma viral oncogene homolog (SRC)/FAK axis in LM20 cells, whereas it was prevalently activated in the MET axis in LM38 cells (Figure 4*C*). These data were consistent with *MET* gene amplification in LM38 cells and *CTNNB1* amplification in LM20 cells for the role of SRC activity in regulating CTNNB1 signaling. Immunoblot analysis confirmed the presence of the phosphorylated MET receptor in LM38 cells, whereas the phosphorylated form of STAT3, which is activated downstream of SRC, was detectable in LM20 cells. The MET and STAT3 proteins were present but not phosphorylated in the other cell line. In particular, high levels of non–tyrosine-phosphorylated STAT3 were detected in LM38 cells, and both lines showed high pSRC levels, which were not reduced by PLX4032 treatment (Figure 4*B*).

To define whether PLX4032 resistance was mediated by the increased expression of ABC transporters, we assessed protein expression of ABCB1/Gp170, ABCC1/MRP1, ABCC2/MRP2, ABCC4/MRP4, and ABCG2/BCRP in the resistant melanoma cell lines. Differential expression was observed for BCRP and MRP4 (Figure W4). However, BCRP overexpression did not result in resistance to PLX4032 as shown by using a mutant BRAF isogenic model system [22]. In addition, topotecan, a well-known MRP4 substrate, displayed a similar effect in LM17 and LM17R cells despite increased MRP4 levels (data not shown). Thus, PLX4032 resistance is not determined by ABC transporters.

MET and SRC as Additional Targets for Combined Treatment with PLX4032

On the basis of the results of molecular profiling, MET and SRC represented new candidate targets expressed at high levels and activated in LM38 and LM20 melanoma cells intrinsically resistant to PLX4032. We thus tested the effect of combining PLX4032 with drugs that inhibited MET and SRC kinases.

The MET inhibitor SU11274 significantly inhibited the proliferation of most of the melanoma cell lines that were examined, including PLX4032-resistant lines, with IC_{50} values of approximately 10 μM (data not shown). The combined treatment with SU11274 and PLX4032 produced a synergistic interaction when tested in LM38 cells (interaction index = 2.5), and growth inhibition was associated with an accumulation of cells in G_1 and AK release in the absence of caspase 3 activation (Figure 5*A* and not shown). The potentiating effect that was obtained by the concomitant inhibition was evident also when other MET inhibitors were tested (Figure 5*B*). After the cotreatment with SU11274 and PLX4032, pERK and pAKT were not downregulated; in contrast, we found a strong down-regulation of MET signaling through pFAK and pSHC (Figure 5*C*).

Because MET is involved in tumor invasion, we evaluated the effects of the combined treatment on the ability of melanoma cells to invade Matrigel and migrate *in vitro*. LM38 melanoma cells were highly responsive to the MET ligand hepatocyte growth factor (HGF), as the addiction of HGF determined a significant increase in the number of cells that migrated through the Matrigel layer (not shown), further confirming the role of MET signaling in mediating the invasive capacity in these cells. Indeed, blocking MET signaling by treatment with SU11274 alone or in combination with PLX4032 strongly inhibited Matrigel invasion. Notably, a moderate effect was observed after treatment with PLX4032, indicating that BRAF inhibition, although not affecting cell growth, may alter the invasive activity of melanoma cells, even in the presence of exogenous HGF (Figure 5*D*). Moreover, LM38 cells produced HGF (data not shown), thus suggesting that an autocrine loop contribute to MET pathway constitutive activation. In addition, the combined drugs downregulated the expression of β_1-integrin, the receptor for extracellular matrix laminin that is involved in adhesive and invasive cellular processes (Figure 5*E*). Scratch wound assays showed that the combination of PLX4032 with SU11274 prevented wound closure, whereas the single drugs impaired wound healing to a limited extent, confirming the effect of the combination on cell migration (Figure 5*F*).

To confirm that MET inhibition can cooperate with BRAF inhibition siRNA silencing of MET was tested. A synergic effect on cell proliferation was detected (interaction index = 1.36), and down-regulation of MET and SHC signal was shown, whereas pERK and pAKT levels were maintained (Figure 6, *A* and *B*).

