

Targeting the PI3K/AKT/mTOR pathway overcomes the stimulating effect of dabrafenib on the invasive behavior of melanoma cells with acquired resistance to the BRAF inhibitor

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Abstract. BRAF inhibitors (BRAFi) have proven clinical benefits in patients with BRAF-mutant melanoma. However, acquired resistance eventually arises. The effects of BRAFi on melanoma cell proliferation and survival have been extensively studied, and several mechanisms involved in acquired resistance to the growth suppressive activity of these drugs have been identified. Much less is known about the impact of BRAFi, and in particular of dabrafenib, on the invasive potential of melanoma cells. In the present study, the BRAF-mutant human melanoma cell line A375 and its dabrafenib-resistant subline A375R were analyzed for invasive capacity, expression of vascular endothelial growth factor receptor (VEGFR)-2, and secretion of VEGF-A and matrix metalloproteinase (MMP)-9, under basal conditions or in response to dabrafenib.

The consequences of inhibiting the PI3K/AKT/mTOR pathway on A375R cell responses to dabrafenib were also evaluated. We found that A375R cells were more invasive and secreted higher levels of VEGF-A and MMP-9 as compared with A375 cells. Dabrafenib reduced invasiveness, VEGFR-2 expression and VEGF-A secretion in A375 cells, whereas it increased invasiveness, VEGF-A and MMP-9 release in A375R cells. In these latter cells, the stimulating effects of dabrafenib on the invasive capacity were markedly impaired by the anti-VEGF-A antibody bevacizumab, or by AKT1 silencing. A375R cells were not cross-resistant to the PI3K/mTOR inhibitor GSK2126458A. Moreover, this inhibitor given in combination with dabrafenib efficiently counteracted the stimulating effects of the BRAFi on invasiveness and VEGF-A and MMP-9 secretion. Our data demonstrate that melanoma cells with acquired resistance to dabrafenib possess a more invasive phenotype which is further stimulated by exposure to the drug. Substantial evidence indicates that continuing BRAFi therapy beyond progression produces a clinical benefit. Our results suggest that after the development of resistance, a regimen combining BRAFi with bevacizumab or with inhibitors of the PI3K/AKT/mTOR pathway might be more effective than BRAFi monotherapy.

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Abbreviations: BRAFi, BRAF inhibitors; BSA, bovine serum albumin; CM, complete medium; DMSO, dimethyl sulfoxide; ECL, enhanced chemiluminescence; ECM, extracellular matrix; EGFR, epidermal growth factor receptor; IC, inhibitory concentration; MMP, matrix metalloproteinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; siRNA, small interfering RNA; PBS, phosphate-buffered saline; RTK, receptor tyrosine kinase; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SEM, standard error of the mean; SNV, single nucleotide variation; STAT3, signal transducers and activators of transcription-3; TBST, Tris-buffered saline/Tween-20; VEGFR, vascular endothelial growth factor receptor

Key words: melanoma, BRAF, dabrafenib, GSK2126458A, bevacizumab, drug resistance, invasion

Introduction

Mutations in the *BRAF* oncogene are present in over 50% of cutaneous melanomas (1), with the vast majority of the mutations leading to the V600E substitution in the BRAF oncoprotein and the constitutive activation of the MAPK/ERK signaling pathway, which is crucial for melanoma growth, survival and invasion (reviewed in refs. 2 and 3). In patients with BRAF-mutant melanoma, inhibition of the oncogenic BRAF kinase with vemurafenib or dabrafenib, or its downstream effector kinases MEK1/2 with trametinib, yields objective response rates of 50-60 and of 20-30%, respectively, and significantly prolongs progression-free and overall survival with respect to dacarbazine chemotherapy (4-6). Moreover, the combination

of dabrafenib and trametinib is well tolerated and associated with a higher response rate and improved disease-free and overall survival as compared with single-agent therapy (7-9).

Despite the impressive initial responses to BRAF inhibitors (BRAFi), the long-term efficacy of these compounds is limited by the emergence of drug resistance (reviewed in refs. 10-13). Multiple mechanisms of acquired resistance to BRAFi have been described, involving both reactivation of MAPK/ERK signaling and/or activation of alternative pro-survival pathways. Restoration of MAPK/ERK signaling in the presence of BRAFi has been shown to occur by activating mutations in *NRAS*, *MAP2K1* or *MAP2K2*, loss of *NF1*, *BRAF*^{V600E} amplification, overexpression of the CRAF or COT1 kinases, as well as by the expression of splice variants of *BRAF*^{V600E} (10-13). Alternative mechanisms of secondary resistance to BRAFi mainly involve alterations leading to upregulation of the PI3K/AKT/mTOR signaling axis. In this regard, mutations in *AKT1*, *AKT3*, *PIK3CA*, *PIK3CG*, *PIK3R2* and *PHLPP1*, *PTEN* loss as well as overexpression and/or activation of the receptor tyrosine kinases (RTKs) platelet-derived growth factor receptor α and β , insulin-like growth factor 1 receptor, fibroblast growth factor receptor-3, and epidermal growth factor receptor (EGFR), have been reported (10-16). Notably, RTK signaling can also bypass mutant BRAF and activate ERK via RAS (10,14,15).

Although a large body of experimental data is available on the effects of BRAFi on melanoma proliferation and survival, and on the molecular mechanisms involved in acquired resistance to the growth-suppressive effects of these agents, much less is known about the impact of BRAFi on melanoma cell migration and invasion. In this regard, Halaban *et al* (17) showed that while vemurafenib inhibited migration of BRAF-mutant melanoma cells, it increased that of BRAF wild-type cells, as a result of drug-induced CRAF activation. Girotti *et al* (18) demonstrated that BRAF-mutant melanoma cells with acquired resistance to PLX4720, a drug structurally related to vemurafenib (19), were more invasive, both *in vitro* and *in vivo*, than the corresponding drug-sensitive counterparts as a consequence of hyperactivation of the EGFR-SRC family kinase-signal transducers and activator of transcription 3 (STAT3) signaling pathway. Increased invasiveness of BRAF-mutant melanoma cells that had been rendered resistant to the BRAFi SB590885 was reported by Vultur *et al* (20), who also showed activation of the STAT3 pathway in these cells under basal conditions and upon exposure to SB590885. Similarly, Zubrilov *et al* (21) found that *in vitro* induction of vemurafenib resistance in BRAF-mutant melanoma cells was associated with increased potential for metastasis. The vemurafenib-resistant cells showed, indeed, enhanced migratory and adherence functions, higher expression levels of several genes involved in promotion of metastasis and increased capacity to form lung and liver micro-metastasis in nude mice. Sanchez-Laorden *et al* (22) demonstrated that PLX4720 stimulated the invasive capacity of RAS-mutant melanoma cells by a mechanism involving activation of the MEK/ERK pathway, increased expression and secretion of IL-8 and induction of protease-dependent invasion. Moreover, increased levels of *IL-8* mRNA and similar responses to PLX4720 were observed by these authors in BRAF-mutant melanoma cells which had developed resistance to the drug. On the contrary, vemurafenib

was reported to exert a moderate inhibition of the *in vitro* invasive activity of a BRAF^{V600E}/NRAS wild-type melanoma cell line showing primary resistance to the antiproliferative effects of the drug as a consequence of MET amplification and activation (23).

