Targeting the Sphingosine 1-Phosphate Axis Exerts Potent Antitumor Activity in BRAFi-Resistant Melanomas

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Abstract

BRAF inhibitors (BRAFi) are used to treat patients with melanoma harboring the V600E mutation. However, resistance to BRAFi is inevitable. Here, we identified sphingosine 1-phosphate (S1P) receptors as regulators of BRAFV600E mutant melanoma cell-autonomous resistance to BRAFi. Moreover, our results reveal a distinct sphingolipid profile, that is, a tendency for increased very long-chain ceramide species, in the plasma of patients with melanoma who achieve a response to BRAFi therapy as compared with patients with progressive disease. Treatment with BRAFi resulted in a strong decrease in S1PR1/3 expression in sensitive but not in resistant cells. Genetic and pharmacologic interventions, that increase ceramide/S1P ratio, down-regulated S1PR expression and blocked BRAFi-resistant melanoma cell growth. This effect was associated with a decreased expression of MITF and Bcl-2. Moreover, the BH3 mimetic ABT-737 improved the antitumor activity of approaches targeting S1P-metabolizing enzymes in BRAFi-resistant melanoma cells. Collectively, our findings indicate that targeting the S1P/S1PR axis could provide effective therapeutic options for patients with melanoma who relapse after BRAFi therapy.

Introduction

Changes in lipid profiles are often associated with an altered metabolic phenotype in tumor cells, which has been recognized as a hallmark of cancers (1). Therefore, lipid metabolism can be exploited to develop therapeutic strategies to treat cancer. We recently showed that sphingolipid (SL) metabolism is strongly altered in cutaneous melanoma in favor of metabolites that promote tumor progression (2–4). SLs are a class of lipids, which regulate key biological processes underlying cancer development including cell death, proliferation, migration, and tumor stroma remodeling (5). Both the expression and activity of several SL-metabolizing enzymes are dysregulated in melanoma, limiting the accumulation of the anti-oncometabolite ceramide and, conversely, facilitating the production of the oncometabolite sphingosine 1-phosphate (S1P; ref. 6). Moreover, in preclinical melanoma models, a S1P-neutralizing antibody could impair tumor angiogenesis (7) and lung metastasis (8).

Melanoma is the deadliest form of skin cancer and one of the most rapidly growing cancers worldwide (9). The BRAF inhibitors (BRAFi) vemurafenib and dabrafenib or the MEK inhibitors trametinib and cobimetinib achieve remarkable antitumor responses in patients with BRAFV600E melanoma with improvements in overall survival. However, initial tumor shrinkage is transient and the vast majority of patients with metastatic tumors develop secondary resistance (10). In addition, about 20% to 40% of patients exhibit primary resistance to BRAFi (11).

Interestingly, dysregulations in key signaling pathways of cell metabolism have been associated to melanoma cell-autonomous resistance to BRAFi. For instance, alterations in the PI3K–AKT–mTORC (12) or MITF–PGC-1α (13) pathways can lead to resistance of melanoma cells to vemurafenib. Dysregulation of SL metabolism has been reported to modulate resistance to conventional chemotherapy in several types of cancer. Indeed, increasing the ceramide/S1P ratio by sphingosine kinase 1 (SPHK1)–specific siRNA can sensitize cancer cells to chemotherapies (14). Moreover, high expression levels of SPHK1 correlate with reduced cumulative disease-specific survival in tamoxifen-treated patients with ER-positive breast cancer (15). Elevated SPHK1 expression has also been associated with resistance of non–small cell lung cancer cells to docetaxel or doxorubicin (16) as well as chronic...
myeloid leukemia cells to imatinib (17). Despite an altered metabolism in melanoma cells, the role of SLs in the resistance of tumor cells to BRAFi is currently unknown.

S1P, which is generated either by SPPH1 or SPPH2, is the natural ligand of a family of five specific G protein–coupled receptors, termed S1P Rs. These receptors play a critical role in chemoresistance of different cancers. For instance, tamoxifen resistance is associated with high expression of S1PR1 and S1PR3 in ER-positive breast cancer patients (15). Moreover, FTY720, a structural analog of sphingosine that inhibits S1PR1 signaling and induces proteasomal degradation of SPPH1, reduces resistance of prostate cancer cells to camptothecin (18, 19).