To assess the functional relevance of the SRC pathway in LM20 cells, the BMS-354825 multikinase inhibitor targeting SRC family kinases was used. When tested in the panel of melanoma cell lines, BMS-354825 displayed a poor inhibitory effect on cell growth, and its antiproliferative effect was not related to the expression of KIT protein, which is one of the kinases targeted by the compound (not shown). BMS-354825 showed a weak inhibitory effect on cell growth in LM20 cells, whereas the combination of BMS-354825 with PLX4032 displayed significant antiproliferative and cytotoxic effects (interaction index = 2.1). Another SRC inhibitor, E804, exerted an additive effect with PLX4032, further corroborating the role of SRC signaling in LM20 cells (Figure 7*A*). Treatment with BMS-354825 downregulated the levels of phosphorylated SRC protein and of the downstream targets paxillin and p130CAS; in addition, BMS-354825 reduced pFAK levels. In contrast, no effect was detectable on pERK and pAKT levels also with this drug combination, suggesting that it is not a necessary requirement to impair cell proliferation (Figure 7*B* and data not shown). The combined treatment with PLX4032 and BMS-354825 decreased MMP-2 production by LM20 melanoma cells, which was measured using gelatin-gel zymography (Figure 7*C*), and reduced the expression of β_1-integrin (Figure 7*D*).

Discussion

It is not yet known how other concurrent genetic alterations in addition to BRAF mutations may affect the clinical efficacy of the BRAF inhibitor PLX4032 in metastatic melanoma and whether a classification level can be defined for the molecular profiles that are

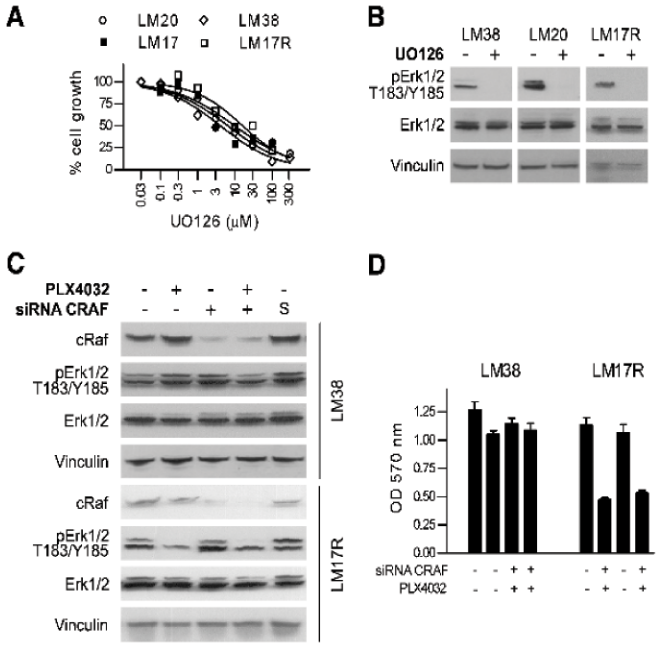
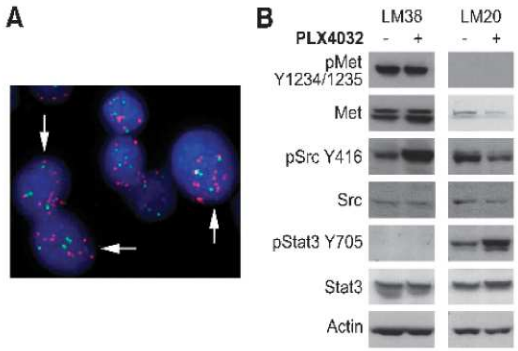


Figure 3. Resistance to PLX4032 is independent of MEK and CRAF. (A) The proliferation of melanoma LM20, LM38, LM17R, and LM17 cells was similarly inhibited by 72 hours of treatment with UO126 (IC_{50} values: 9.7, 4, 6.3, and 16.1 μM, respectively). (B) Levels of pERK are downregulated after 24 hours of incubation with UO126 (25 μM). (C) Western blot analysis of LM38 and LM17R cells that were transfected with CRAF or control siRNA for 24 hours before being treated for 24 hours with PLX4032 (3.2 μM), showing CRAF protein levels downregulated to 14% and to 20%, respectively, as determined by quantification of the signal by Image Quant v5.2 software. (D) LM38 and LM17R cells were treated for 24 hours with CRAF or control siRNA before 72 hours of treatment with PLX4032 (3.2 μM). Absorbance at 570 nm after MTT staining is shown, indicating that CRAF inhibition does not increase sensitivity to PLX4032.