To the best of our knowledge, no data are presently available concerning the effects of dabrafenib on the metastatic potential of BRAF-mutant melanoma cells. In the present study we, therefore, evaluated the effect of dabrafenib on the *in vitro* invasive capacity of melanoma cells sensitive or with acquired resistance to the growth suppressive effects of the drug. We also investigated whether inhibition of the PI3K/AKT/mTOR signaling pathway could modulate the effects of dabrafenib on the invasive behavior of the resistant cells.

Materials and methods

Cell cultures. The human melanoma cell line A375 was purchased from the European Collection of Cell Cultures (Salisbury, UK) and cultured in BioWhittaker™ RPMI-1640 medium (Lonza, Verviers, Belgium) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA), 2 mM BioWhittaker™ L-glutamine (Lonza), and 50 μ g/ml BioWhittaker™ gentamicin (Lonza) (hereafter referred to as complete medium, CM). The A375R subline with acquired resistance to dabrafenib was generated by growing the parental A375 cells in gradually increasing concentrations of dabrafenib (from 1 nM up to 1.5 μ M) over a period of 4 months. After selection, A375R cells were maintained in CM supplemented with 1.5 μ M dabrafenib.

Drugs, chemicals and antibodies. Dabrafenib (GSK2118436A) and GSK2126458A were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich) at a final concentration of 1.92 and 1 mM, respectively. Both drugs were stored as stock solutions at -80°C and diluted in CM just before use.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich, dissolved at a concentration of 5 mg/ml in Gibco™ phosphate-buffered saline (PBS; Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) and stored at 4°C.

For western blot analysis, the following rabbit polyclonal antibodies were used: anti-human ERK1/2 (GTX17942; GeneTex, Irvine, CA, USA), anti-human phospho-ERK1/2 (Thr185/Tyr187) (44-680G; Invitrogen), anti-human β -tubulin (sc-9104; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-vascular endothelial growth factor receptor-2 (VEGFR-2) (sc-6251; Santa Cruz Biotechnology), anti-human AKT (9272; Cell Signaling Technology, Inc., Beverly, MA, USA) and anti-human phospho-AKT (Ser473) (9271; Cell Signaling Technology).

Reagents for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) were all purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA).

DNA extraction and whole exome sequencing. DNA extraction from A375 and A375R cell lines was performed using the DNeasy Blood & Tissue kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol, and sheared with a Bioruptor sonicator (Diagenode, Liege,

Belgium). DNA libraries were prepared and captured using the SureSelect Human All Exon kit (50 Mb; Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions. DNA libraries were sequenced by Genomix4life S.r.l., (Baronissi, Salerno, Italy) on Illumina NextSeq 500 platform (Illumina, San Diego, CA, USA) with 100-bp paired-end reads.

Exome sequence analysis. Sequence reads were mapped to the reference human genome assembly hg19 using Burrows-Wheeler Aligner (BWA-MEM) version 0.7.12 (24). PCR duplicates were removed using Picard 1.130 (<http://broadinstitute.github.io/picard>). The Genome Analysis Toolkit (GATK) version 3.4 was used for realignment of insertions and deletions, quality recalibration and variant calling (25). Then, to create a sub-selection of high-quality single nucleotide variations (SNVs), a series of filtering options was applied, including minimum read depth of 8x, SNV call quality score above 30, and non-reference allele frequency above 20%. Variants were annotated with information from RefGenes using wANNOVAR web-based tool (26).

Chemosensitivity assay. A375 and A375R cells were suspended in CM, seeded (50 μ l/well) into BD Falcon™ 96-well plates (BD Biosciences, Bedford, MA, USA) and allowed to adhere at 37°C in a 5% CO₂ atmosphere for 18 h. Graded amounts of dabrafenib or GSK2126458A were then added to the cells in 50 μ l of CM. As a control, A375 and A375R cells were treated with DMSO alone. The plates were incubated at 37°C for five days and cell proliferation was then evaluated by the MTT assay, as previously described (27). Three replica wells were used for each group. Drug concentration producing 50% inhibition of cell growth (i.e. IC₅₀), was calculated on the regression line in which absorbance values at 595 nm were plotted against the logarithm of drug concentration. In a different set of experiments, A375R cells, plated as described above, were incubated with DMSO alone, 100 nM dabrafenib, 20 nM GSK2126458A or a combination of the two drugs for 5 days and then assayed for proliferation using the MTT assay.

Cell treatment for western blot analysis and invasion assay. A375 and A375R cells were suspended in CM, seeded into BD Falcon™ 10-cm-dishes (BD Biosciences) and allowed to adhere at 37°C in a 5% CO₂ atmosphere for 18 h. Thereafter, dabrafenib (100 nM) or DMSO alone was added to the cultures and the dishes incubated at 37°C for additional 6 or 48 h. In a different set of experiments, A375R cells, plated as described above, were incubated with DMSO alone, 100 nM dabrafenib, 20 nM GSK2126458A or a combination of the two drugs for 48 h. At the end of the incubation period, melanoma cells were processed for western blot analysis or tested for their ability to invade the extracellular matrix (ECM) *in vitro*.

Western blot analysis. For analysis of ERK1/2, phosphorylated ERK1/2, AKT and phosphorylated AKT expression, control and drug-treated cells were recovered from culture, washed and total cellular extracts were prepared as described previously (28). For analysis of VEGFR-2 expression, melanoma cells were left in the culture dishes, washed, lysed directly in SDS sample buffer (50 mM Tris-HCl pH 6.8, 100 mM

dithiothreitol, 2% SDS, 0.1% bromophenol blue and 10% glycerol) and then boiled for 5 min. Proteins per sample (15 μ g) were run on a 6% (VEGFR-2) or 12% SDS-polyacrylamide gels, transferred to nitrocellulose membranes (Amersham Biosciences, Buckinghamshire, UK) and blocked with 5% non-fat milk in Tris-buffered saline supplemented with 0.1% Tween-20 (TBST) for 1 h at room temperature. The membranes were then incubated in the same solution, or in TBST containing 5% bovine serum albumin (BSA), overnight at 4°C with primary antibodies at the following dilutions: anti-ERK1/2, anti-phospho-ERK1/2, anti-AKT and anti- β -tubulin 1:1,000; anti-phospho-AKT 1:500; anti-VEGFR-2 1:200. The anti- β -tubulin antibody was used as an internal standard for loading. Immunodetection was carried out using appropriate horseradish peroxidase-linked secondary antibodies and enhanced chemiluminescence (ECL) or ECL Prime (phospho-AKT) detection reagents (Amersham Biosciences). Where indicated, film was scanned on a GS-710 Calibrated Imaging Densitometer and analyzed by means of Quantity One Software Version 4.1.1 (Bio-Rad Laboratories).