In this study, we used bioinformatic and lipidomic approaches to analyze how SLs are related to the human melanoma cell-autonomous resistance to BRAFi. We also investigated how S1P-dependent signaling pathways are affected in resistant tumor cells, to identify novel therapeutic targets for patients who relapse on first-line targeted therapies.

Materials and Methods

Cell culture and generation of stable SPL-overexpressing cells

Human melanoma cell lines were grown either in RPMI (M249), DMEM (A375, WM1346, WM-266.4, and WM9), or MEM (SK-MEL28) medium supplemented with 7% (WM9) or 10% heat-inactivated FBS in the presence of 5% CO2 in a humidified atmosphere at 37°C. A375, SK-MEL28, and WM-266.4 cells were obtained from ATCC (LGC). WM1346 and WM9 cells were kindly provided by Dr. M. Herlyn (The Wistar Institute, Philadelphia, PA). All melanoma cell lines carry the BRAFV600E mutation, except WM1346 cells, which express the oncogenic N-RASQ61K mutation. PLX-resistant melanoma cells M249R (20), WM9R (21), and A375R (22) were generated by chronic PLX exposure of parental M249, WM9, and A375 cells, respectively. All three resistant sublines showed elevated levels of p-ERK1/2 upon PLX exposure. To guarantee cell line authenticity, melanoma cell lines were used for a limited number of passages and routinely tested for the expression of MelanA/MART1. Cell lines were tested for the absence of mycoplasma contamination by PCR once every 2 weeks.

A375-vector and A375-SPL cells were generated by transduction using an empty and a human SPL-encoding lentiviral pTRIP-DU3-CMV-MCS plasmid, respectively. Selection was started 48 hours after transfection using 1 µg/mL puromycin. WM9-vector and WM9-SPL were obtained after transfection with an empty pcDNA5/TO plasmid (Invitrogen) and a plasmid containing the cDNA encoding human SPL, respectively. Transfection was carried out using the Lipofectamine 2000 reagent (Invitrogen). Stable transfectants were then selected using 3 µg/mL blasticidin.

Quantitative RT-PCR

Nonconfluent cells were harvested, and RNA was extracted (RNasy Kit; Qiagen) according to the manufacturer’s protocol and treated with RNase-free DNase (Qiagen). RNA quality was assessed by automated gel electrophoresis (Experion; Bio-Rad). One microgram of RNA was reverse transcribed (SuperScript II; Invitrogen) and the cDNA used as a template for qPCR. The reactions were performed in duplicate on the StepOne instrument (Applied Biosystems) using SYBR Green PCR Kit and primer assay (Quantitect; Qiagen). Results were quantified using the StepOne system software. GAPDH and β-actin transcripts were analyzed for normalization.

Mass spectrometric analysis of cellular and plasma sphingolipids

Blood samples of patients with BRAFV600E-positive metastatic melanoma treated with vemurafenib or dabrafenib were obtained from the Institut Universitaire du Cancer de Toulouse-Oncopole. All patients gave written informed consent for blood acquisition according to an Institutional Review Board approved protocol. Blood was centrifuged (2,400 × g, 20 minutes, 4°C) to obtain plasma and then stored at −70°C until analysis. Lipidomic analyses were performed on 0.2 mL of plasma from six patients (median age at the diagnosis: 60-60 females), for whom clinical data and response to BRAFi were known. All patients received at least three treatment cycles (each cycle is 4 weeks) with BRAFi and were classified as “responders” when the tumor regressed or remained stable or “nonresponders” when progression of the tumor was observed according to RECIST criteria.

Plasma and melanoma cell SLs were analyzed after lipid extraction and quantified by ultra-performance liquid chromatography using an Agilent 1290 UPLC system coupled to a G6460 triple quadrupole spectrometer (Agilent Technologies; ref. 3).

Cell viability and flow cytometry

For cytotoxicity assays, cells were incubated with medium containing or not FBS and vemurafenib (PLX; Selleckchem), ABT-737 (A-779024.0)/ABT-793 (A-793440.4; Abbott Laboratories, Chicago, IL), C16-ceramide (Avanti Polar Lipids), FTY-720 (Selleckchem), SKI-1 (Enamine, Riga, Latvia), or WI46 (Sigma-Aldrich) for the indicated times at 37°C. Control conditions always included the same organic solvent at the same concentration as that used to dissolve the pharmacologic compound tested. Cell viability was assessed using the MTT assay (Euromedex).

