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 kinase; MET, met proto-oncogene; MIF, microphthalmia-associated transcription factor; MLPA, multiple ligation-dependent probe amplification; MMP-2, matrix metalloproteinase 2; MRPA, multidrug resistance protein 4; NRAS, neuroblastoma RAS viral (v-ras) oncogene homolog; p70S6K, 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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\(^2\)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.

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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 BRAFV600E 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 MEKI1, 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 BRAFV600E-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 ToxLight 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 Lakes, 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 β1-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.
Melanoma Gene Alterations Including PTEN Loss

Melanoma cells are not associated with other common gene alterations, and independent of other gene alterations. In fact, 18 of 20 BRAF V600E-mutated melanoma cell lines were sensitive to the compound, with IC50 values ranging between 0.01 and 1 μM, whereas 2 cell lines displayed a poor sensitivity and showed IC50 values that were approximately 10 μM. The different IC50 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 G1 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 p70S6K, 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 G1 phase of the cell cycle. In contrast, pAKT, pERK, pp70S6K, 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 1C).

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 G1 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 in the 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.

<table>
<thead>
<tr>
<th>Melanoma*</th>
<th>IC_{50} PLX4032 (μM)†</th>
<th>BRAF‡</th>
<th>nBRAF 7q34§</th>
<th>nMITF 3p14¶</th>
<th>NRAS‡</th>
<th>PTEN</th>
<th>TP53</th>
<th>p16</th>
<th>p14/ARF</th>
<th>cKIT§</th>
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<td>wt</td>
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<td>del</td>
<td>+</td>
<td></td>
</tr>
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<td>−</td>
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<td>V600E</td>
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<td>10</td>
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<td>+</td>
<td>1.862LJ</td>
<td></td>
</tr>
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<td>3</td>
<td>wt</td>
<td>P385S</td>
<td>S127F</td>
<td>A1487L</td>
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<td>Y234C</td>
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<td>−</td>
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<td>V600E</td>
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<td>wt</td>
<td>wt</td>
<td>E258K</td>
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<td>wt</td>
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<td>wt</td>
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<td>V600E</td>
<td>7</td>
<td>4</td>
<td>wt</td>
<td>IVS2–2A/G</td>
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<td>+</td>
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<td>wt</td>
<td>+</td>
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<td>+</td>
<td>−</td>
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<td>wt</td>
<td>+</td>
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<td>wt</td>
<td>+</td>
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<td>4</td>
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<td>P385S</td>
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<td>4</td>
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<td>−</td>
<td>+</td>
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<td>3</td>
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<td>wt</td>
<td>−</td>
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<td>+</td>
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<td>3</td>
<td>9</td>
<td>wt</td>
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<td>wt</td>
<td>−</td>
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<td>+</td>
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<td>wt</td>
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*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 S, G1, S, and G2/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).
and at 3p21 was detected in LM20 gene amplification in LM38 cells and CCND1 Neoplasia at 11q13 and of Selection of PLX4032-resistant variant LM17R by long- Vol. 13, No. 12, 2011 B). These data were consistent and data not shown) and by using quantitative PCR MET and SRC in Primary Resistance to PLX4032 Vergani et al. values of approximately 10 < .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 4A 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 4C). 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 4B).

To define whether PLX4032 resistance was mediated by the increased expression of ABC transporters, we assessed protein expression of ABCB1/Gp170, ABC1/MPR1, ABC2/MPR2, ABC4/MPR4, and ABCC2/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 IC50 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 G1 and AK release in the absence of caspase 3 activation (Figure 5A and not shown). The potentiating effect that was obtained by the concomitant inhibition was evident also when other MET inhibitors were tested (Figure 5B). 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 5C).
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 5D). 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 β1-integrin, the receptor for extracellular matrix laminin that is involved in adhesive and invasive cellular processes (Figure 5E). 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 5F).

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 7A). 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 7B 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 7C), and reduced the expression of β1-integrin (Figure 7D).

**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
Associated with primary resistance. Although \textit{BRAF}, \textit{NRAS}, and \textit{KIT} mutations are mutually exclusive, mutated \textit{BRAF} melanoma may carry common alterations in \textit{CDKN2A}, \textit{PTEN}, and \textit{TP53} genes, as well as alterations of \textit{CDK4}, \textit{CTNNB1}, \textit{FGFR2}, \textit{MITF}, \textit{ERBB4}, \textit{MMP}, and \textit{GRIN2A} genes [3,15,23], and other potential driver mutations still poorly characterized [24]. Here, we show that, apart from \textit{BRAF} mutation, the gene alterations that are common in melanoma, such as \textit{PTEN} and \textit{TP53} mutations, and \textit{BRAF} and \textit{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 \textit{BRAF} gene and have reported the overgrowth of \textit{NRAS} mutated [11], \textit{PTEN} deleted [13], and C121S \textit{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 \textit{de novo} mutations.

The \textit{in vitro} studies on primary resistance to \textit{BRAF} inhibitors have detected \textit{CCND1} gene amplification in cell lines that were resistant to the \textit{BRAF} inhibitor SB590885 [25]. Other studies have identified different changes in \textit{MEK1} and \textit{BRAF} T529N causing resistance to PLX4720 [26,27]. Melanoma cell lines carrying homozygous \textit{BRAF}\textit{V600E} mutation were shown to be more sensitive to PLX4032 than those carrying heterozygous \textit{BRAF}\textit{V600E} mutation [28–30]. Although homozygosity is rare, the 7q34 chromosomal region where the \textit{BRAF} gene is located is frequently amplified in melanoma lesions and especially in \textit{BRAF}\textit{V600E}-mutated melanomas [31]. Amplification of the mutated \textit{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, \textit{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 \textit{in vitro}, the \textit{BRAF} gene was not amplified compared with the parental cell line.

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

The PLX4032-resistant line LM20 harbored amplified \textit{MITF} gene. \textit{MITF} gene amplification was detected in 30% of our \textit{BRAF}\textit{V600E}-mutated cell lines. Unexpectedly, however, melanomas with amplified \textit{MITF} (≥4 copies) showed lower IC\textsubscript{50} values than melanomas without \textit{MITF} amplification when only cell lines carrying two gene copies were considered (0.05 to 0.4 \mu{}M, \textit{P} = .0013), suggesting that \textit{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 \textit{MET} gene in LM38 cells and of \textit{CCND1} and \textit{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 signals. The amplification of the \textit{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 IGFR1 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 β1-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 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 < .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 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.
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 β1-integrin, suggesting that the 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 β1-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 groups 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

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References


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). G1 and G2/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.

<table>
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</tr>
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<tr>
<td>LM17</td>
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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.