Invasion assay in Boyden chambers. This assay was performed as previously described (29). Briefly, control and drug-treated melanoma cells were removed from culture, washed, suspended in invasion medium (1 μ g/ml heparin/0.1% BSA in RPMI-1640) and loaded (2x10⁵ cells) into the upper compartment of Boyden chambers equipped with 8- μ m pore diameter polycarbonate filters (Nuclepore, Whatman Inc., Clifton, NJ, USA) coated with 20 μ g of BD Matrigel™ Basement Membrane Matrix (BD Biosciences, hereafter referred to as Matrigel). Invasion medium or, where indicated, invasion medium containing 20 ng/ml VEGF-A (ImmunoTools, Friesoythe, Germany) was added to the lower compartment of the chambers. For each experimental condition, three Boyden chambers were set up. After incubation of the Boyden chambers at 37°C in a 5% CO₂ atmosphere for 4 h, the filters were removed from the chambers and the cells were fixed in ethanol for 5 min and stained in 0.5% crystal violet for 15 min. The cells from the upper surface of the filter were removed by wiping with a cotton swab and the migrated cells, attached to the lower surface of the filters, were counted under the microscope. Twelve microscopic fields (magnification, x200), randomly selected on triplicate filters, were scored for each experimental condition.

In a set of experiments, invasion assays were performed in the presence of the anti-human VEGF-A monoclonal antibody bevacizumab (5 μ g/ml) (Avastin; Roche, Welwyn Garden City, UK) or the corresponding control IgG₁ immunoglobulins (MAB002; R&D Systems, Minneapolis, MN, USA). A375R cells were pre-incubated with the antibodies for 30 min at room temperature in a rotating wheel. The cells were then loaded in the Boyden chambers without removing the antibodies.

Three-dimensional spheroid invasion assay. A375 and A375R spheroids were generated as described by Naber *et al* (30) with minor modifications. Briefly, melanoma cells were suspended (4x10⁴ cells/ml) in CM containing 0.24% methylcellulose (Sigma-Aldrich) and seeded (100 μ l/well) into non-adhesive, round-bottom 96-well plates (Corning Inc., Corning, NY, USA). Plates were centrifuged at 1250 x g for 90 min and

then incubated at 37°C in a 5% CO₂ atmosphere for 18 h to allow the cells to organize into three-dimensional spheroids (one spheroid in each well). Spheroids were then harvested and individually embedded between two layers of Matrigel as previously described (31) with minor modifications. Briefly, 50 µl of Matrigel diluted in RPMI-1640 medium (4 µg/ml), were added to the wells of a flat-bottom 96-well plate, that was then incubated at 37°C for 15 min to let the Matrigel solidify. Melanoma spheroids, suspended in 100 µl of Matrigel diluted in invasion medium (4 µg/ml), were then seeded in the Matrigel-precoated plate (one spheroid/well, five replicates). After a 30-min incubation at 37°C to allow Matrigel polymerization, spheroids were overlaid with 100 µl of invasion medium and the plate incubated at 37°C for 48 h. At the end of incubation, images of the invading spheroids were taken using a Leica inverted microscope and a Canon digital camera PowerShot G5.

Transient transfection with small interfering RNA (siRNA) targeting AKT1 and drug treatment of the transfected cells. Oligonucleotide siRNA targeting AKT1 (CUCACAGCC CUGAAGUACU) (siAKT) and the corresponding scramble oligonucleotide (GAUCCUAUAUUCGGUAGU) (siCTRL) to be used as a control were purchased from Sigma-Proligo (The Woodlands, TX, USA).

A375R cells were suspended in CM without antibiotics, seeded into BD Falcon™ 6-well plates (BD Biosciences), allowed to adhere at 37°C in a 5% CO₂ atmosphere for 18 h, and then transfected with 10 nM siAKT or siCTRL using Lipofectamine™ RNAiMAX reagent (Invitrogen) according to the manufacturer's protocol. Twenty-four hours after transfection, the cells were incubated with 100 nM dabrafenib or DMSO alone. After 48 h of culture, the cells were collected and processed for western blot analysis or tested for ability to invade the ECM.

Evaluation of VEGF-A and matrix metalloproteinase-9 (MMP-9) secretion. A375 and A375R cells were suspended in CM, seeded into BD Falcon 10 cm-dishes (BD Biosciences) and allowed to adhere at 37°C in a 5% CO₂ atmosphere for 18 h. Thereafter, A375 cells were incubated with DMSO alone or 100 nM dabrafenib, whereas A375R were incubated with DMSO alone, 100 nM dabrafenib, 20 nM GSK2126458A or a combination of the two drugs. After 48 h of drug exposure, culture supernatants were collected, centrifuged at 600 x g for 10 min to remove cells in suspension and debris, and frozen at -20°C until use. Cells were detached with a solution of 1.5 mM EDTA in PBS and counted to determine the total number of cells in the culture. Quantification of the VEGF-A amount in the culture supernatants was performed as previously described (32). For MMP-9 evaluation, culture supernatants were concentrated at least 5-fold in Centrplus concentrators (Amicon, Beverly, MA, USA). The amount of active MMP-9 in the culture supernatants was then determined using the Quantikine ELISA Human MMP-9 immunoassay (R&D Systems) according to the manufacturer's protocol, and calibrated against a standard curve. The amount of VEGF-A and MMP-9 was normalized to the number of total cells counted in each culture at the time of supernatant collection.

Statistical analysis. Statistical significance among different values of drug IC₅₀, different number of ECM invading cells and different levels of VEGF-A and MMP-9 secretion, was assessed using two-sided Student's t-test analysis.

Results

Generation and characterization of the A375R subline with acquired resistance to dabrafenib. To generate a cellular model of acquired resistance to dabrafenib, the human melanoma cell line A375, which harbors the BRAF^{V600E} mutation and is highly sensitive to the growth suppressive effects of BRAF inhibitors, was cultured with gradually increasing concentrations of dabrafenib until a subline (i.e. A375R) able to growth in the presence of 1.5 µM of the drug was obtained. This concentration of dabrafenib corresponds to the peak plasma concentration reported for patients treated with the drug at the recommended dose of 150 mg BID (33).

A375 and A375R cells were initially analyzed for sensitivity to the antiproliferative effects of dabrafenib, and for expression of total and phosphorylated ERK1/2, either under basal conditions or following a 6-h exposure to 100 nM of the drug. MTT assays, performed after five days of cell culture with graded concentrations of dabrafenib, revealed that the A375R subline was 10,000-fold more resistant to the growth inhibitory effect of this drug than the parental cell line (Fig. 1A). A375R cells also expressed higher basal levels of phosphorylated ERK1/2 as compared with A375 cells (Fig. 1B). Moreover, while dabrafenib, as expected, markedly downregulated the level of phosphorylated ERK1/2 in A375 cells, it further increased it in the drug-resistant subline (Fig. 1B).

Previous studies have shown that melanoma cells with acquired resistance to the BRAFi vemurafenib, PLX4720 or SB590885 possess a more invasive phenotype (18,20-22), whereas no data are available regarding the metastatic potential of dabrafenib-resistant cells. Therefore, we evaluated the ability of A375 and A375R cells to spontaneously invade the ECM using the Boyden chamber assay. The results illustrated in Fig. 1C show that the dabrafenib-resistant A375R cells were ~2-fold more invasive than the dabrafenib-sensitive A375 cells.