C

kDa Mw LM38

185
115
80
65
1
2

Band	Score	Swiss Prot ID	Entry name	MW	Coverage
1	167.81	P08581	MET_HUMAN (C_1)	153	29
2	220.91	Q05397	FAK1_HUMAN (C_1)	119	39

kDa Mw LM20

130
95
72
1
2
3
4

Band	Score	Swiss Prot ID	Entry name	MW	Coverage
1	108.71	Q05397	FAK1_HUMAN (C_1)	119	23
2	19.24	O75815	BCAR3_HUMAN (C_1)	93	12
3	32.87	P49023	PAXI_HUMAN (C_1)	65	26
4	48.07	P12931	SRC_HUMAN (C_1)	60	23

Figure 4. Molecular characteristics of the PLX4032-resistant LM20 and LM38 cell lines. (A) FISH analysis with orange labeled probe targeting MET gene and green control probe in LM38 cells. Four green and more than eight orange signals are shown in cells indicated by the arrows. (B) Detection of pMET, pSRC, and pSTAT3 signals after 24 hours of treatment with PLX4032 (3.2 μM) in LM38 and LM20 cells by Western blot analysis. (C) Identification of some relevant immunoaffinity-purified proteins from LM38 and LM20 cell lines. Top: Coomassie blue staining of anti-pTyr affinity-purified proteins from LM38 cells. Bottom: Silver staining of anti-pTyr affinity-purified proteins from LM20 cells. Protein extracts were incubated with anti-pTyr agarose-conjugated antibody. Bound proteins were washed, eluted, and resolved by 4% to 12% SDS-PAGE. Mw indicates molecular weight markers. Swiss Prot ID indicates accession number.

associated with primary resistance. Although *BRAF*, *NRAS*, and *KIT* mutations are mutually exclusive, mutated BRAF melanoma may carry common alterations in *CDKN2A*, *PTEN*, and *TP53* genes, as well as alterations of *CDK4*, *CTNNB1*, *FGFR2*, *MITF*, *ERBB4*, *MMP*, and *GRIN2A* genes [3,15,23], and other potential driver mutations still poorly characterized [24]. Here, we show that, apart from *BRAF* mutation, the gene alterations that are common in melanoma, such as *PTEN* and *TP53* mutations, and *BRAF* and *MITF* amplification, are not associated with PLX4032 sensitivity in a large panel of genetically characterized short-term melanoma cell lines.

Studies performed on melanoma tissue from few patients relapsing on treatment with PLX4032 have ruled out the occurrence of additional secondary mutations in the *BRAF* gene and have reported the overgrowth of *NRAS* mutated [11], *PTEN* deleted [13], and C121S *MEK1* mutated [14] metastases in different individual cases. These results suggest that the mechanisms that mediate acquired resistance rely on different genetic alterations that may include the overgrowth of preexisting genetic variants selected by the treatment as well as *de novo* mutations.

The *in vitro* studies on primary resistance to BRAF inhibitors have detected *CCND1* gene amplification in cell lines that were resistant to the BRAF inhibitor SB590885 [25]. Other studies have identified different changes in *MEK1* and *BRAF* T529N causing resistance to PLX4720 [26,27]. Melanoma cell lines carrying homozygous $BRAF^{V600E}$ mutation were shown to be more sensitive to PLX4032 than those carrying heterozygous $BRAF^{V600E}$ mutation [28–30]. Although homozygosity is rare, the 7q34 chromosomal region where the *BRAF* gene is located is frequently amplified in melanoma lesions and especially in $BRAF^{V600E}$-mutated melanomas [31]. Amplification of the mutated *BRAF* allele was detected in association with acquired resistance to MEK inhibitors in a melanoma cell line in a previous study [32]. In our panel of melanoma cell lines, *BRAF* gene amplification was detected in 30% of the cell lines, including the resistant LM38 melanoma model, whereas in the resistant variant LM17R, which was obtained by long-term exposure to PLX4032 *in vitro*, the *BRAF* gene was not amplified compared with the parental cell line.

