

Conjunctival Melanoma Targeted Therapy: MAPK and PI3K/mTOR Pathways Inhibition

Ikram El Zaoui,¹ Maya Bucher,² Donata Rimoldi,³ Michael Nicolas,⁴ Gurkan Kaya,⁵ Rosanna Pescini Gobert,⁶ Nicola Bedoni,¹ Ann Schalenbourg,⁴ Ezziat Sakina,⁴ Leonidas Zografos,⁴ Serge Leyvraz,⁷ Nicolo Riggi,⁶ Carlo Rivolta,^{1,8} and Alexandre P. Moulin⁴

¹Department of Computational Biology, Unit of Medical Genetics, Lausanne University, Lausanne, Switzerland

²Dermatology Unit, CHUV, Lausanne University, Lausanne, Switzerland

³Ludwig Institute for Cancer Research, Epalinges, Switzerland

⁴Jules-Gonin Eye Hospital, Lausanne University, FAA, Lausanne, Switzerland

⁵Dermatology and Venerology Division, Dermatopathology Laboratory, Geneva University Hospital, Geneva, Switzerland

⁶Experimental Pathology, Lausanne University Pathology Institute, Lausanne, Switzerland

⁷Charité Cancer Comprehensive Center, Charité - Universitätsmedizin Berlin, Berlin, Germany

⁸Department of Genetics and Genome Biology, University of Leicester, Leicester, United Kingdom

Correspondence: Alexandre P. Moulin, Jules-Gonin Eye Hospital, Lausanne University, FAA, Avenue de France 15, Lausanne 1004, Switzerland;

Alexandre.Moulin@fa2.ch.

IEZ and MB contributed equally to the work presented here and should therefore be regarded as equivalent authors.

Submitted: December 22, 2018

Accepted: May 3, 2019

Citation: El Zaoui I, Bucher M, Rimoldi D, et al. Conjunctival melanoma targeted therapy: MAPK and PI3K/mTOR pathways inhibition. *Invest Ophthalmol Vis Sci.* 2019;60:2764-2772. <https://doi.org/10.1167/iovs.18-26508>

PURPOSE. To analyze the activity of mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinases/mechanistic target of rapamycin (PI3K/mTOR) pathways in benign and malignant conjunctival melanocytic proliferations and explore whether specific inhibitors can suppress growth of conjunctival melanoma (CJM) cells.

METHODS. The presence of a BRAF V600E mutation and activation of ERK, MEK, S6, and AKT were assessed with immunohistochemistry in 35 conjunctival nevi and 31 melanomas. Three CJM cell lines were used: CRMM1, carrying the BRAF V600E mutation; CRMM2, harboring the NRAS Q61L mutation; and T1527A, with a BRAF G466E mutation. WST-1 assays were performed with a BRAF inhibitor (vemurafenib), two MEK inhibitors (trametinib, selumetinib), a PI3K inhibitor (pictilisib), and a dual PI3K/mTOR inhibitor (dactolisib). The phosphorylation of ERK, MEK, and S6 were tested with western blots and apoptosis with cleaved caspase-3 immunostaining.

RESULTS. A BRAF V600E mutation was detected in 42.6% of nevi and in 35.5% of CJM. MEK and ERK activation were higher in CJM, occurring in 62.9% and 45.7% of the nevi and 90.3% and 96.8% of the CJM, respectively. There was also a significant increase in S6 activation in CJM (90.3%) compared with the nevi (20%). CRMM1 was sensitive to trametinib and the PI3K inhibitors but only marginally to vemurafenib. CRMM2 was moderately sensitive to pictilisib, whereas T1527A was resistant to all drugs tested.

CONCLUSIONS. The MAPK pathway activity in CJM is increased, not only as a consequence of the BRAF V600E mutation. Targeted therapy may be useful for patients with CJM, especially those with activating BRAF mutations, whereas NRAS-mutated melanomas are relatively resistant.

Keywords: melanoma, conjunctiva, mutations, MAPK pathway, BRAF

Conjunctival melanoma, whose incidence increased from 1990 to 1999 in the United States (0.46/million),¹ is associated with a high recurrences rate, ranging from 32% to 62%.^{2,3} The therapeutic management of metastatic conjunctival melanoma is challenging and metastatic death occurs in 9% to 35% of patients at 10 years postdiagnosis.⁴⁻⁶

Conjunctival and cutaneous melanomas not only share common clinical features, such as metastatic spread through the lymphatic system, but also share some molecular features. The mutation spectrum appears to be similar to cutaneous melanoma, with a majority of cytosine to thymine transitions (C>T)^{7,8} suggesting a high impact of UV light-induced damage. The high mutation load with ~90,000 mutations in the entire genome⁷ is also similar to skin melanoma⁹ and contrasts sharply with the low mutation load identified in uveal melanoma.¹⁰ Mutations commonly observed in cutaneous melanoma, such as

V600E in exon 15 of BRAF or Q61L in exon 3 of NRAS^{8,11} are also identified in conjunctival melanoma, with BRAF mutations being found in 29% to 35%^{12,13} and NRAS mutations in 18% of the cases.¹² More recently, NFI mutations could be identified in 33% of the patients.¹⁴

Numerous studies have assessed the importance of the mitogen-activated protein kinase (MAPK) pathway in cutaneous melanoma.¹⁵ Small-molecule inhibitors, binding to the active conformation of BRAF and blocking access to ATP, disrupt MAPK signaling, reduce proliferation in vitro, and induce tumor regression in vivo in BRAF^{V600E} melanomas.^{16,17} In patients with metastatic melanoma carrying the BRAF^{V600E} mutation, these inhibitors were associated with an impressive complete or partial tumor response.¹⁸⁻²⁰

Direct MEK targeting downstream of BRAF represents an alternative way to overcome tumor resistance in patients with



BRAF-mutated skin melanomas. Trametinib, an allosteric and highly selective MEK inhibitor, significantly improved progression-free survival and overall survival in patients with *BRAF* mutant metastatic melanoma.²¹

NRAS mutations, usually mutually exclusive with *BRAF* mutations, occur in 20% to 30% of skin melanomas and more commonly in older patients.^{11,22} The *RAS*^{Q61L} mutation impairs hydrolysis of guanosine-5'-triphosphate (GTP) to guanosine diphosphate (GDP) and the transition of *NRAS* from an active to an inactive state. Downstream single inhibition of the MAPK pathway through MEK inhibition,²² alone or in combination with phosphoinositide 3-kinases/mechanistic target of rapamycin (PI3K/mTOR) inhibition,²³ has been attempted in *NRAS*-mutated melanoma. Although in vitro MEK inhibitors appeared more successful than PI3K inhibitors, the combined use of MEK and PI3K reduced cell viability and tumor size in vivo models more efficiently.²⁴