The invasive behavior of A375 and A375R cells was also investigated using an invasion assay based on Matrigel-embedded spheroids, that mimics the three-dimensional tumor architecture and incorporates components of the ECM (34). Consistent with results obtained in Boyden chamber assays A375R spheroids invaded into Matrigel to a higher extent as compared with parental A375 cells (Fig. 1D).

Finally, to investigate whether reactivation of the MAPK/ERK pathway in A375R cells could be dependent on mutations in *HRAS*, *KRAS*, *NRAS*, *MAP2K1*, *MAP2K2* or on *NFI* loss, whole exome sequencing was performed in both A375 and A375R cell lines. No mutations in the three *RAS* genes or in *MAP2K1* and *MAP2K2*, and no loss of *NFI* were detected in A375 and A375R cells.

Dabrafenib impairs invasiveness and VEGF-A secretion in A375 cells, whereas it stimulates these functions in A375R cells. We previously showed that an autocrine loop sustained by the interaction of VEGF-A with VEGFR-2 promoted melanoma cell ability to migrate and invade the ECM *in vitro* (35).

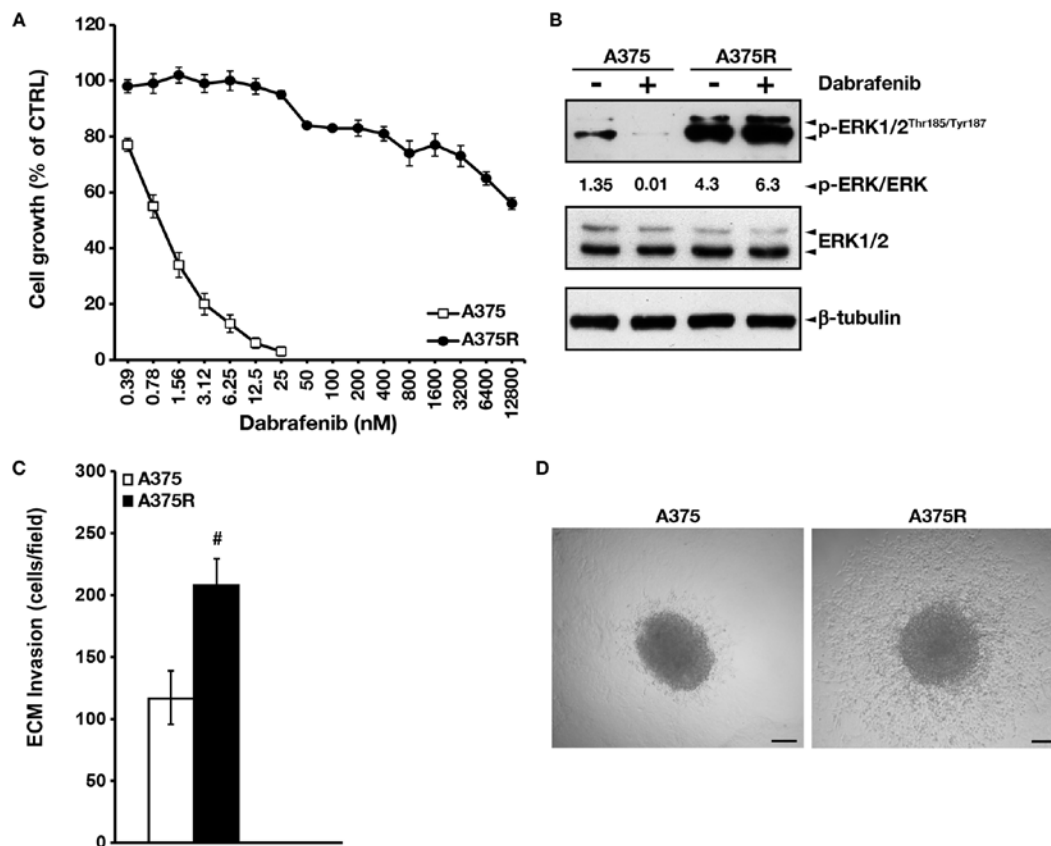


Figure 1. Characterization of the dabrafenib-resistant A375R cells. (A) A375 and A375R cells were incubated with increasing concentrations of dabrafenib or with DMSO alone for five days and then proliferation was assessed by the MTT assay. Data are expressed in terms of percentage of growth of cells treated with dabrafenib with respect to cells treated with DMSO alone (CTRL). Each value represents the arithmetic mean of six (A375) or three (A375R) independent experiments performed with triplicate samples. Bars, standard error of the mean (SEM). For each experiment, the dabrafenib IC_{50} value was calculated as described in Materials and methods. The IC_{50} mean value \pm SEM for A375 cells was 1.041 ± 0.150 nM. The IC_{50} value for A375R was >10 μ M. (B) A375 and A375R cells were treated with 100 nM dabrafenib or DMSO alone (-) for 6 h. Equal amounts of total proteins were analyzed by immunoblotting using antibodies against phospho-ERK1/2 (Thr185/Tyr187) (p-ERK1/2), ERK1/2 and anti- β -tubulin. For each sample, the ratio between the densitometric level of phospho-ERK1/2 and ERK is shown. The results are representative of three independent experiments. (C) A375 and A375R cell ability to spontaneously invade the ECM was evaluated using Boyden chambers equipped with Matrigel-coated filter. Data are expressed as number of invading cells per microscopic field. Each value represents the arithmetic mean \pm SEM of three independent experiments. [#] $P < 0.05$ A375R vs. A375. (D) A375 and A375R spheroids were prepared and embedded into Matrigel as described in Materials and methods. After 48 h of culture, images depicting invasive outgrowth of spheroids were taken under a Leica inverted microscope at low magnification. Depicted spheroids are representative of three independent experiments. Scale bar represents 200 μ m.

Recently, Liu *et al* (36) demonstrated that PLX4720 inhibited VEGF production in a melanoma cell line and xenografts, and that downregulation of VEGF expression occurred in the tumor of melanoma patients treated with PLX4720. Furthermore, Beazley-Long *et al* (37) reported that vemurafenib decreased VEGF-A expression in BRAF-mutant and drug-sensitive melanoma cells, whereas it increased this cytokine in BRAF-wild-type melanoma cells. Therefore, to investigate the effects of dabrafenib on the metastatic potential of melanoma cells, A375 and A375R cells were treated with 100 nM of the drug (or with DMSO as vehicle control) for 48 h and then analyzed for the ability to invade the ECM under basal conditions and in response to exogenously added VEGF-A, as well as for VEGF-A secretion and VEGFR-2 expression.

The results illustrated in Fig. 2A show that both cell lines responded to VEGF-A with an increase of ECM invasion, and that not only spontaneous but also VEGF-A-induced invasive capacity was higher in the A375R cell line as compared with the parental counterpart. Dabrafenib significantly inhibited spontaneous and VEGF-A-induced ECM invasion in A375

cells. In contrast, A375R cell invasiveness was markedly stimulated by the drug in both conditions.