In addition to *BRAF* gene amplification, LM38 melanoma cells resistant to PLX4032 lacked PTEN. We detected lower levels of cytotoxicity in PTEN-negative melanoma cells after exposure to PLX4032 compared with melanomas with intact PTEN, but a similar block of cell cycle, suggesting a role for PTEN in the cytotoxic effect of PLX4032. This finding is in agreement with studies reporting that PTEN loss contributes to PLX4720 resistance by suppressing BIM-mediated apoptosis [33].

The PLX4032-resistant line LM20 harbored amplified *MITF* gene. *MITF* gene amplification was detected in 30% of our $BRAF^{V600E}$-mutated cell lines. Unexpectedly, however, melanomas with amplified *MITF* ($\geq$4 copies) showed lower IC_{50} values than melanomas without *MITF* amplification when only cell lines carrying two gene copies were considered (0.05 *vs* 0.4 μM, $P = .0013$), suggesting that *MITF* amplification does not contribute to PLX4032 resistance.

Because it has been shown that kinase inhibitors are able to interact with members of the ABC family of transporters and that ABC transporters can mediate resistance to kinase inhibitors [34,35], we tested whether BCRP and MRP4 showing overexpression in resistant cells play a role in PLX4032 resistance. The results of these experiments do not indicate a role for BCRP or MRP4 in resistance to PLX4032.

By expanding the genetic characterization to the analysis of altered chromosomal regions by MLPA, the amplification of *MET* gene in LM38 cells and of *CCND1* and *CTNNB1* genes in LM20 cells was detected. This pattern was consistent with the pTyr profiling analysis as detected by MALDI-TOF indicating activated MET and SRC signaling. The amplification of the *MET* gene has been reported in melanoma [36] along with chromosome 7 polysomy [31]. The amplification of

CCND1 was detected in approximately 25% melanoma bearing mutated *BRAF* [37]. Although *CTNNB1* mutations have been reported in melanoma, gene amplification was not formerly shown, although it was detected by MLPA in melanoma lesions [38].

Epigenetic changes providing compensatory signaling to bypass BRAF blockade and activate ERK are associated with acquired resistance to BRAF inhibitors. Several different mechanisms have been described, including the activation of a platelet-derived growth factor receptor β, IGF1R/phosphoinositide 3-kinase and MAP3K8/COT signaling [11–13]. Moreover, increased CRAF protein levels and switching from BRAF to CRAF dependency has been associated with the *in vitro* acquired resistance to AZ628 BRAF inhibitor [21]. Although our data do not support a role for CRAF in resistance to PLX4032, in the current study, LM17R cells with acquired resistance to PLX4032 showed increased IGFR1 signaling and consistently higher levels of pAKT compared with that of the parental LM17 cell line (data not shown). Up-regulation of IGF1R signaling was reported to occur in two of four melanoma cell variants that were selected *in vitro* for resistance to the 885 BRAF inhibitor [13], therefore appearing as a rather common mechanism by which melanoma cells compensate BRAF inhibition.

Targeting other signaling molecules in crucial pathways may represent an approach to enhance the clinical impact of treatment with PLX4032. Preclinical studies showed that MEK inhibitors in combination with PLX4720 reduced cell growth and pERK expression [12] and may prevent the emergence of resistant clones [26].

We show that simultaneously targeting multiple pathways may represent a promising option for treating PLX4032-resistant melanomas.