For cytometry analyses, cells were treated or not with PLX (5 µmol/L) for 48 hours in medium containing 7% FBS in the absence or presence of 5 µmol/L SKI-1, JTE-013 (Sigma-Aldrich), WI46, or CAY-10444 (Cayman Chemical). Phosphatidylycerine externalization was evaluated after labeling with Annexin-V-FITC (250 ng/mL) and propidium iodide (12.5 µg/mL, AbCys) and analyzed using a BD LSRFortessa flow cytometer.

Western blot analyses

Equal amounts of proteins were electrophoresed on a 10% to 15% SDS-PAGE, transferred to a nitrocellulose membrane (Perkin-Elmer) and blotted with monoclonal anti-S1PR1, anti-S1PR3 (Epitomics), or polyclonal anti-S1PR2 (Abgent). Monoclonal anti-Bcl-2 (50E3), anti-MITF (DSG7V), anti-β-actin, and polyclonal anti-SPPH1 antibodies were from Cell Signaling Technology. Polyclonal anti-SPL was from Abcam. Proteins were detected using an ECL detection system (Bio-Rad).

Animal studies

All procedures and animal housing were approved by the ethical committee according to European legislation translated to French law as Décret 2013-118 on February 1, 2013. Five millions WM9 cells were intradermally injected into the flank of 6-week-old female nude mice (Janvier). When tumors reached a volume of 50 to 100 mm3, mice were randomly divided into three experimental groups (n = 6–10 per group). The control group received excipient/vehicle (10% DMSO/5% Cremophore/5%
Bioinformatics
Gene expression data for melanoma cell lines (log2 transformed) were downloaded from the Cancer Cell Line Encyclopedia (CCLE) cohort (https://genome-cancer.ucsc.edu/). In addition, the BRAFi response profile of 28 BRAFV600E-mutated melanoma cell lines was also analyzed (23).

**SIPR1, SIPR3, and SPHK1 mRNA levels were assessed in patients with metastatic melanoma of the Cancer Genome Atlas (TCGA) skin cutaneous melanoma (SKCM) cohort (n = 369).** Level 3 data were interpreted using gene transcription estimates as in RSEM normalized and centered log2 counts (https://genome-cancer.ucsc.edu/). Expression levels were correlated using Pearson product–moment correlation.

Statistical analyses
All statistical analyses were performed using GraphPad Prism 6 software. Results are expressed as mean ± SEM, and group comparisons were performed with an unpaired two-tailed Student t test for comparison of 2 groups, or one-way ANOVA followed by the post hoc Tukey test for comparison of experiments that consisted of ≥3 groups. The Mann–Whitney U test was used to test statistical significance of differences in mean tumor growth between independent groups after treatment. A P value less than 0.05 was considered statistically significant (*, P < 0.05; **, P < 0.01; ***, P < 0.001).

**Results**
Melanoma cell expression of SIPR correlates with resistance to BRAFi
To test whether SIP-dependent signaling pathways are related to the resistance of melanoma cells to BRAFi, we examined a series of BRAFV600E-mutant melanoma cell lines from the Cancer Cell Line Encyclopedia (CCLE) for which IC50 values, that is, drug concentrations causing 50% growth inhibition, of the selective BRAFi PLX-4720 (the closely related precursor of PLX-4032) were available (23). Bioinformatic analysis revealed that the levels of SIPR1 and SIPR3 transcripts positively correlate with resistance of melanoma cells to the BRAFi (Fig. 1A). In contrast, no significant relationship was detected between the expression of other SIPR, that is, SIPR2, SIPR24, and SIPR25, and the sensitivity of melanoma cells to the BRAFi.

To confirm these results, SIPR expression was monitored in BRAFi-resistant sublines derived from BRAFV600E-positive melanoma cells. M249R (Fig. 1B), WM98 (Fig. 1C), and A375S (Supplementary Fig. S1A) cells displayed strong resistance to PLX-4032 (PLX) compared with their PLX-sensitive parental counterparts. Of interest was the finding that whereas the expression of SIPR1 and, to a lesser extent, SIPR3 was reduced in parental melanoma cells upon PLX incubation, it was not affected in the corresponding PLX-resistant sublines treated or not with the inhibitor (Fig. 1D and E; Supplementary S1B). In contrast, the expression of SIPR2 was not significantly reduced in all PLX-treated melanoma cells. These results indicate that SIPR1 and SIPR3 expression remains unchanged in PLX-resistant cells upon exposure to PLX, and could represent a potential therapeutic target for overcoming acquired BRAFi resistance.