VEGF-A secretion in the culture supernatants of A375 and A375R cells, and VEGFR-2 expression in the two cell lines were evaluated by specific ELISA and western blot analysis, respectively. We found that basal secretion of VEGF-A was higher in A375R cells as compared with parental A375 cells (Fig. 2B). Moreover, VEGF-A secretion was markedly impaired in dabrafenib-treated A375 cells, whereas it was significantly increased in drug-treated A375R cells. Exposure to dabrafenib caused a reduction of VEGFR-2 expression in A375 cells but it did not substantially affect the receptor levels in A375R cells (Fig. 2C). Of note, the effects of 1.5 μ M dabrafenib on A375R invasiveness, VEGF-A secretion and VEGFR-2 expression were comparable to those produced by 100 nM of the drug (data not shown).

Bevacizumab inhibits dabrafenib-induced invasion in A375R cells. The findings that A375R cells were more invasive and secreted higher levels of VEGF-A than A375 cells, and

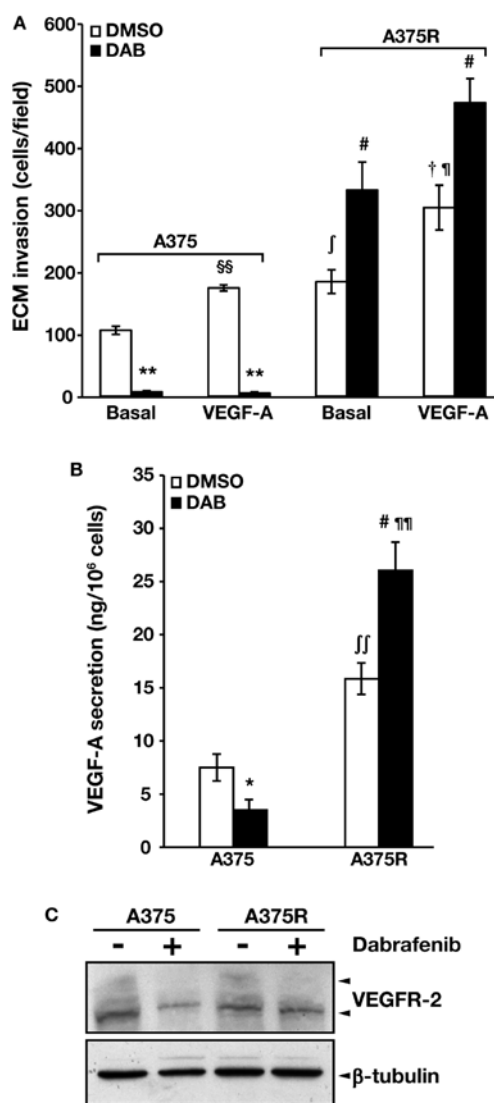


Figure 2. Dabrafenib inhibits ECM invasion and VEGF-A secretion in A375 cells whereas it enhances these functions in A375R cells. (A) The cells were cultured in the presence of 100 nM dabrafenib (DAB) or DMSO alone for 48 h. Thereafter, cell ability to invade the ECM, either spontaneously (Basal) or in response to VEGF-A (20 ng/ml) was evaluated using Boyden chambers equipped with Matrigel-coated filter. Data are expressed as number of invading cells per microscopic field. Each value represents the arithmetic mean \pm SEM of three independent experiments. ^{§§} $P < 0.01$ A375/VEGF-A vs. A375/Basal; ^{**} $P < 0.01$ A375/DAB vs. A375/DMSO; [†] $P < 0.05$ A375R/VEGF-A vs. A375R/Basal; [#] $P < 0.05$ A375R/DAB vs. A375R/DMSO; [‡] $P < 0.05$ A375R/basal vs. A375R/basal; [§] $P < 0.05$ A375R/VEGF-A vs. A375/VEGF-A. (B) A375 and A375R cells were cultured with 100 nM dabrafenib or DMSO alone for 48 h. Culture supernatants were then collected and the amount of VEGF-A quantified using ELISA. Each value represents the arithmetic mean \pm SEM of four independent experiments. ^{*} $P < 0.05$ A375/DAB vs. A375/DMSO; [#] $P < 0.05$ A375R/DAB vs. A375R/DMSO; [‡] $P < 0.01$ A375R/DMSO vs. A375/DMSO; ^{§§} $P < 0.01$ A375R/DAB vs. A375/DAB. (C) A375 and A375R cells were treated with 100 nM dabrafenib or DMSO alone (-) for 48 h. Equal amounts of total proteins were analyzed by immunoblotting using antibodies against VEGFR-2 or β -tubulin. The results are representative of two independent experiments.

responded to dabrafenib with a further increase of ECM invasion and VEGF-A secretion, prompted us to investigate whether inhibition of VEGF-A binding to its cognate RTKs could impair spontaneous and dabrafenib-induced invasiveness of A375R cells. To this end, the cells were treated with 100 nM dabrafenib or DMSO alone for 48 h, and then assayed

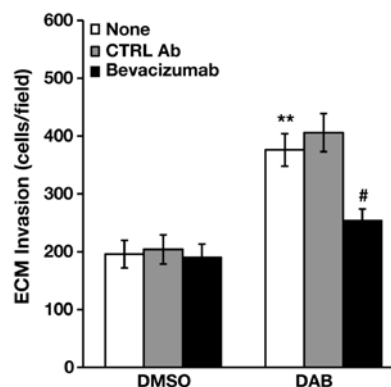


Figure 3. Bevacizumab inhibits dabrafenib-induced stimulation of A375R cell invasiveness. A375R cells were treated with 100 nM dabrafenib (DAB) or DMSO alone for 48 h and then assayed for their ability to invade the ECM in the presence of the anti-VEGF-A monoclonal antibody bevacizumab (5 μ g/ml) or a control antibody (CTRL Ab) or in the absence of antibodies (None). Data are expressed as number of invading cells per microscopic field. Each value represents the arithmetic mean \pm SEM of three independent experiments. ^{**} $P < 0.01$ None/DAB vs. None/DMSO; [#] $P < 0.05$ DAB/Bevacizumab vs. DAB/None and vs. DAB/CTRL Ab.

for ECM invasion in the absence or in the presence of the anti-VEGF-A monoclonal antibody bevacizumab (38,39), or of a control antibody. As illustrated in Fig. 3, bevacizumab did not affect ECM invasion of DMSO-treated cells, whereas it abrogated dabrafenib-induced stimulation of A375R cell invasiveness.

AKT silencing prevents dabrafenib-induced stimulation of the invasive capacity of A375R cells. VEGF-A binding to VEGFR-2 can activate a number of downstream signaling pathways, including the MAPK/ERK and PI3K/AKT cascades (40,41). Moreover, previous studies (42) demonstrated that VEGF-A overproduction and concomitant expression of its receptors promote melanoma cell growth and survival through the activation of the MAPK/ERK and PI3K/AKT signaling pathways. We therefore investigated whether, in addition to reactivation of the MAPK/ERK signaling in the presence of dabrafenib, A375R cells also showed hyperactivation of the PI3K/AKT pathway. Western blot analysis of total and phosphorylated AKT in A375 and A375R cells, showed that the drug-resistant subline expressed higher basal levels of phosphorylated AKT as compared with the parental cell line (Fig. 4A). Moreover, in both cell lines exposure to 100 nM dabrafenib for 48 h enhanced the levels of phosphorylated AKT (Fig. 4A). Exome sequencing performed in A375 and A375R cells did not reveal mutations in the *AKT1*, *AKT3*, *PIK3CA*, *PIK3CG*, *PIK3R2* and *PHLPP1* genes, or *PTEN* loss.