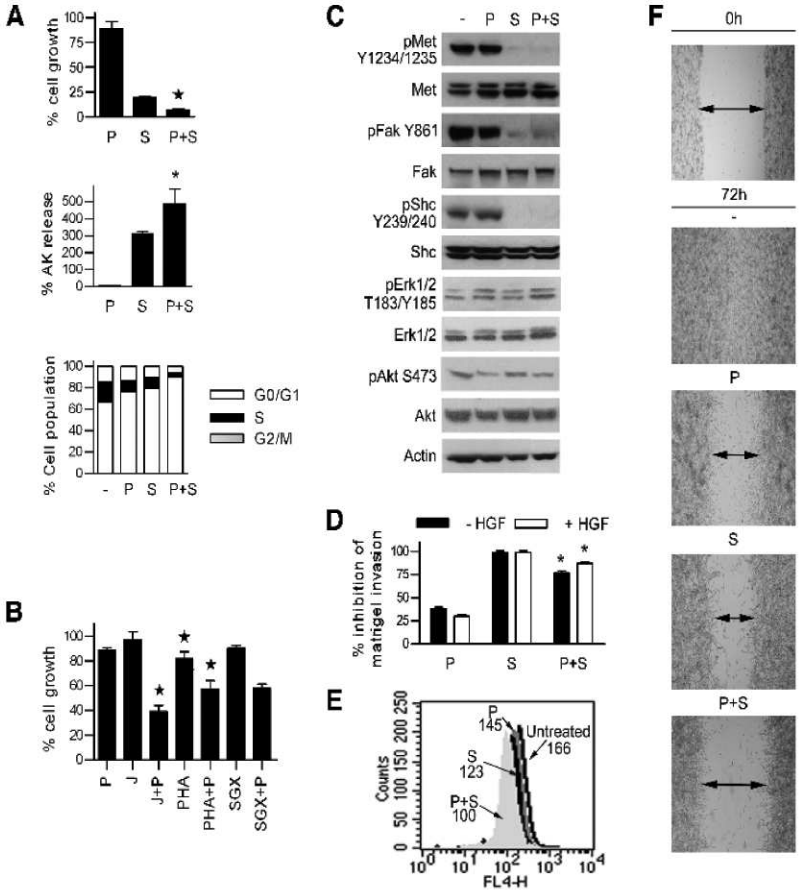


Figure 5. Cotreatment with MET inhibitor and PLX4032 inhibits growth, invasion, and migration of LM38-resistant melanoma. (A) Growth inhibition (72 hours), AK release (72 hours), and cell cycle (24 hours) in LM38 cells treated with PLX4032 and/or SU11274. *$P < .0001$ by one-way ANOVA followed by the Bonferroni correction. ★: interaction index = 2.5. (B) The inhibitory effect of PLX4032 combined with JNJ-38877605 (J), PHA-665752 (PHA), and SGX-523 (SGX) on proliferation is shown. ★: interaction index = 2.2, 1.22, and 1.33, respectively. $P < .0001$ by one-way ANOVA followed by the Bonferroni correction. (C) Western blot analysis showing the regulation of downstream MET targets in LM38 cells after 24 hours of treatment with PLX4032 and/or SU11274. (D) The Matrigel cell invasion assay showing the effect of exposure to PLX4032, SU11274 or both in LM38 cells. The percent inhibition of migration at 24 hours with or without HGF compared with that of untreated cells is shown. *$P < .0001$ compared with treatment with PLX4032 by Student's t test. (E) FACS analysis of β_1-integrin expression after 24 hours of exposure to PLX4032 and/or SU11274 in LM38 cells. Mean fluorescence intensity after treatment is indicated. (F) Scratch wound assay showing closure of a scratch wound in cultured LM38 cells under control conditions or in the presence of PLX4032, SU11274, or both for 72 hours. Medium was replaced every day to remove detached dead cells. Magnification, ×2.5. − indicates untreated control; P, PLX4032 (3.2 μM); S, SU11274 (10 μM).

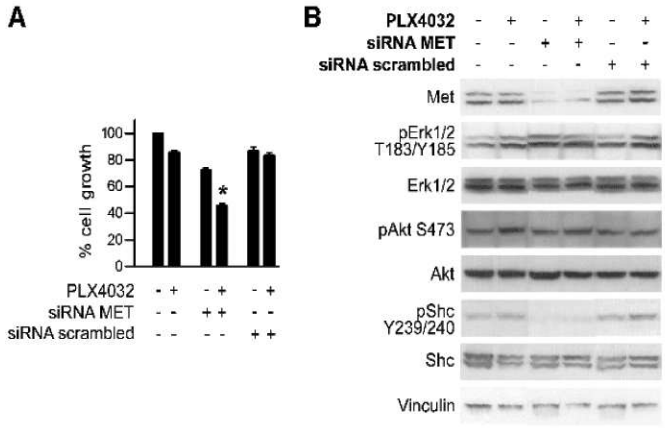


Figure 6. MET silencing increases sensitivity to PLX4032 in LM38 PLX4032 resistant cell line. (A) LM38 cells were treated for 96 hours with MET or control siRNA and with PLX4032 (3.2 μM). After MTT staining, the percentage of cell growth was calculated compared with the untreated control. *$P < .0001$ by one-way ANOVA followed by the Bonferroni correction. (B) Western blot analyses of LM38 cells showing modulation of MET signaling after 96 hours of the indicated treatments. MET protein levels were downregulated to 20%, as determined by quantification of the signal by Image Quant v5.2 software.