Bioinformatic analysis of an RNA-seq dataset obtained from a collection of 369 human metastatic melanomas (TCGA) revealed positive correlations between the mRNA expression of SIPR1 and SIPR3 as well as SPHK1 (Fig. 2A). In accordance with these observations, PLX strongly reduced SPHK1 expression (Fig. 2B) as well as SPHK1 enzymatic activity (Fig. 2C) in WM98 cells as compared with WM99 cells. Moreover, BRAFi treatment led to increased ceramide/SIP ratio (Fig. 2D, right) through the elevation of virtually all ceramide molecular species (Fig. 2D, left) in sensitive cells only. Of note, ceramides containing very long-chain fatty acids (i.e., C24) were the most abundant species.

To determine whether plasma SL concentrations were associated to the response of patients with melanoma to BRAFi, peripheral blood from 19 patients who received at least three cycles of BRAFi was collected for measurement of SLs by mass spectrometry. Among the six patients tested, three patients had progressive disease (nonresponders) and three patients exhibited a partial response (responders). Remarkably, plasma SL analysis revealed higher ceramide/SIP ratios in responders in comparison with nonresponders (Supplementary Fig. S2, right). Levels of very long-chain ceramides species showed a tendency to increase in responders, whereas no change in other ceramide species (Supplementary Fig. S2, left) was observed.

In addition, inhibition of SPHK1 enzymatic activity in BRAFi-resistant cells with the nonlipid SPHK inhibitor SKI-I (Fig. 3A; ref. 28), which increases the ceramide/SIP ratio (Fig. 3B) in BRAFi-resistant cells, was associated with increased apoptosis as compared with untreated cells or cells exposed to PLX (Fig. 3C). No difference was observed between SKI-I alone and the combination SKI-I and PLX in both BRAFi-sensitive and -resistant melanoma cells (Supplementary Fig. S3). To extend our observations in vivo, we next transplanted WM9 cells in the flanks of nude mice and monitored the formation of tumors upon injection of PLX or SKI-I. Although administration of PLX resulted in minimal changes in WM98 tumor growth as compared with WM99 (Fig. 3D), injection of SKI-I led to a significant decrease in the size of WM98 tumors (Fig. 3E).

The SIP lyase (SPL), which irreversibly degrades SIP, is underexpressed in human melanoma cells compared with healthy melanocytes (2). To validate our hypothesis that increasing the
Figure 1.
Differential effect of PLX on S1PR expression in BRAFi-sensitive and BRAFi-resistant cells. 

A, Correlation between relative S1PR expression and sensitivity of melanoma cells to PLX-4720. S1PR1-5 mRNA levels are displayed according to the IC50 values of PLX-4720 (μmol/L) in a panel of BRAFV600 melanoma cells (n = 28, Tukey box plot, Student t test).

B and C, Cell viability was assessed after 48 hours using the MTT test. Results are expressed as percentage of the value determined in the absence of the drug. Data are mean ± SEM of three independent experiments. Statistical differences relative to untreated cells are shown (Student t test).

D and E, Left: Expression of S1PR1, S1PR2, and S1PR3 was assessed by Western blot analysis after 24 hours. Right: Levels of S1PRs normalized to β-actin and relative to untreated cells (mean ± SEM of two to three independent experiments). Statistical differences relative to untreated cells are shown (Student t test).

F and G, WM9R cells were treated or not with PLX (5 μmol/L) for 24 or 48 hours in medium containing 7% FBS in the absence (none) or presence of W146, JTE-013, CAY10444, or FTY-720 (5 μmol/L). F, After 48 hours, cells were labeled with Annexin-V-FITC and propidium iodide, and analyzed by flow cytometry. Percentages (mean ± SEM of three independent experiments) of Annexin-V-positive cells are indicated. G, Cell viability was assessed using the MTT test. Results are expressed as percentage of the value determined at the corresponding time in the absence of the drug. Data are mean ± SEM of three independent experiments. Statistical differences relative to untreated cells are shown (Student t test). NS, not significant.
ceramide/S1P ratio could be an attractive therapeutic strategy for BRAFi-resistant melanoma cells, we next assessed cell viability of SPL-overexpressing WM9R cells following PLX exposure. Overexpression of SPL in WM9R cells (Fig. 3F), which resulted in a two-fold increase in ceramide/S1P ratio (Fig. 3G), significantly sensitized WM9R cells to PLX (Fig. 3H). Interestingly, treatment with exogenous C16-ceramide reduced cell viability of WM9R cells and the wild-type BRAF melanoma cell line WM1346, which is intrinsically resistant to BRAFi (Fig. 3I).