The efficiency of direct *BRAF* inhibition in conjunctival melanoma (CJM) has been evaluated in vitro in two studies.^{25,26} The response to *BRAF* inhibition in advanced metastatic CJM has been variable, ranging from a partial response lasting 1 month²⁷ to significant tumor regression peaking after 4.5 months.²⁸ A complete regression for 38 months of an irradiated metastatic CJM treated with vemurafenib has also been documented.²⁹

We conducted a study to assess the activity of the MAPK and PI3K/mTOR pathways in conjunctival melanomas tissues in relation to the presence or absence of the oncogenic *BRAF*^{V600E} mutation. We also assessed in vitro the response of conjunctival melanoma cell lines containing defined *BRAF* and *NRAS* mutations to *BRAF*, MEK, PI3K, and dual PI3K/mTOR inhibitors.

MATERIALS AND METHODS

Patients

The clinical charts of patients treated for conjunctival melanoma or conjunctival nevi in Jules-Gonin Eye Hospital from 1998 until 2013 where formalin-fixed paraffin-embedded tissue was available were selected. The following clinical information was retrieved from the patients' charts: age, sex, tumor localization, treatment, recurrences, metastases, death, and length of follow-up. The study was approved by the Swiss Federal Department of Health (authorization 032.0003-48) as well as local ethic committee (protocol 340/15) and adhered to the tenets of the Declaration of Helsinki.

Tissues

Formalin-fixed paraffin-embedded tissues were retrieved from the archives of Jules-Gonin Eye Hospital Pathology Laboratory. Sections (5 µm) were cut, and hematoxylin-eosin stains were performed. The sections were incubated with the following anti-human antibodies: anti-phospho-MEK (Ser221, rabbit monoclonal; Cell Signaling, Danvers, MA, USA; dilution 1:200), anti-phospho-ERK (Thr202/Tyr204, rabbit monoclonal; Cell Signaling; dilution 1:600), anti-phospho-AKT (Ser473, rabbit monoclonal, dilution 1:50), and anti-phospho-S6 (Ser235/236, rabbit monoclonal; Cell Signaling; dilution 1:200). Positive controls were found in endothelial cells of conjunctival stroma and conjunctival epithelium for anti-phospho-S6, anti-phospho MEK, and ERK (Supplementary Fig. S1). For phospho-AKT, a phospho-AKT (Ser 473) slide with LNCaP cells treated with or without a PI3K inhibitor, LY294002, was used. A streptavidin/biotin detection method with 3,3'-diaminobenzidine tetrachloride or 3-amino 9-ethyl-

carbazole was used for signal detection (Dako Envision System/HRP Dual Link; Dako Agilent, Santa Clara, CA, USA).

The *BRAF*^{V600E} mutation was assessed in the tissues using a *BRAF*^{V600E}-specific monoclonal antibody (clone VE1, mouse monoclonal; Ventana Medical Systems, Oro Valley, AZ, USA) with a BenchMark Ultra platform (Roche Diagnostics, Rotkreuz, Switzerland).

The proportion of positively stained cells was scored in the following manner: 0% to 10% positive cells: score 1; >10% to 50%: score 2; and >50% to 100%: score 3. Three independent observers (APM, MB, and MN) evaluated the immunostained slides and discordant cases were reviewed simultaneously to reach a concordant agreement.

There was a concordance of 75.8%, 88.6%, 95.4%, 93.9%, and 93.9% among the observers and the evaluation of pMEK, pERK, cytoplasmic pAKT, nuclear pAKT, and pS6, respectively.

Cells

Conjunctival melanoma cell lines CRMM1 and CRMM2, established by Gordon Nareyeck, University of Essen, Germany, were kindly provided by Martine Jager, University of Leiden with the authorization of use granted from Gordon Nareyeck. The cells were cultured in F-12K nutrient mixture, with Kaighn's modification, containing L-glutamine (Gibco, Life Technologies, Carlsbad, CA, USA), 10% heat-inactivated fetal bovine serum (Greiner Bio-one, Kremsmuenster, Austria) and 2% penicillin/streptomycin (Gibco). T1527A was established in our laboratories (Jules-Gonin Eye Hospital and Ludwig Center for Cancer Research, Switzerland) from a perilimbal conjunctival melanoma extending onto the cornea in a 65-year-old man. These cells were grown in a mixture of 50% of RPMI 1640 modified media (Gibco) and 50% Dulbecco's modified Eagle's medium (DMEM) F-12 (nutrient mixture with GlutaMAX; Gibco) supplemented with 10% fetal bovine serum (Greiner Bio-one) and 6 mM *N*-2-hydroxyethyl-1-piperazine-*N'*-2-ethanesulfonic acid (HEPES; Gibco). An analysis of the full length of *BRAF* in the original tumor and the derived cell line T1527A revealed the mutation G466E in exon 11 of *BRAF* in both samples (Supplementary Fig. S2). *NRAS* was found to be wild type in both the cell line and the original tumor. In addition, next-generation sequencing of the tumor, performed as in Rivolta et al.,⁷ did not reveal any mutation in *NFI*. An analysis of *HRAS* revealed the heterozygous mutation Q61R in the T1527A cell line.

Reagents

Vemurafenib (PLX4032, S1267), pictilisib (GDC-0941, S1065), selumetinib (AZD6244, S1008), trametinib (GSK1120212, S2673), and dactolisib (BEZ 235) were purchased from Selleck Chemicals (Huissen, The Netherlands). All drugs were dissolved in dimethyl sulfoxide (DMSO) to reach a stock concentration of 10 mM.

WST-1 Assay

The WST-1 assay, a colorimetric test that was developed to determine the survival and growth of eukaryotic cells in proliferation or to evaluate cytotoxicity,³⁰ was used to evaluate cell viability and calculate the half maximal inhibitory concentration (IC₅₀) of the drugs.

Briefly, cells were seeded in a 96-multiwell plate at a density of 5000 to 6000 cells/well in 200 µl of medium. The following day, cells were treated with increasing concentrations of kinase inhibitors. Twenty four and 72 hours later, Cell Proliferation Reagent WST-1 (20 µl) was added to each well,³¹ including the wells that contained only medium (with corresponding diluted

DMSO). Plates were then incubated for 1 hour at 37°C in the dark and analyzed with a microplate reader (Hidex Sense; Hidex, Turku, Finland) at 420 nm versus 650 nm. Experiments were performed in triplicate and repeated three times. IC₅₀ values were calculated using GraphPad software (GraphPad Software, San Diego, CA, USA).