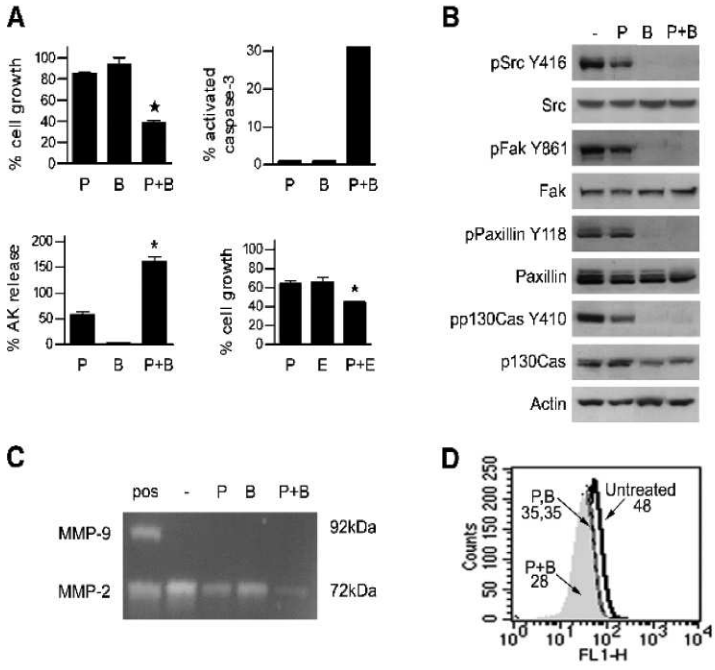


Figure 7. Cotreatment with SRC inhibitors and PLX4032 inhibits LM20 melanoma cell growth and downregulates MMP-2 and β_1-integrin. (A) Growth inhibition, AK release (72 hours), and activated caspase 3 (48 hours) in LM20 cells that were treated with PLX4032 and/or BMS-354825. *$P < .0001$ by one-way ANOVA followed by the Bonferroni correction. ★: interaction index = 2.1. Bottom right, growth inhibitory effect of PLX4032 combined with the SRC inhibitor E804. *$P < .0001$ compared with single treatments by Student's t test. (B) Western blot analysis showing regulation of downstream SRC targets in LM20 cells after 24 hours of treatment with PLX4032 and/or BMS-354825. (C) Gelatin zymography detecting MMP-2 in supernatants from LM20 cells collected after 24 hours of exposure to PLX4032, BMS-354825, or their combination. MMP-2 band was detectable at 72 kDa. (D) FACS analysis of β_1-integrin expression after 24 hours of exposure to PLX4032 and/or BMS-354825 in LM20 cells. Mean fluorescence intensity after treatment is indicated. – indicates untreated control; B, BMS-354825 (100 nM); E, E804; P, PLX4032 (3.2 μM); pos, positive control.

Treatment with the MET inhibitor SU11274 inhibited the growth of LM38 cells harboring constitutively activated MET and the combination with PLX4032 increased this effect. The treatment specifically inhibited MET kinase activity and downstream signaling. It is possible that the effects of SU11274 resulted from the inhibition of additional kinases involved in MET-dependent downstream responses or reduced because of off-target effects. SU11274 was reported to reduce proliferation in some melanoma cell lines [39,40] and HGF-induced motility and invasion in cell models of other tumor types. MET inhibition with other drugs or by specific siRNA confirmed the role of MET signaling in LM38 cells resistant to PLX4032. MET overexpression has been shown to contribute to resistance to cytotoxic drugs in ovarian cancer [41]. Although *MET* gene mutations are very rare [39,40,42], *MET* gene amplification [36] and autocrine production of HGF [43] occur frequently in melanoma. MET activation has been associated to *NRAS* mutation in melanoma [44]. In addition, MET signaling is upregulated by MITF [45].