Given that inhibition of SPHK1 may result in decreased S1PR expression, as previously described in SPHK1 knocked-down MCF-7 breast cancer cells (29), S1PR expression was next assessed in cells incubated with SKI-I. Fig. 4A shows that treatment with SKI-I reduced S1PR1 and S1PR3 expression in both PLX-sensitive and -resistant WM9 cells. In contrast, S1PR2 expression was not altered. Of note, a marked decrease of S1PR1 expression was also observed in SPL-overexpressing WM9R cells upon PLX treatment (Fig. 4B).

Collectively, these data indicate that increasing the ceramide/S1P ratio impairs S1PR expression and exerts both in vitro and in vivo powerful antitumor activity in BRAFi-resistant melanoma cells in a cell-autonomous manner.

Downregulation of S1PR expression due to altered ceramide/ S1P ratio reduces MITF-mediated resistance to PLX

Previous studies have documented the role of ceramide (30) and S1P/S1PR axis (31) in regulating the expression of the key melanocytic transcription factor MITF. Importantly, it was recently shown that MITF expression contributes to BRAFi resistance (13, 32). To explore the molecular mechanisms underlying the role of S1PR in melanoma cell resistance to BRAFi, we evaluated MITF expression in several melanoma cells upon SKI-I treatment. In line with previous reports, MITF expression was not reduced in PLX-resistant melanoma cells upon PLX treatment (Fig. 5A, right) compared with their sensitive counterparts (Fig. 5A, left). In contrast, incubation with SKI-I reduced the transcript levels of MITF and one of its target gene TYR, in both WM9 cell lines. Similar results were obtained by analyzing MITF protein expression (Fig. 5B). Of interest, MITF levels were partially restored in WM9 cells upon incubation with exogenous S1P. Moreover, MITF expression was no longer reduced in S1PR1-overexpressing WM9S cells following PLX exposure as compared with WM9R cells expressing an empty vector. PLX-induced cell death was abolished in these conditions (Supplementary Fig. S4A–S4C). A reduced expression of MITF upon SKI-I treatment was also observed in other melanoma cell lines carrying the BRAFVal600E mutation (Fig. 5C). These modifications were accompanied by a decrease in cell viability of PLX-sensitive (WM9R, M249R, and SK-MEL28) as well as PLX-resistant (WM9R and M249R) cells (Fig. 5D). In accordance, overexpression of SPL, which leads to decreased S1PR1 expression (Fig. 4B), resulted in a reduction of MITF levels (Fig. 5E) and sensitization of melanoma cells to BRAFi (Fig. 5F).

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Figure 3.
Increasing the ceramide/S1P ratio impairs the growth of PLX-resistant WM9 cells. A–C, WM9R cells were treated or not with 5 μmol/L PLX or SKI-I for 24 (A, B) or 48 (C) hours in medium containing 7% FBS. A, SPHK1 enzymatic activity was determined. B, Cellular lipids were extracted, quantified by mass spectrometry and normalized to protein content. Ratios of total ceramide/S1P are presented and are representative of three different experiments. C, Cells were labeled with Annexin-V-FITC and propidium iodide, and analyzed by flow cytometry. Percentages (mean ± SEM of two independent experiments) of Annexin-V-positive cells are indicated. Statistical differences relative to untreated WM9R cells are indicated. Statistical differences relative to untreated WM9R cells were determined (one-way ANOVA with post hoc Tukey test). D and E, Four days after intradermal injection of 5 10⁵ WM9 cells, melanoma-bearing mice (n = 6–10 per group) were treated with vehicle, SKI-I or PLX for 2 weeks and final tumor volumes were monitored. Statistical differences relative to vehicle treated WM9S- (D) or WM9R- (E) bearing mice were determined using the Mann–Whitney U test. F–H, Control (Vector) or SPL-overexpressing WM9R melanoma cells were treated (H) or not (F, G) with the indicated concentrations of PLX for 48 hours in medium containing 7% FBS. F, SPL expression was determined by Western blot analysis. G, Cellular lipids were extracted, quantified by mass spectrometry, and normalized to protein content. Ratios of total ceramide/S1P are presented and are representative of three different experiments. H, Cell viability was assessed using the MTT test. Results are expressed as percentage of the value determined in the absence of the drug (DMSO) and are mean ± SEM of three independent experiments. I, Cell viability was monitored on PLX-resistant WM9 or WM1346 melanoma cells treated or not with C₁₆-ceramide at the indicated concentrations for 48 hours. C₁₆-ceramide was dissolved in dodecane/ethanol (dod-EtOH; 2:98, v/v) and added to the medium without FBS. Results are expressed as percentage of the value determined in the absence of the drug. Statistical differences were determined using Student’s t test.
Downregulation of S1PR expression due to altered ceramide/S1P ratio potentiates Bcl-2 inhibitor-induced apoptosis