To establish whether hyperactivation of the PI3K/AKT pathway could underlie the increased invasive capacity of A375R cells, AKT1 was silenced in these cells using specific siRNAs. The results illustrated in Fig. 4B show that total AKT levels were efficiently reduced in the siAKT-transfected cells exposed to DMSO or to 100 nM dabrafenib for 48 h. Notably, DMSO-treated and dabrafenib-treated siAKT-transfected cells also showed a marked reduction of phospho-AKT levels. This finding indicates that in A375R cells, basal and dabrafenib-induced phosphorylation of AKT mainly involve the AKT1 isoform. As observed for bevacizumab, AKT1 silencing

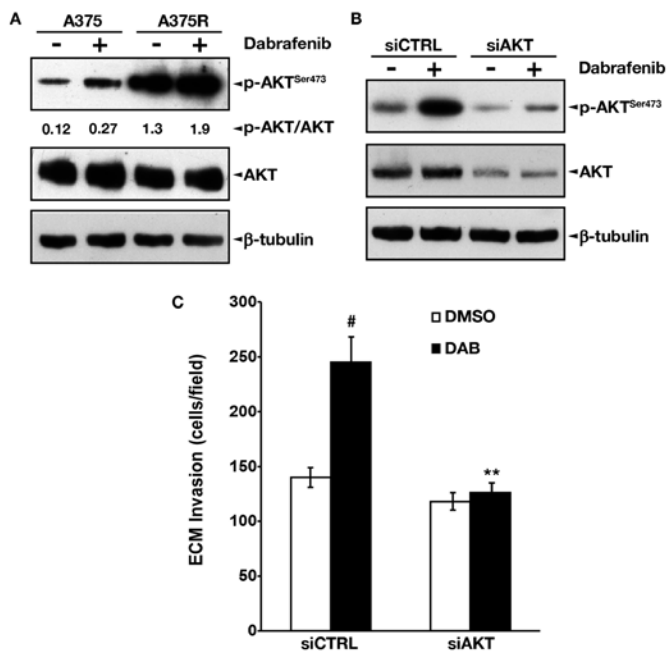


Figure 4. AKT silencing inhibits dabrafenib-induced stimulation of A375R invasiveness. (A) A375 and A375R cells were treated with 100 nM dabrafenib or DMSO alone (-) for 48 h. Equal amounts of total proteins were analyzed by immunoblotting using antibodies against phospho-AKT (Ser473) (p-AKT), AKT or β -tubulin. For each sample, the ratio between the densitometric level of phospho-AKT and AKT is shown. The results are representative of three independent experiments. (B) A375R cells were transiently transfected with 10 nM of siAKT or siCTRL and 24 h later incubated with 100 nM dabrafenib or DMSO alone (-). After 48 h of culture, equal amounts of total proteins were analyzed by immunoblotting using antibodies against phospho-AKT (Ser473) (p-AKT), AKT or β -tubulin. The results are representative of two independent experiments. (C) A375R cells were transfected and treated as described in (B) and then assayed for spontaneous invasion of ECM. Data are expressed as number of invading cells per microscopic field. Each value represents the arithmetic mean \pm SEM of three independent experiments. [#]P<0.05 siCTRL/DAB vs. siCTRL/DMSO; ^{**}P<0.01 siAKT/DAB vs. siCTRL/DAB.

failed to reduce basal invasiveness of A375R cells, whereas it completely abrogated upregulation of ECM invasion induced by dabrafenib (Fig. 4C).

The PI3K/mTOR inhibitor GSK2126458A impairs invasiveness and VEGF-A secretion induced by dabrafenib in A375R cells. Based on the key role played by MAPK/ERK signaling reactivation and PI3K/AKT/mTOR signaling upregulation in acquired resistance to BRAFi, several therapeutic approaches co-targeting the MAPK/ERK and the PI3K/AKT/mTOR pathways in patients with BRAF-mutant melanoma are underway to improve the efficacy and durability of clinical response (12; <https://www.clinicaltrials.gov/>).

We therefore investigated whether co-treatment of A375R cells with the dual PI3K/mTOR inhibitor GSK2126458A could antagonize the stimulating effects exerted by dabrafenib on ECM invasion and VEGF-A secretion. Preliminary experiments were also performed to evaluate A375R cell sensitivity to the growth inhibitory effect of GSK2126458A and to select the drug concentration to be used in combination with dabrafenib.

In agreement with previous findings (43), MTT proliferation assays, performed after five days of cell exposure to

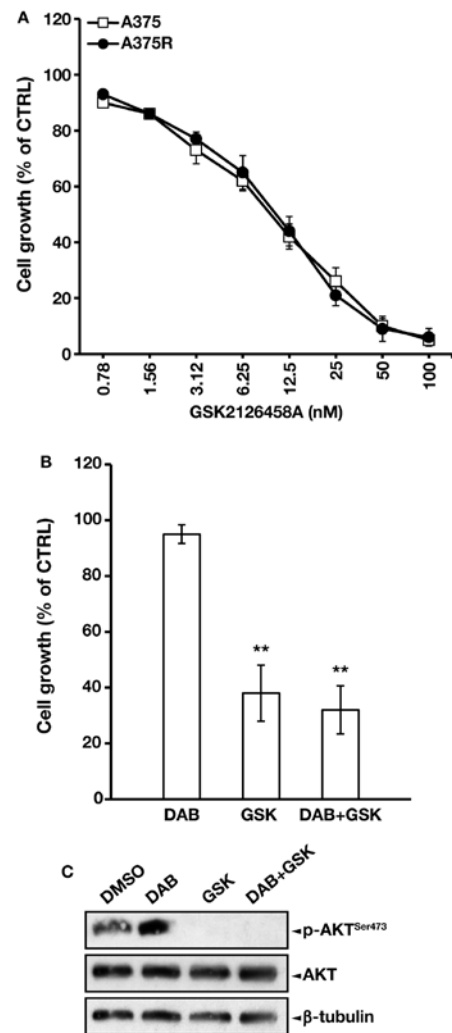


Figure 5. GSK2126458A alone or in combination with dabrafenib inhibits proliferation and AKT phosphorylation in A375R cells. (A) A375 and A375R cells were incubated with increasing concentrations of GSK2126458A or with DMSO alone for five days and then proliferation was assessed by the MTT assay. Data are expressed in terms of percentage of growth of cells treated with GSK2126458A with respect to cells treated with DMSO alone (CTRL). Each value represents the arithmetic mean of three independent experiments performed with triplicate samples. Bars, SEM. GSK2126458A IC₅₀ mean value \pm SEM was 7.836 \pm 0.597 nM for A375 cells and 8.940 \pm 1.654 nM for A375R cells. The difference between the IC₅₀ values was not statistically significant. (B) A375R cells were cultured with DMSO alone, 100 nM dabrafenib (DAB), 20 nM GSK2126458A (GSK) or a combination of the two drugs for five days and then assayed for proliferation using the MTT assay. Data are expressed in terms of percentage of growth of drug-treated cells with respect to DMSO-treated cells (CTRL). Each value represents the arithmetic mean \pm SEM of three independent experiments performed with triplicate samples. Bars, SEM. ^{**}P<0.01 GSK and DAB+GSK vs. DAB. (C) A375R cells were cultured with DMSO alone, 100 nM dabrafenib (DAB), 20 nM GSK2126458A (GSK) or a combination of the two drugs for 48 h. Equal amounts of total proteins were analyzed by immunoblotting using antibodies against phospho-AKT (Ser473) (p-AKT), AKT or β -tubulin. The results are representative of two independent experiments.