BMS-354825, which is a multikinase inhibitor targeting the SRC family kinases, induced apoptosis in LM20 cells when combined with PLX4032. BMS-354825 was reported to downregulate activated SRC, FAK, and EphA2 in melanoma cells and to inhibit proliferation in some melanoma cell lines [46,47]. However, BMS-354825 alone did not significantly affect the growth of LM20 cells. Likely, STAT3 activation regulated an oncogenic signaling in LM20 cells. Moreover, the combination of PLX4032 with SU11274 or with BMS-354825 reduced the invasive and migratory capacities, consistently with inhibition of MMP-2 activity and the expression of β_1-integrin, suggesting that the drug combination may result in an inhibitory effect on melanoma growth and dissemination. These results are consistent with a regulatory role of MAPK signaling on the expression of MMPs [48] and β_1-integrin [49]. Furthermore, these data revealed that cell functions other than proliferation and survival are reduced by exposure to PLX4032, suggesting that they are governed by signaling molecules affected by PLX4032 treatment. Because of these effects, we can hypothesize that synergic inhibition of cell proliferation of PLX4032 with MET or SRC inhibitors results from some inhibitory effects on MAPK signaling exerted by PLX4032, which are overridden by compensatory routes exerted by other MEK activators when used as a single treatment.

SRC and MET have been implicated in the development and progression of several types of tumors as a result of the interaction with receptor tyrosine kinases and their downstream effectors leading to proliferation, cell growth, survival, motility, migration, and angiogenesis. In particular, aberrant MET activation, due to overexpression, mutations, or gene amplification, has been associated with poor clinical outcome and drug resistance in lung, hepatic, renal, and colorectal carcinoma [50]. The nonreceptor protein tyrosine kinase SRC acts as a signal transducer from the cell surface receptors by sequential phosphorylation of tyrosine residues on different substrates. SRC is a key molecule in tumor progression providing oncogenic signals for cell survival, epithelial-mesenchymal transition, mitogenesis, invasion, angiogenesis, and metastasis. Aberrant expression and activation of SRC occur in breast, prostate, lung, and colorectal carcinomas, in association with poor clinical outcome, and have stimulated interest in using SRC kinase inhibitors as therapeutic cancer agents, some of which have entered clinical experimentation [51].

Our results highlight the complexity of signaling in melanoma and support the relevance of genetic and proteomic profiling to build rational combination treatments with targeted agents.

Acknowledgments

The authors thank Donata Penso, Enrica Favini, Elisabetta Corna, and Andrea Lampis for their contribution to the study. The authors thank Plexxikon Inc. (Berkeley, CA) and Gideon Bollag for providing us PLX4032.

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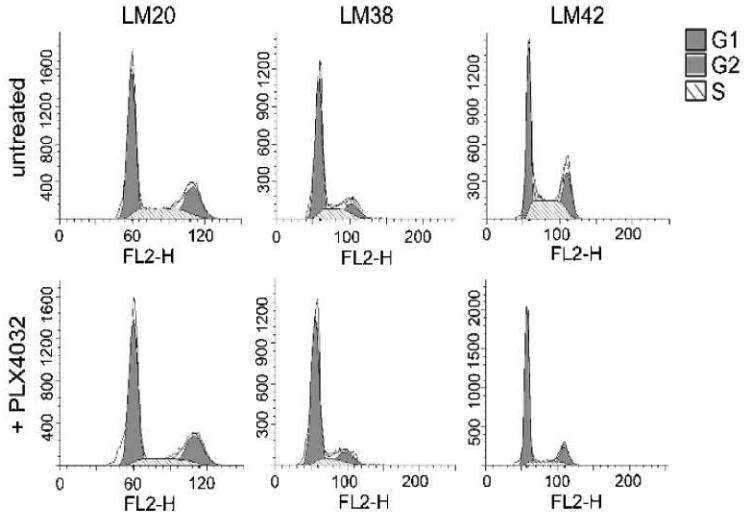

Figure W1. Histograms for cell cycle of the resistant cell lines LM20, LM38, and the sensitive cell line LM42 after 24 hours of treatment with PLX4032 (3.2 μM). G_1 and G_2/M are represented by the two filled peaks, with the dashed peak in between corresponding to the S phase.