Because MITF directly regulates Bcl-2 (33), which contributes to melanoma cell resistance to BRAFi (34), we next assessed Bcl-2 expression in cells treated or not with SKI-I. As expected, upon PLX treatment WM9R cells (Fig. 6) or S1PR1-overexpressing WM9S cells (Supplementary Fig. S4C) did not exhibit a decrease in Bcl-2 mRNA (Fig. 6A) and protein (Fig. 6B; Supplementary S4C) upon PLX as compared with WM9S cells. In contrast, expression of Bcl-2 was significantly reduced in WM9R cells incubated with SKI-I (Fig. 6A and B) whereas that of Bcl2A1, Bcl-xL, and Mcl-1 did not differ significantly (Fig. 6A). To ensure Bcl-2 could regulate apoptosis in melanoma upon S1PR downregulation, we next examined cell death following cotreatment with the BH3-mimetic ABT-737 (35) and SKI-I. As shown in Fig. 6C, whereas ABT-737 did not sensitize WM9R cells to PLX, it enhanced apoptosis induced by SKI-I as compared with untreated cells or cells incubated with the inactive enantiomer ABT-793 (35). This result was confirmed in SPL-overexpressing A375 cells, in which ABT-737 displayed strong synergistic lethality when combined with SPL overexpression whereas ABT-737 alone was not cytotoxic (Fig. 6D). Taken together, these data suggest that combination of S1PR downregulation and BH3-mimetics may represent a promising strategy for treating PLX-resistant melanomas.

Discussion

SLs are pleiotropic lipids, which are integrated into the wider cellular metabolic network (36). Altered SL metabolism contributes to cancer progression and represents an exploitable target for the development of new treatments. A BRAFV600E-dependent increase of S1P levels was reported in melanoma cells (4, 37); however, the effect of therapies targeting the S1P axis on the response of melanoma cells to BRAFi has never been investigated.

Here, we describe, for the first time, changes in the plasma SL profile of patients with melanoma with progressive disease as compared with patients who respond to BRAFi therapy. In particular, our data reveal a tendency for increased plasma C24-ceramide in responders versus nonresponders. To further determine the relationship between SL changes and timing of response or progression during BRAFi therapy, measurement of plasma SLs should be carried out on larger patient cohorts, before treatment and more frequently during the treatment. Previous studies reported that changes in circulating SL levels may serve as novel biomarkers for monitoring response to therapy. For instance,

Figure 4. Downregulation of S1PR expression by SKI-I or overexpression of SPL. WM9S/R (A) or SPL-overexpressing WM9R (B) melanoma cells were treated or not with 5 μmol/L of PLX or SKI-I for 24 hours in medium containing 7% FBS. Left: Expression of S1PR1, S1PR2 and S1PR3 was assessed by Western blot analysis. Right: Levels of S1PRs normalized to β-actin and relative to nontreated cells (mean ± SEM of three independent experiments). Statistical differences relative to nontreated cells are shown (Student t test).
Figure 5.
Targeting of S1P/S1PR axis inhibits MITF expression and exerts antitumor effects on melanoma cells. A, WM9 cells were treated or not with 5 μmol/L PLX for 24 hours in medium containing 7% FBS in the absence or presence of 5 μmol/L SKI-I. Relative mRNA levels are depicted for MITF and TYR. Results are mean ± SEM of three independent experiments. B and C, Melanoma cell lines were incubated or not with 5 μmol/L SKI-I for 24 hours in medium containing FBS in the presence or absence of 2 μmol/L S1P. Expression of MITF was assessed by Western blot analysis. D, Melanoma cells were incubated or not with PLX (5 μmol/L) or the indicated concentrations of SKI-I for 48 hours. Cell viability was assessed using the MTT test. Results are expressed as percentage of the value determined at the corresponding time in the absence of the drug (DMSO). Data are mean ± SEM of two independent experiments. Statistical differences relative to untreated cells were determined (Student t test). E and F, Control (Vector) or SPL-overexpressing A375 or WM266.4 melanoma cells were incubated or not with 5 μmol/L (E) or the indicated concentrations (F) of PLX for 24 (E) or 48 (F) hours. E, Left: Expression of SPL and MITF was assessed by Western blot analysis. Right: Levels of proteins normalized to β-actin and relative to non-treated cells. F, Cell viability was assessed using the MTT test. Results are expressed as percentage of the value determined in the absence of the drug. Data are means ± SEM of three independent experiments. Statistical differences relative to nontreated control melanoma cells were determined (Student t test).
increased serum C18-ceramide levels were described in head and neck squamous cell carcinoma patients responding to a therapy combining gemcitabine with doxorubicin in comparison with nonresponders (38).