graded concentrations of GSK2126458A, revealed that the dabrafenib-resistant subline was responsive to the PI3K/mTOR inhibitor, displaying a drug IC₅₀ value comparable to that of the parental cells (Fig. 5A).

The clinically achievable concentration of 20 nM GSK2126458A was then selected for the subsequent experiments. This drug concentration inhibited A375R proliferation

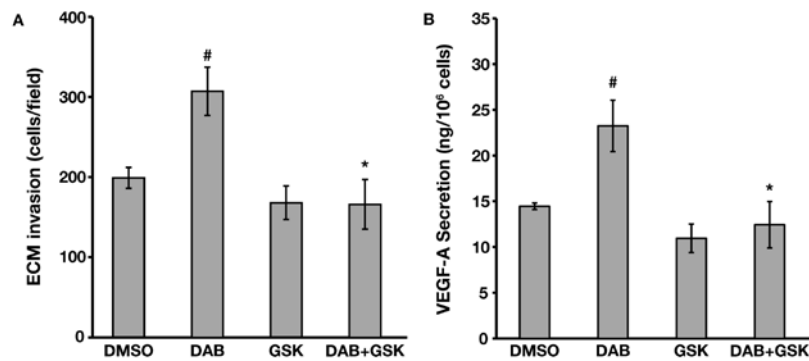


Figure 6. GSK2126458A abrogates dabrafenib-induced stimulation of ECM invasion and VEGF-A secretion in A375R cells. (A) The cells were cultured with DMSO, 100 nM dabrafenib (DAB), 20 nM GSK2126458A (GSK) or a combination of the two drugs for 48 h and then assayed for their ability to spontaneously invade the ECM. Data are expressed as number of invading cells per microscopic field. Each value represents the arithmetic mean \pm SEM of three independent experiments. # P <0.05 DAB vs. DMSO; * P <0.05 DAB+GSK vs. DAB. (B) A375R cells were treated as described in (A). Culture supernatants were then collected and the amount of VEGF-A quantified using ELISA. Each value represents the arithmetic mean \pm SEM of four independent experiments. # P <0.05 DAB vs. DMSO; * P <0.05 DAB+GSK vs. DAB.

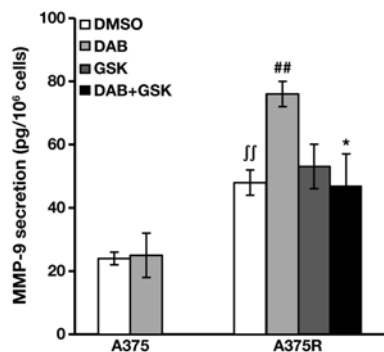


Figure 7. GSK2126458A abrogates dabrafenib-induced stimulation of MMP-9 secretion in A375R cells. A375 cells were cultured with DMSO alone or 100 nM dabrafenib (DAB), whereas A375R were cultured with DMSO alone, 100 nM dabrafenib, 20 nM GSK2126458A (GSK) or a combination of the two drugs. After 48 h of drug exposure, culture supernatants were collected and the amount of MMP-9 determined by ELISA. Each value represents the arithmetic mean \pm SEM of four independent experiments. ## P <0.01 A375R/DAB vs. A375R/DMSO; * P <0.05 A375R/DAB+GSK vs. A375R/DAB; \ddagger P <0.01 A375R/DMSO vs. A375/DMSO.

by ~70%, either alone or in combination with dabrafenib (Fig. 5B). Moreover, a 48 h-exposure to 20 nM GSK2126458A completely abrogated AKT phosphorylation in both DMSO-treated and dabrafenib-treated A375R cells (Fig. 5C) without affecting cell viability (data not shown).

A375R cells were exposed to DMSO, 20 nM GSK2126458A, 100 nM dabrafenib or a combination of the two drugs for 48 h and then tested for ECM invasion and VEGF-A secretion. Invasiveness of A375R cells was not substantially affected by GSK2126458A alone (Fig. 6A). However, the PI3K/mTOR inhibitor abrogated dabrafenib-induced stimulation of ECM invasion (Fig. 6A). In agreement with the invasion assays, VEGF-A secretion was not significantly altered in A375R exposed to GSK2126458A alone (Fig. 6B). On the other hand, the PI3K/mTOR inhibitor was able to completely counteract the upregulation of VEGF-A release caused by the BRAFi (Fig. 6B).

The PI3K/mTOR inhibitor GSK2126458A impairs MMP-9 secretion induced by dabrafenib in A375R cells. MMPs, a

large family of proteolytic enzymes able to degrade the ECM, play a critical role in many physiological processes and are also implicated in tumor angiogenesis and metastatic spreading of cancer cells (reviewed in refs. 44,45). In melanoma, increased expression of MMP-1, MMP-2 and MMP-9 has been associated with an invasive phenotype (46). Moreover, VEGF-A has been reported to upregulate the expression of certain MMPs, including MMP-9, in endothelial cells and ovarian cancer cells (47,48).

We, therefore, determined MMP-9 levels in culture supernatants of A375 and A375R cells, treated with DMSO or 100 nM dabrafenib for 48 h. In A375R cells, MMP-9 secretion was also evaluated after treatment with 20 nM GSK2126458A, alone or in combination with dabrafenib.

The results illustrated in Fig. 7 show that A375 cells secreted low amounts of MMP-9, which were not affected by dabrafenib treatment. A375R displayed levels of MMP-9 secretion 2-fold higher than those detected in A375 cells. Moreover, in the resistant subline the release of MMP-9 was significantly stimulated by dabrafenib. Similarly to the results obtained with VEGF-A, GSK2126458A alone did not alter basal MMP-9 secretion in A375R cells, but abrogated the upregulation caused by dabrafenib treatment (Fig. 7).

Discussion

In the present study we describe for the first time the effects of dabrafenib on the invasive capacity of BRAF-mutant melanoma cells sensitive or with acquired resistance to the antiproliferative activity of this BRAFi, as well as the modulating effect of GSK2126458A, a dual PI3K/mTOR inhibitor, on the responses of the resistant cells to dabrafenib.