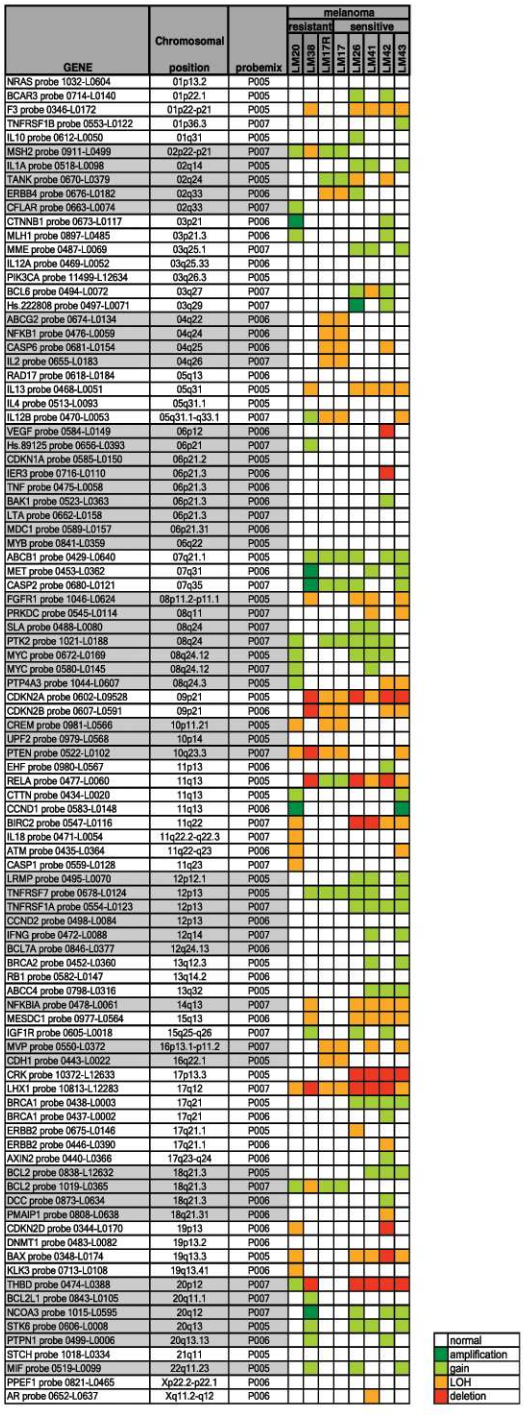

Figure W2. Gene gain or loss as detected by MLPA analysis. Results obtained with PLX4032-resistant LM20, LM38, and LM17R melanoma cells and PLX4032-sensitive LM17, LM26, LM41, LM42, and LM43 melanoma cells are shown.

Table W1. TaqMan Copy Number Analysis of *MET*, *CCND1*, and *CTNNB1* Genes.

Melanoma	nMET 7q31	nCCND1 11q13	nCTNNB1 3p21
LM20	2	8	7
LM38	6	2	2
LM17R	3	2	2
LM17	2	2	2

Gene copy number as evaluated by quantitative PCR as detailed in Materials and Methods.

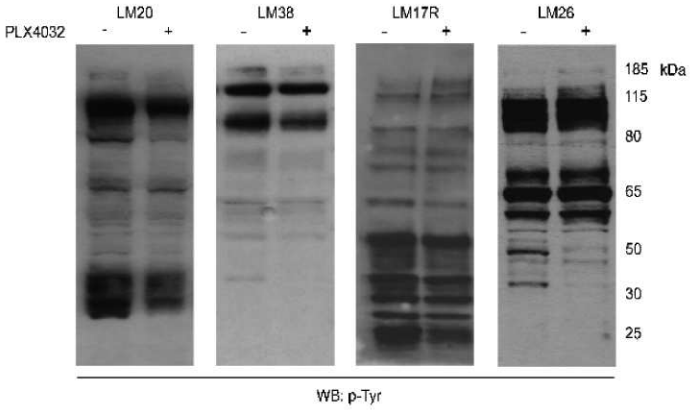

Figure W3. Phosphotyrosine protein separation of melanoma cell lines before and after PLX4032 treatment. Anti-pTyr immunoblot of whole-cell extracts isolated from control cells or cells that were treated with PLX4032 (3.2 μM for 24 hours) and resolved by 4% to 12% SDS-PAGE is shown.

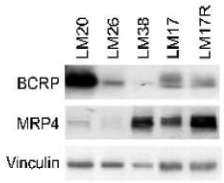

Figure W4. Expression of membrane BCRP and MRP4 transporters of the ABC superfamily in PLX4032-resistant and PLX4032-sensitive cell lines.