Western Blot Analysis

Cells were disrupted in radioimmunoprecipitation assay buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 1% sodiumdodecyl sulfate) in the presence of protease and phosphatase inhibitors. Protein concentration was measured using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA).³²

Proteins were boiled in Laemmli buffer (62 mM Tris-HCl [6.8 pH], 2% SDS, 10% glycerol, 5% β-mercapto-ethanol, and 0.01% bromophenol blue). After blocking with 5% BSA in PBS for 1 hour at room temperature, membranes were incubated overnight at 4°C with the following primary antibodies: anti-ERK and anti-phospho-ERK (Cell Signaling; catalog no. 4695 and catalog no. 4370S, used at 1:1000); anti-MEK and anti-phospho-MEK (Cell Signaling; catalog no. 9126S and catalog no. 2338S, 1:1000); anti-S6 ribosomal protein and anti-phospho-S6 ribosomal protein (Cell Signaling; catalog no. 2217S and catalog no. 4858 S, 1:1000); and anti-alpha-tubulin (Sigma; catalog no. T5168, 1:4000). Horseradish peroxidase-conjugated anti-rabbit (Dako; P0399) or anti-mouse (Dako; P0447) antibodies, diluted 1:5000, were used as secondary reagents. Visualization of the immunoreactive bands was obtained by a chemoluminescent substrate, Super Signal West Pico Chemiluminescent Substrate (Thermo Fischer Scientific, Waltham, MA, USA) using fusion solo chemiluminescence and optional fluorescence imaging system.

Indirect Immunofluorescence Experiments

Cells were seeded at a density of 10/well in 1 ml of complete medium in Lab-Tek devices (4 wells, glass slide; Thermo Fisher Scientific). Cells were treated with various kinase inhibitors at their respective IC₅₀ concentrations. The activation of caspase-3 was evaluated 24 hours later by immunofluorescence with cleaved caspase-3 (catalog no. AP3725a; Abgent, San Diego, CA, USA; dilution 1:100, overnight incubation at 4 °C; secondary antibody [Invitrogen, Carlsbad, CA, USA]; A11037; Alexa Fluor; dilution 1:300). Cells were observed using a fluorescence microscope, Olympus BX51 (Olympus, Shinjuku, Tokyo, Japan), equipped with 40× and 60× objectives.

Statistical Analysis

Statistical analysis was performed using JMP 8.0 software (SAS, Cary, NC, United States), GraphPad Prism 5 program (GraphPad Software) and R statistical software, version 3.5.1 (R Foundation for Statistical Computing, Vienna, Austria).

Tests of significance were two-tailed, and results were considered as significant at an alpha level of $P < 0.05$, and a P value of 0.05 was considered to be borderline significant. Fisher's exact tests were used to establish an association between two qualitative variables. Mann-Whitney U test was used to compare continuous variables.

RESULTS

Clinicopathologic Characteristics

The nevi group ($n = 35$) comprised 13 subepithelial and 22 compound nevi. The male/female patient ratio was 21/14, with

TABLE 1. MAPK and pi3K Pathway Activation in Conjunctival Nevi and Melanomas

Protein	n (%)		P Value*
	Nevus, n = 35	Melanoma, n = 31	
pMEK			0.0008
Score 1	13 (37)	3 (10)	
Score 2	14 (40)	9 (29)	
Score 3	8 (23)	19 (61)	
pERK			<0.0001
Score 1	19 (54)	1 (3)	
Score 2	11 (31)	12 (39)	
Score 3	5 (14)	18 (58)	
pAKTc			0.002
Score 1	2 (6)	0	
Score 2	18 (51)	6 (32)	
Score 3	15 (43)	25 (68)	
pAKTn			0.316
Score 1	2 (6)	1 (3)	
Score 2	9 (26)	14 (45)	
Score 3	24 (69)	16 (52)	
pS6			<0.0001
Score 1	28 (80)	3 (10)	
Score 2	7 (20)	18 (58)	
Score 3	0	10 (32)	

Significant P values < 0.05 are highlighted in bold.

* Cochran-Armitage test.

a mean age of 36.9 (SD, 21.3 years). None of the nevi occurred in the tarsal conjunctiva and nine nevi arose in the caruncula. None of the nevi recurred (mean follow up, 31.5 months [SD, 29.9 months]).

The melanoma group ($n = 31$) consisted of 15 males and 16 females, with a mean age of 66.25 (SD, 20.4 years). According to the last American Joint Committee on Cancer TNM classification, 12 tumors belonged to the T1, 7 to the T2, and 12 to the T3 category. Melanomas arose mostly from primary acquired melanosis (67.8%) and less frequently from a nevus (19.3%) or de novo (12.9%). The origin of melanoma was determined by a combination of clinical and histopathologic factors.

Mean follow-up was 55 months (SD, 76.3 months). Local recurrences were found in 58.6% of the cases. Metastases developed in 10 patients, of whom 4 died.

Pathway Activation in the Tissues

The BRAF mutation was detected with immunohistochemistry in 42.6% (15/35) of the nevi and 35.5% (11/31) of the melanomas. In the nevi, BRAF mutation was more frequent in the subepithelial nevi (69%, 9/13) than in the compound nevi (27%, 6/22; Fisher $P = 0.032$). In the melanomas, BRAF mutation was not significantly correlated with any clinicopathologic factor (sex, age, tumor localization, melanoma origin [nevus, primary acquired melanosis, de novo], depth of invasion, proliferative activity, local lymphatic invasion, local recurrence, and metastases; Supplementary Table S1), but our study included only 31 melanomas.