The invasive behavior of melanoma cells with acquired resistance to dabrafenib, the only BRAFi presently approved for melanoma therapy beside vemurafenib, has not been investigated so far. However, increased invasiveness of melanoma cells with secondary resistance to the BRAFi PLX4720 (18,22), SB590885 (20), or vemurafenib (21) has been previously reported and linked to hyperactivation of the SRC family kinase-STAT3 signaling pathway (18,20), upregulation of several gene products involved in the metastatic process (21),

or increased expression of IL-8 (22). Our results showing that the dabrafenib-resistant cell line A375R displayed higher invasive capacity and higher production of VEGF-A and MMP-9 as compared with the drug-sensitive parental cell line A375 are consistent with these previous findings and provide further insight into melanoma biological characteristics associated with acquired resistance to BRAFi. Notably, in A375 cells exposure to dabrafenib impaired invasiveness, VEGFR-2 expression and VEGF-A secretion, and did not affect MMP-9 release. In contrast, in A375R cells treatment with dabrafenib caused an increase of the invasive capacity, as well as of VEGF-A and MMP-9 release, and did not substantially alter VEGFR-2 expression. These data point out that cells with acquired resistance to dabrafenib, not only are more invasive than the sensitive parental cells, but also respond to the drug with a further increase of their metastatic potential.

In the present study, we focused our investigation on the possible mechanisms underlying the effects of dabrafenib on the invasive capacity of the resistant cells, and on the possibility to modulate those effects. Indeed, there are some contrasting data regarding the clinical outcome that could be achieved by continuing BRAFi monotherapy in patients who had developed secondary resistance. Carlino *et al* (49) showed that melanoma cells with *in vitro* or *in vivo* acquired resistance to dabrafenib still responded to the BRAFi with reduced proliferation, even though they did not undergo apoptosis. These authors also reported a rapid acceleration of the disease upon dabrafenib suspension in a melanoma patient who had been treated with the drug for about six additional months after progression. Accordingly, clinical benefit of continuing BRAFi beyond RECIST disease progression was demonstrated in a large group of melanoma patients by Chan *et al* (50). In contrast, a preclinical study conducted with two different primary human melanoma xenograft models demonstrated that tumors with acquired resistance to vemurafenib were dependent on this drug for continuous proliferation, and that withdrawal of vemurafenib caused regression of established drug-resistant tumors (51). Furthermore, Hartsough *et al* (52) showed that melanoma cells with acquired resistance to PLX4720 due to the expression of mutant BRAF splice variants grow more efficiently, both *in vitro* and *in vivo*, in the presence of PLX4720 than in the absence of the inhibitor. Although randomized prospective trials are necessary to establish the role that prolonged BRAF inhibition may have in patients undergoing disease progression, the findings that dabrafenib stimulated ECM invasion and VEGF-A and MMP-9 production in A375R cells, cast, at least, a note of caution for the continuation of BRAFi monotherapy beyond progression. *In vivo* release of VEGF-A by melanoma cells can promote tumor growth and metastasis by both paracrine and autocrine mechanisms, namely supporting angiogenesis and stimulating melanoma cell proliferation, migration and invasion (35,40-42). Accordingly, elevated expression of VEGF-A and its receptors has been associated with melanoma progression (53). Moreover, MMP-9 is an important regulator of tumor angiogenesis and vasculogenesis, and has also been implicated in the formation of the metastatic niche (44). Although continuing single agent dabrafenib beyond progression could suppress the growth of those cells that within a heterogeneous tumor cell population have remained sensitive to the drug, it

might accelerate the metastatic spreading of the drug-resistant cells. A better clinical benefit might be achieved by combining dabrafenib with a drug able to counteract the stimulating effects of the BRAFi on melanoma cell invasiveness.

In the present study, we show that dabrafenib-induced stimulation of A375R invasive capacity was markedly inhibited in the presence of bevacizumab, a therapeutic anti-VEGF-A mAb which prevents the interaction of the angiogenic factor with its receptors VEGFR-1 and VEGFR-2 (38,39). This finding supports the hypothesis that dabrafenib-induced stimulation of ECM invasion in A375R cells mainly relies on drug-mediated upregulation of VEGF-A secretion and the subsequent engagement of signaling pathways regulating melanoma cell invasiveness. In this regard, the PI3K/AKT/mTOR signaling cascade, which is one of the signaling pathways activated upon binding of VEGF-A to VEGFR-1 and VEGFR-2 (40), appears to play a crucial role. Indeed, AKT1 silencing by specific siRNAs abrogated the stimulating effect of dabrafenib on A375R cell invasive capacity. Moreover, when A375R cells were treated with dabrafenib in combination with GSK2126458A, phosphorylation of AKT was entirely suppressed and this molecular event was accompanied by abrogation of dabrafenib-induced stimulation of ECM invasion and of VEGF-A and MMP-9 production. Several studies have demonstrated that co-targeting the PI3K/AKT/mTOR is an effective strategy to reverse acquired resistance to the growth suppressive effects of BRAFi (43,54-58). Our data, showing that A375R cells were highly responsive to GSK2126458A, as a single agent or in combination with dabrafenib, are consistent with those studies, and show that inhibition of the PI3K/AKT/mTOR pathway can also efficiently counteract dabrafenib-induced stimulation of the invasive capacity of melanoma cells with required resistance to this agent. Of note, neither bevacizumab, nor anti-AKT1 siRNA were able to impair basal invasiveness of A375R. Moreover, GSK2126458A alone, at the concentration selected for the combination with dabrafenib, did not alter basal ECM invasion and VEGF-A and MMP-9 secretion in A375R cells even if efficiently impaired AKT phosphorylation. Taken together, these data suggest that the higher basal invasive capacity displayed by A375R cells does not depend on VEGF-A engagement of its cognate RTKs or on AKT hyperactivation observed in these cells. It is possible that one or more mechanisms previously described to be involved in the increased aggressiveness of melanoma cells resistant to other BRAFi also underlie the elevated invasive behavior of the dabrafenib-resistant cell line generated in the present study. However, further investigations are required to address this issue.

In conclusion, our results demonstrate that dabrafenib effectively impairs the metastatic potential of BRAF-mutant melanoma cells sensitive to the antiproliferative activity of the drug. They also provide evidence that melanoma cells with acquired resistance to dabrafenib possess a more invasive phenotype which is further stimulated by exposure to the inhibitor via upregulation of VEGF-A secretion and activation of the PI3K/AKT/mTOR signaling pathway. Our data suggest that in patients progressing on dabrafenib therapy, a regimen combining the BRAFi with bevacizumab or with an inhibitor of the PI3K/AKT/mTOR pathway might provide a clinical benefit higher than that of extended BRAFi monotherapy. Notably, the

combination of vemurafenib and cobimetinib (MEK inhibitor) with or without bevacizumab is being testing in a randomized phase 2 clinical trial in patients with unresectable or metastatic BRAF-mutant melanoma (NCT01495988; <https://www.clinicaltrials.gov/>). A phase II clinical study is also ongoing to assess the efficacy and safety of the combination of LGX818 (BRAFi) with the PI3K inhibitor BKM120, or other targeted agents, in patients with unresectable or metastatic BRAF-mutant melanoma after relapse on LGX818 (NCT01820364; <https://www.clinicaltrials.gov/>).

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