Figure 6. Combined targeting of S1P/S1PR axis and Bcl-2 synergistically reduces PLX-resistant melanoma cell viability. A–C, WM9 cells were treated or not with PLX (5 μmol/L) for 24 (A, B) or 48 (C) hours in medium containing 7% FBS in the absence or presence of 5 μmol/L SKI-I, ABT-793, or ABT-737. A, Relative mRNA level is depicted for Bcl-2, Bcl2A1, Bcl-xL, and Mcl-1. Results represent mean ± SEM of three independent experiments. Statistical differences relative to untreated cells were determined (Student t test). B, Left: Expression of MITF and Bcl-2 was assessed by Western blot analysis. Right: Levels of proteins normalized to β-actin and relative to untreated cells. C, Cells were labeled with Annexin-V-FITC and propidium iodide, and analyzed by flow cytometry. Percentages (mean ± SEM of three independent experiments) of Annexin-V-positive cells are indicated. Statistical differences were determined using one-way ANOVA with post hoc Tukey test. D, Control (Vector) or SPL-overexpressing A375 melanoma cells were incubated or not with the indicated concentrations of ABT-793 or ABT-737 for 48 hours. Cell viability was assessed using the MTT test. Results are expressed as percentage of the value determined in the absence of the drug. Data are mean ± SEM of two independent experiments.

Our data also show that sensitivity of BRAFV600-mutated melanoma cells to PLX is negatively correlated with S1PR1/3 expression. S1PR expression was not affected by PLX in cells that exhibit acquired resistance to the BRAFi compared with their sensitive
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Counterparts. Accordingly, PLX-resistant melanoma cells could be re-sensitized to BRAFi by cotreatment with S1PR1 or S1PR3 antagonists (Fig. 7). Of note, expression of those receptors is also strongly increased in chemoresistant breast cancer cells (15), whereas that of other S1PR subtypes is rather reduced. Moreover, S1PR1 expression is also associated to unfavorable clinicopathologic features in nonmuscle invasive urothelial carcinoma (39). FTY720, which acts as a functional antagonist of S1PR1 by inducing its internalization and degradation (40), was reported to stimulate apoptosis in drug-resistant multiple myeloma cells (41) and sensitize radio-resistant breast cancer cells (42). However, FTY720 could exert its antitumoral effects through indirect inhibition of SPHK1 (43). The SKI-I inhibitor induces the proteasomal degradation of SPHK1 and increases the ceramide/SIP ratio and apoptosis (44). Our results show that elevation of ceramide/SIP ratio, induced by SKI-I treatment or SPL overexpression, causes a decrease in S1PR expression in melanoma cells (Fig. 7). The molecular mechanism by which the ceramide/SIP ratio influences S1PR expression is currently under investigation. siRNA knockdown of SPHK1 was reported to reduce S1PR expression and signaling in S1P-treated breast cancer cells. In addition, S1P binding to S1PR3 activates SPHK1, suggesting that SPHK1, ceramide/SIP ratio, and S1PR function in an amplification loop to promote cancer progression (29).