Activation of the MAPK and AKT pathways in nevi and conjunctival melanomas was assessed by immunohistochemistry using antibodies specific for the phosphorylated forms of MEK, ERK, AKT, and S6 proteins, as detailed in the Materials and Methods section. The results are summarized in Table 1 and Figure 1 (nevi) and Figure 2 (melanomas), as well as Supplementary Table S1. Considering as positive those lesions containing at least 10% stained melanocytic cells (i.e., those

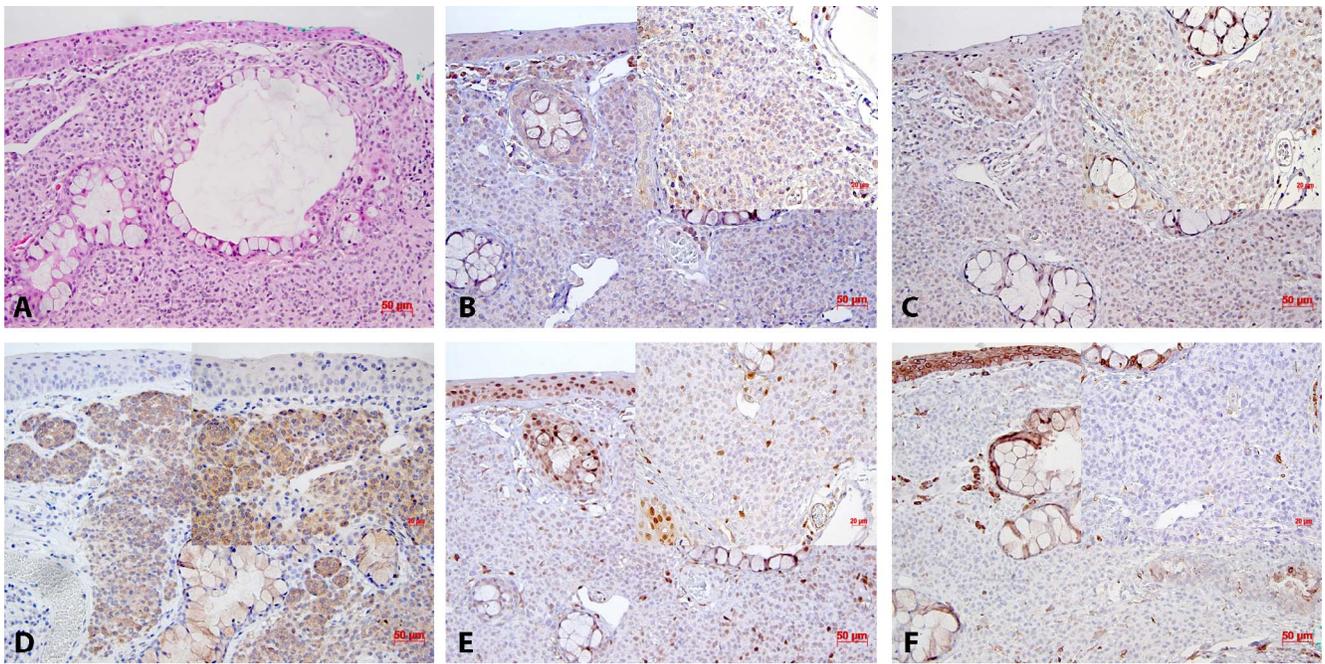


FIGURE 1. Activation status of the MAPK and PI3K pathways in a conjunctival subepithelial nevus. A BRAF V600E mutation can be identified with immunohistochemistry (D). There is no significant activation of MEK (B) or ERK (E) or S6 (F), as indicated by immunostaining with antibodies to the respective phosphoproteins. AKT (C) activation can be observed in some cells. (A) Corresponding area demonstrating a subepithelial nevus with pseudoglands containing goblet cells (stain: hematoxylin-eosin). (B) phospho-MEK. (C) phospho-AKT. (D) BRAF^{V600E}. (E) phospho-ERK. (F) phospho-S6. (A-F) Magnification: ×126; insets (B-F): magnification: ×252.

scored as 2 and 3 in our scoring method), cytoplasmic MEK activation was found in 62.9% of the nevi and in 90.3% of the melanomas (Cochran-Armitage test, $P = 0.0008$). ERK phosphorylation was detected both in the nucleus and the cytoplasm in 45.7% of the nevi and in 96.8% of the melanoma

cases (Cochran-Armitage test, $P < 0.0001$). There was good correlation between the activation of ERK in the cytoplasm and nucleus (Cochran-Armitage test, $P < 0.0001$).

Cytoplasmic pAKT was positive (score 2 or 3) in 94.3% of the nevi and in all melanomas, and similarly, nuclear pAKT was

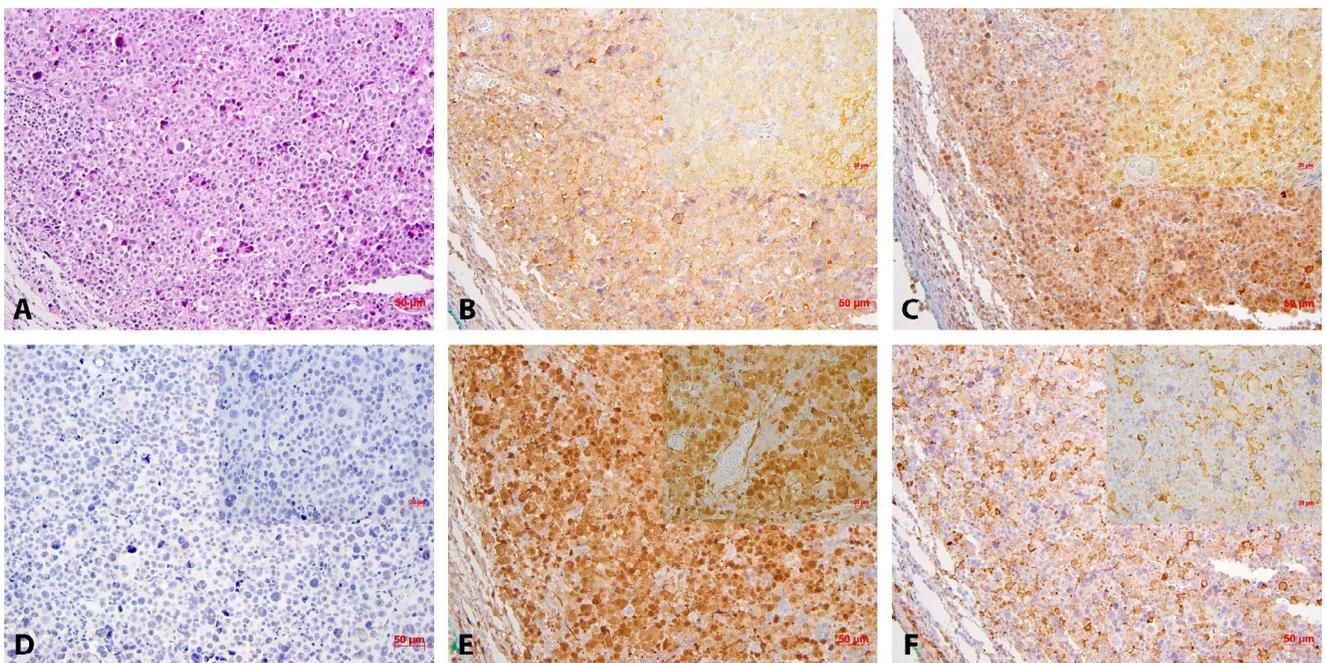


FIGURE 2. MAPK and PI3K pathways in an advanced conjunctival melanoma. Despite the absence of the BRAF V600E mutation (D), there is an activation of MEK (B) and ERK translocating to the nucleus (E) as well as an activation of AKT (C) and S6 (F). (A) Corresponding area with sheets of pleomorphic, atypical melanoma cells (stain: hematoxylin-eosin). (B) phospho-MEK. (C) phospho-AKT. (D) BRAF^{V600E}. (E) phospho-ERK. (F) phospho-S6. (A-F) Magnification: ×126; insets (B-F) magnification: ×252.

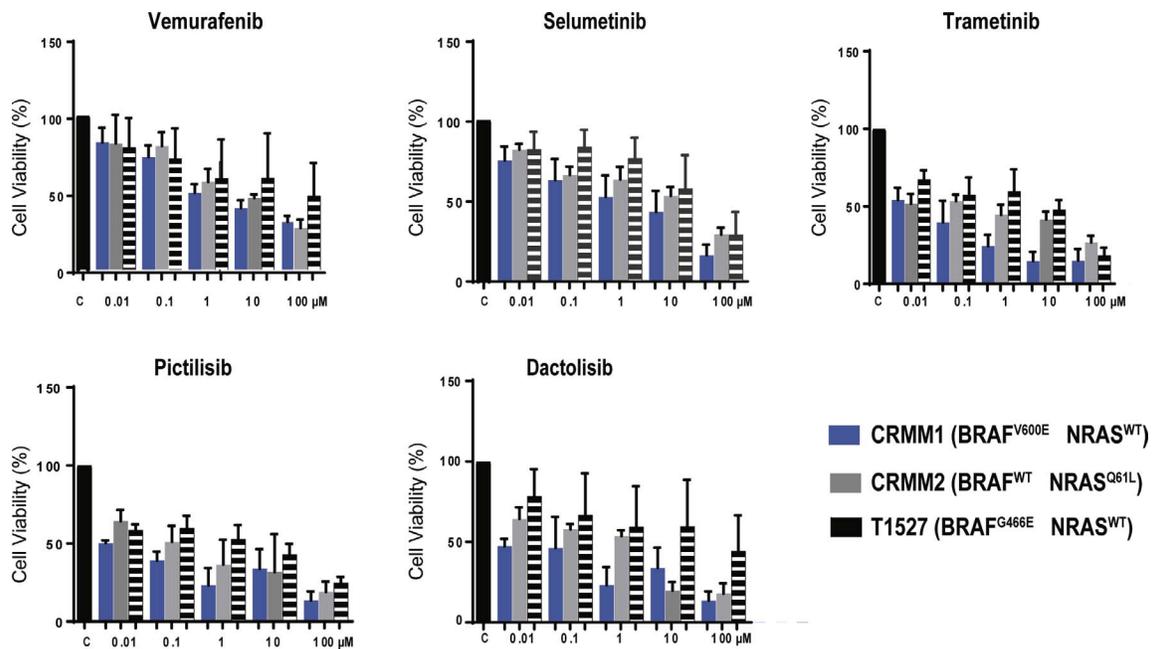


FIGURE 3. Dose-dependent effect of different MAPK and PI3K/mTOR pathway inhibitors on the growth of CJM cells (CRMM1, CRMM2, and T1527A). Cells were treated with the indicated inhibitors at the indicated concentrations for 72 hours. Cell viability was evaluated using the WST-1 assay. Values show mean \pm SD of triplicate measurements. These are representative results of three different experiments.

identified in 94.3% of the nevi and in 96.8% of the melanomas. However, the proportion of CJM samples with a more extensive staining (score 3) was higher (80.6%) than observed in nevi (42.9%; Cochran-Armitage test, $P = 0.002$). pS6 was found in 20% of nevi and in 90.3% of melanoma (Cochran-Armitage test, $P < 0.0001$).

A significant correlation was found between the activation of MEK and ERK in both melanomas and nevi (Fisher, $P = 0.022$). There was no correlation between the activation of AKT and the downstream activation of S6.

There was no correlation between the presence of BRAF mutation detected with immunohistochemistry and the activation of MEK, ERK, AKT, and S6.

In the melanomas, there was no correlation between the activation of AKT, S6, MEK, or ERK and the TNM stage, depth of invasion, proliferative activity, local lymphatic invasion or local recurrences, metastases, and death (Supplementary Table S1).

In Vitro Responses to MAPK and AKT Inhibitors

To evaluate the sensitivity of conjunctival melanoma cells to inhibitors targeting the signaling pathways implicated in tumorigenesis, cultured conjunctival melanoma cell lines containing characterized BRAF and NRAS mutations were treated with different concentrations of vemurafenib (BRAF

inhibitor), selumetinib and trametinib (MEK inhibitors), pictilisib (AKT inhibitor), and dactolisib (a dual PI3K/mTOR inhibitor). The effect on viability was measured after 24 and 72 hours of treatment using the WST-1 assay (Fig. 3; Supplementary Fig. S3). Figure 3 shows that the cell lines had different sensitivity to the drugs. IC₅₀ values calculated for the inhibitors are summarized in Table 2. CRMM1 was the only cell line sensitive to inhibitors of both MAPK (vemurafenib and trametinib) and AKT (pictilisib and dactolisib) pathways. However, sensitivity to vemurafenib was weak (just above 1 μ M), despite the presence of BRAF^{V600E} mutation. CRMM2 was moderately sensitive to pictilisib, whereas T1527A was resistant to all inhibitors tested. Our analysis revealed that T1527A was harboring not only a BRAF^{G466E} mutation but also a HRAS^{Q61R} mutation. The HRAS^{Q61R} mutation was possibly already present at a lower level in the primary tumor (CM3-T) from which it was derived (Supplementary Fig. S2).

To assess the involvement of apoptosis in the drug-induced effect on viability, we asked whether the caspase-3 apoptosis pathway was activated in the more sensitive CRMM1 cells treated with the various inhibitors at IC₅₀ concentrations. Immunocytochemistry of cleaved caspase-3 showed cells with increased perinuclear staining following a 24-hour treatment with the inhibitors but not in CRMM1 cells without the inhibitors (Supplementary Fig. S5).