Although little is known about the regulation of S1PR3 in cancer, S1PR1 has been extensively studied. The oncogenic K-Ras mutant has recently been shown to stimulate S1PR3 expression through the binding of the transcription factor SMAD3 to the promoter region of S1PR3 in human lung adenocarcinomas (45). S1PR1 is transcriptionally regulated by the STAT3 and reciprocally, S1PR1 participates in a positive feedback loop with STAT3 pathway in cancer, thereby promoting tumor progression and metastasis (46). Consistent with this notion, FTY720, which decreases the expression of SPHK1 and S1PR1, could inhibit the NF-kB/IL-6/STAT3 amplification cascade linked to the development of colitis-associated cancer (27). siRNA knockdown of SPHK1 also led to a significant reduction of leptin-induced STAT3 phosphorylation in ER-negative breast cancer cells (47). In addition, the loss of spl elicits STAT3 activation (48). Importantly, STAT3 activation signaling is a negative prognostic factor in human melanoma (49) and inhibition of STAT3 signaling reduces inducible melanoma cell growth (50). Future studies on the relationship between the intracellular ceramide/SIP ratio controlled by SPHK1 and S1PR1/3, and STAT3 may reveal additional insights into the molecular mechanism underlying the acquired resistance of melanoma cells to BRAFi.

We also provide evidence that downregulation of S1PR expression mitigates MITF-mediated resistance to PLX (Fig. 7). A growing body of evidence indicates that a fraction of patients relapse with tumors having greatly increased MITF (51). Consequently, targeting MITF, which plays a key role in the initial phases of drug-induced tolerance, can sensitize acquired resistant cells to BRAF and MEK inhibitors (32). A previous study has shown that FTY720 downregulates MITF and tyrosinase expression without ERK activation (52). In contrast, topical application of a purified SL fraction, containing some sphingoid bases, to age-onset gray-haired C57BL/6 mouse skin, induced the re-growth of black hair with differentiated melanocytes expressing MITF (6, 53). In line with these observations, we show that SIP itself is able to activate MITF expression when added to melanoma cells (Fig. 6B). However, MITF expression is also increased in B16 murine melanoma at late stages of progression, i.e., when ceramide levels are reduced by downregulation of acid sphingomyelinase (30). Here, we describe, for the first time, that the SIP axis represents a critical regulator of the MITF-mediated signaling pathways that control melanoma cell-autonomous resistance to BRAFi. However, the SIP axis is also a key player of the tumor microenvironment remodeling in melanoma, as it promotes angiogenesis (7), stromagenesis (4) and the macrophage M2 phenotype (54). In particular, it elicits the release of TGF-β from melanoma cells, thus promoting the recruitment and phenotypic shift of the tumor macrophages (54) as well as the differentiation of quiescent fibroblasts into activated fibroblasts (4). Interestingly, activated fibroblasts and macrophages were shown to provide resistance of melanoma cells to targeted therapies by secreting HGF (55) or TNF (56), respectively. Whether the “inside-out” signaling of SIP is associated to these phenomena is of particular interest and is currently under investigation.

An important hallmark of melanoma is resistance to apoptosis, which is mediated by low levels of B13-only proteins and/or high levels of Bcl-2-like prosurvival proteins (57). Here, we demonstrate that the ceramide/SIP ratio controls Bcl-2 levels without significantly modifying Bcl2-A1 expression, both being regulated by MITF. Consistent with these results, high levels of SPHK1 activity were associated with high expression of Bcl-2 and reported to counteract Fas and ceramide-mediated cell death in A375 human melanoma cells (58). Moreover, we show that strategies leading to S1PR downregulation cooperate with ABT-737 to induce apoptosis in PLX-resistant melanoma cells. This combination was more effective than the combination of PLX and ABT-737. Combining Bcl-2 inhibitors and BRAFi led to higher levels of caspase-dependent apoptosis and reduced tumor growth in vivo.
more efficiently than monotherapy (34). However, this combination was not always effective against cell lines established from patients with acquired resistance to vemurafenib (59). Collectively, our results propose an alternative combination drug therapy based on S1PR downregulation in order to exert potent antitumor activity against BRAF-resistant melanomas.

Disclosure of Potential Conflicts of Interest

N. Meyer has received speaker bureau honoraria from Roche, BMS, MSD, Novartis. Pierre Fabre is a consultant/advisory board member of Roche, BMS, MSD, Novartis, and Pierre Fabre: French pharmaceutical laboratory. No potential conflicts of interest were disclosed by the other authors.

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Targeting the Sphingosine 1-Phosphate Axis Exerts Potent Antitumor Activity in BRAFi-Resistant Melanomas

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