TABLE 2. IC₅₀ Values of Inhibitors in Conjunctival Melanomas

Cell Line	Vemurafenib (μ M)	Selumetinib (μ M)	Trametinib (nM)	Pictilisib (nM)	Dactolisib (nM)
CRMM1	1.08	7	9.8	10	14
CRMM2	>10	10	360	500	1700
T1527A	>100	>10	>10,000	>10	>10,000

IC₅₀ values were calculated from viability assays (as shown in Fig. 3) performed on cells treated with the indicated inhibitors for 72 hours. Values are the mean of three independent experiments. IC₅₀ values demonstrate that CRMM1 is at least partially sensitive to vemurafenib, trametinib, pictilisib, and dactolisib. CRMM2 is only sensitive to pictilisib and trametinib, whereas T1527a is not sensitive to any of the drugs tested.

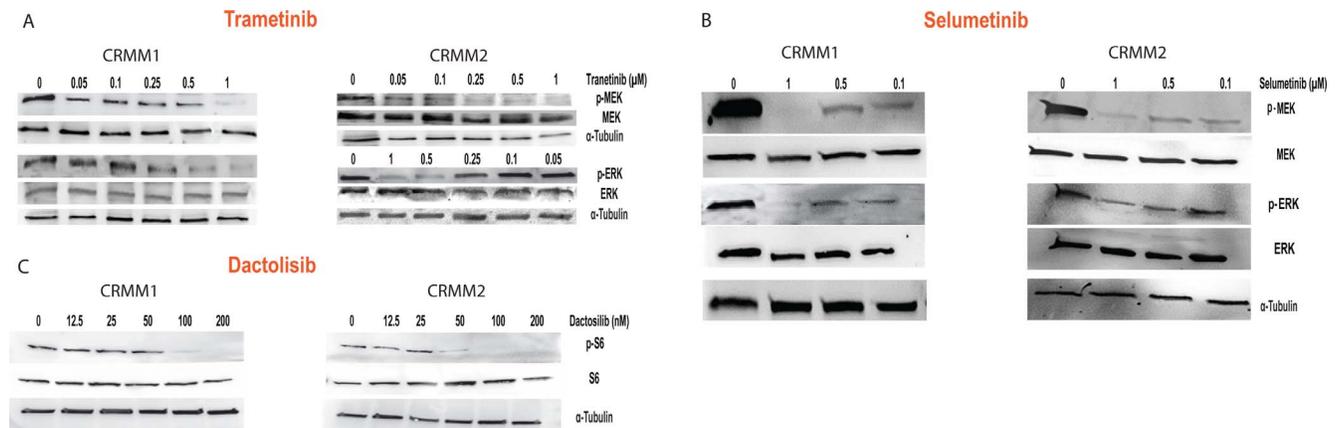


FIGURE 4. Effect of BRAF, MAPK, and PI3K/mTOR inhibitor treatment on MEK, ERK, and S6 protein phosphorylation of CJM cells. (A–C) Western blots of lysates from CRMM1 and CRMM2 cells treated with increasing doses of selumetinib, trametinib, and dactolisib, respectively, for 24 hours, showing changes in the phosphorylated forms of downstream substrates (p-ERK, p-MEK, and p-S6). Total levels of the respective proteins are shown as control. α -Tubulin was used as a gel loading control. The figure shows the results of a representative experiment.

Effect of Kinase Inhibitors on Phospho-MEK, -ERK, and -S6 Levels

To understand the different sensitivity of the cell lines tested to the inhibitors, Western blot analyses were first performed to assess the basal levels of phosphorylated (activated) and total ERK, MEK, and S6 proteins in CJM cell lines. All three cell lines displayed a constitutive activation of the above proteins (Supplementary Fig. S4).

To assess whether the responses to inhibitors were pathway-specific, we treated the cell lines harboring activating *BRAF* and *NRAS* mutations, that is CRMM1 and CRMM2, with MEKi (trametinib and selumetinib) or dual PI3K-mTORi (dactolisib) for 24 hours and assessed the effects on the activation of MEK, ERK, and S6 with Western blotting. In CRMM1 and CRMM2 (Fig. 4A), the lowest dosage of selumetinib (0.1 μ M) greatly inhibited the phosphorylation of both MEK and downstream ERK. Trametinib caused similar concentration-dependent decreases in the phosphorylation of MEK and ERK in the two cell lines. In both cases, levels of total MEK and ERK remained unchanged (Fig. 4B). Phosphorylation of S6 was inhibited by dactolisib in both cell lines (Fig. 4C). Altogether, the drugs showed activity on relevant targets in both cell lines.

DISCUSSION

As a significant proportion of conjunctival melanomas contains *BRAF* or *NRAS* mutations, we directly tested in the tumor tissues the activation of the downstream pathways, that is the MAPK and PI3k/mTOR pathways. The *BRAF*^{V600E} mutation was found in 42.6% of the nevi and 35.5% of melanomas. The presence of this *BRAF* mutation in conjunctival nevi has been documented in 19%²⁵ to 50%³³ of cases, a lower rate than in cutaneous nevi, where *BRAF*^{V600E} has been identified in 60%³⁴ to 87.5%.^{35–37} Based upon this study and previous reports, a *BRAF*^{V600E} mutation occurs in 26%²⁵ to 35%^{13,28} of conjunctival melanomas. Although a *BRAF*^{V600E} occurred at an earlier age in our study with respect to what has been previously reported in Denmark¹³ (62.09 years versus 68.55 years), this was not significant.

Our results demonstrate that the MAPK pathway is constitutively activated in both conjunctival nevi and melanomas. Moreover, the fact that a *BRAF*^{V600E} mutation is less frequent in melanomas than in nevi implies that the increased

activation of this pathway identified in our study may not depend only upon a *BRAF* oncogenic mutation. It is understood that the modulation of the MAPK pathway results from a fine balance between positive and negative regulators. Apart from *BRAF*^{V600E}, the loss of the negative control by *NF1* may partially explain this enhanced MAPK activity. In cutaneous melanoma, an *NF1* mutation is an important event, identified within 14% of cases by the tumor genome atlas project.¹¹ Recently, *NF1* mutations could be identified in 21 of 63 conjunctival melanomas (33%).¹⁴ Alternatively, the increased MAPK activity may also be directly explained by the presence of other oncogenic mutations, such as *NRAS* mutations, discovered in 18% of conjunctival melanoma cases.²⁸ By analogy, in 32 *NRAS*-mutated cutaneous melanomas, increased activity of the MAPK pathway was observed in more than 60% of cases.²⁴

In the conjunctival melanomas, we encountered an almost significantly increased nuclear localization of AKT compared with the nevi, as well as a concomitant increased phosphorylation of S6, suggesting an increased activity of the PI3k/mTOR pathway, which could be partially explained by the presence of an *NRAS* mutation or a loss of *NF1* function.

Considering that an activation of both the MAPK and PI3K/mTOR pathways occur in conjunctival melanoma, we tested their inhibition *in vitro* in cell lines with a *BRAF*^{V600E} or an *NRAS*^{Q61L} mutation or without these mutations. Direct inhibition of *BRAF* with vemurafenib in the *BRAF*^{V600E}-mutated cell line CRMM1 resulted in a moderate effect. The IC₅₀ value of 1.08 μ M, calculated for a 72-hour treatment, is very similar to that of previously published data on the same cell line.²⁵ However, significant inhibition of growth was not seen at 24 hours. Interestingly, live cell imaging of the effect of vemurafenib on *BRAF*-mutated cutaneous melanoma cell lines demonstrated that at this time a significant proportion of the cells was still dividing.³⁸ Vemurafenib was not effective in the *NRAS*-mutated cell line or the cell line with the *BRAF*^{V466E} mutation. Our data are in line with the paradoxical MAPK activation and increased ERK phosphorylation observed in cutaneous and conjunctival melanomas with an *NRAS* mutation and treated with a *BRAF* inhibitor.^{25,39}

Our results demonstrate that the MEK inhibitors trametinib and selumetinib significantly decreased phosphorylation of MEK and ERK in both the *BRAF*^{V600E} and the *NRAS*-mutated cell lines. However, significant growth inhibition after 72 hours was only achieved in the *BRAF*^{V600E} mutant with trametinib. The IC₅₀ of 10 nM obtained in our study with the *BRAF*-

mutated conjunctival melanoma cell line is similar to the values found in primary cultures of *BRAF*-mutated cutaneous melanoma, ranging from <0.1 nM to 374 nM.⁴⁰ On the other hand, the IC₅₀ identified in the *NRAS*-mutated conjunctival melanoma cell line is much higher than previously reported in *NRAS*-mutated skin melanoma.⁴⁰ This may be due to the fact that *NRAS* can potentially activate the PI3K/mTOR pathway, resulting in less sensitivity of the CRMM2 cell line to trametinib inhibition. Trametinib, as a single agent, was also effective in other *NRAS*-mutated cutaneous melanoma cell lines.²⁴ In patients with advanced cutaneous melanomas harboring *BRAF* mutations, trametinib was partially effective as a single agent but not in those with *NRAS* mutations.⁴¹ Although selumetinib suppressed the growth of *BRAF*-mutated skin melanoma cell lines,⁴² similar to what we observed in our study after 72 hours, it did not significantly improve disease-free survival in a phase II prospective study, including patients with advanced melanoma treated with either selumetinib or temozolomide, regardless of the mutational *BRAF* or *NRAS* status.⁴³

Targeting the PI3K/mTOR pathway resulted in a significant growth inhibition in both the *BRAF*^{V600E}- and *NRAS*-mutated cell lines. In our study, the single inhibition of PI3K with pictilisib appeared to be more efficient than a dual inhibition of PI3K and downstream mTOR with dactolisib. Pictilisib was also the only drug with a small effect on the class 3 *BRAF*-mutant cell line. In cutaneous melanoma, the efficiency of pictilisib as a single agent has been variable: although it is partially effective in several cell lines,⁴⁴ in other cell lines with either the *BRAF*^{V600E} mutation⁴⁵ or *NRAS* mutations,²⁴ an effective growth inhibition could not be observed. However, in a phase I clinical study including 60 patients with solid tumors (among whom 5 skin melanoma patients), the best, partial, clinical response was achieved in a patient with a *BRAF*^{V600E}-mutated metastatic cutaneous melanoma.⁴⁶ Although the dual inhibition of PI3K and mTOR with dactolisib resulted in an inactivation of S6 in both our *BRAF*^{V600E}- and *NRAS*-mutated cell lines, cell viability was only significantly reduced in the *BRAF*^{V600E}-mutated line. In skin melanoma, dactolisib had a cytostatic effect in vitro and vivo,⁴⁷ but in a phase I clinical trial including several solid tumors, no objective response could be found by using dactolisib as a single agent.⁴⁸

None of the drugs used in our study induced a significant growth reduction in T1527A with a *BRAF*^{G466E} mutation. This mutation, occurring in the P-loop domain of BRAF, confers impaired kinase activity⁴⁹ and reduced interaction with MEK.⁵⁰ It is one of the so-called class 3 *BRAF* mutants described recently by Yao et al.⁵¹ with low kinase activity that are insensitive to vemurafenib. BRAF with P-loop mutations can bind and activate CRAF in the cytosol, leading to consecutive MEK activation.⁵² This finding is in agreement with the constitutive MAPK kinase pathway activation in T1527A, as also described in cutaneous melanoma cell lines with *BRAF*^{P-loop} mutations.⁵³ The lack of efficiency of MEK inhibitors in T1527A is also consistent with MEK resistance to MEK inhibition described in cell lines with *BRAF*^{P-loop} mutations.⁵³ In contrast to activating *BRAF* mutations, class 3 *BRAF* mutations usually coexist in melanomas with RAS mutations or *NF1* mutations/deletion, whereas in lung and colorectal cancers they often depend on receptor tyrosine kinase activation for RAS activity.⁵¹ As T1527A cells were established from a conjunctival melanoma lacking *NF1* mutations and harboring wild-type *NRAS*, it is interesting to note the presence of the oncogenic *HRAS* Q61R mutation in T1527A, possibly through the selection of a minor clonal subpopulation from the primary tumor already containing *HRAS* Q61R mutations

In our study, the use of a single drug allowed growth reduction, more in the *BRAF*-mutated and less the *NRAS*-mutated cell lines. In a study by Cao et al.,²⁵ both MEK inhibition and AKT inhibition resulted in a synergistic effect in the same cell lines used in our study. Similarly, in cutaneous melanoma, synergistic inhibition of the MAPK and PI3K pathways has been demonstrated in cell lines with an *NRAS* mutation²⁴ and in *BRAF*-resistant cell lines.⁵⁴ In metastatic cutaneous melanoma cell line spheroids, the combination of MEK and AKT inhibition prevented growth and invasion.⁵⁵ However, in four phase I clinical trials assessing the use of PI3K and MEK inhibitors in solid tumors, the overall response rate was limited to 4.7%, with a disease control rate of 19.2%, although a partial response was noted in a few patients with *BRAF*- or *NRAS*-mutated melanomas.⁵⁶

Over the last years, modulation of the tumor immune microenvironment with checkpoint inhibitors has significantly improved the survival of patients with cutaneous melanoma.⁵⁷⁻⁶¹ The response to checkpoint inhibitors depends on the local tumor immune microenvironment, that is notably the expression of T CD8⁺³² or PD-L1^{32,62} within the tumor. In conjunctival melanoma, the expression of PD-L1, found in 19% (5/27) of cases, has been associated with a worse tumor-related survival⁶³ and might be an argument for the use of checkpoint inhibitors in advanced conjunctival melanoma, especially considering the relative resistance of some CJM cell lines to MAPK or PI3K/mTOR inhibition.

In conclusion, in this study the increased activity of the MAPK and PI3K/mTOR pathways in conjunctival melanoma tumor progression may not rely solely on the presence of a BRAF mutation. We further demonstrate that the responsiveness of the *NRAS*^{Q61L} and the *BRAF*^{G466E}/*HRAS*^{Q61R} conjunctival melanoma cell lines to single MAPK or PI3K inhibition is limited. Altogether, our findings suggest that in advanced conjunctival melanoma, the molecular genetic background of the tumor should be determined to guide the choice of the best therapeutic option. Further investigations into mechanisms of intrinsic resistance and combination targeted therapies should also be pursued.

Acknowledgments

Supported by the "Fond'Action Contre le Cancer" Foundation, Switzerland.

Disclosure: **I. El Zaoui**, None; **M. Bucher**, None; **D. Rimoldi**, None; **M. Nicolas**, None; **G. Kaya**, None; **R. Pescini Gobert**, None; **N. Bedoni**, None; **A. Schalenbourg**, None; **E. Sakina**, None; **L. Zografos**, None; **S. Leyvraz**, None; **N. Riggi**, None; **C. Rivolta**, None; **Alexandre P. Moulin**, None

References

1. Yu GP, Hu DN, McCormick S, Finger PT. Conjunctival melanoma: is it increasing in the United States? *Am J Ophthalmol*. 2003;135:800-806.
2. Seregard S, Kock E. Conjunctival malignant melanoma in Sweden 1969-91. *Acta Ophthalmol (Copenh)*. 1992;70:289-296.
3. Tuomaala S, Toivonen P, Al-Jamal R, Kivela T. Prognostic significance of histopathology of primary conjunctival melanoma in Caucasians. *Curr Eye Res*. 2007;32:939-952.
4. Paridaens AD, Minassian DC, McCartney AC, Hungerford JL. Prognostic factors in primary malignant melanoma of the conjunctiva: a clinicopathological study of 256 cases. *The Br J Ophthalmol*. 1994;78:252-259.
5. Shields CL, Markowitz JS, Belinsky I, et al. Conjunctival melanoma: outcomes based on tumor origin in 382 consecutive cases. *Ophthalmology*. 2011;118:389-395.

6. Missotten GS, Keijser S, De Keizer RJ, De Wolff-Rouendaal D. Conjunctival melanoma in the Netherlands: a nationwide study. *Invest Ophthalmol Vis Sci.* 2005;46:75–82.
7. Rivolta C, Royer-Bertrand B, Rimoldi D, et al. UV light signature in conjunctival melanoma; not only skin should be protected from solar radiation. *J Hum Genet.* 2016;61:361–362.
8. Hodis E, Watson IR, Kryukov GV, et al. A landscape of driver mutations in melanoma. *Cell.* 2012;150:251–263.
9. Berger MF, Hodis E, Heffernan TP, et al. Melanoma genome sequencing reveals frequent PREX2 mutations. *Nature.* 2012;485:502–506.
10. Royer-Bertrand B, Torsello M, Rimoldi D, et al. Comprehensive genetic landscape of uveal melanoma by whole-genome sequencing. *Am J Hum Genet.* 2016;99:1190–1198.
11. Cancer Genome Atlas Network. Genomic classification of cutaneous melanoma. *Cell.* 2015;161:1681–1696.
12. Griewank KG, Westekemper H, Murali R, et al. Conjunctival melanomas harbor BRAF and NRAS mutations and copy number changes similar to cutaneous and mucosal melanomas. *Clin Cancer Res.* 2013;19:3143–3152.
13. Larsen AC, Dahl C, Dahmcke CM, et al. BRAF mutations in conjunctival melanoma: investigation of incidence, clinicopathological features, prognosis and paired premalignant lesions. *Acta Ophthalmol.* 2016;94:463–470.
14. Scholz SL, Cosgarea I, Susskind D, et al. NF1 mutations in conjunctival melanoma. *Br J Cancer.* 2018;118:1243–1247.
15. Wellbrock C, Arozarena I. The complexity of the ERK/MAP-kinase pathway and the treatment of melanoma skin cancer. *Front Cell Dev Biol.* 2016;4:33.
16. Sondergaard JN, Nazarian R, Wang Q, et al. Differential sensitivity of melanoma cell lines with BRAFV600E mutation to the specific Raf inhibitor PLX4032. *J Transl Med.* 2010;8:39.
17. Tsai J, Lee JT, Wang W, et al. Discovery of a selective inhibitor of oncogenic B-Raf kinase with potent antimelanoma activity. *Proc Natl Acad Sci U S A.* 2008;105:3041–3046.
18. Flaherty KT, Puzanov I, Kim KB, et al. Inhibition of mutated, activated BRAF in metastatic melanoma. *N Engl J Med.* 2010;363:809–819.
19. Sosman JA, Kim KB, Schuchter L, et al. Survival in BRAF V600-mutant advanced melanoma treated with vemurafenib. *N Engl J Med.* 2012;366:707–714.
20. Chapman PB, Hauschild A, Robert C, et al. Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *N Engl J Med.* 2011;364:2507–2516.
21. Flaherty KT, Robert C, Hersey P, et al. Improved survival with MEK inhibition in BRAF-mutated melanoma. *N Engl J Med.* 2012;367:107–114.
22. Ascierto PA, Schadendorf D, Berking C, et al. MEK162 for patients with advanced melanoma harbouring NRAS or Val600 BRAF mutations: a non-randomised, open-label phase 2 study. *Lancet Oncol.* 2013;14:249–256.
23. Greger JG, Eastman SD, Zhang V, et al. Combinations of BRAF, MEK, and PI3K/mTOR inhibitors overcome acquired resistance to the BRAF inhibitor GSK2118436 dabrafenib, mediated by NRAS or MEK mutations. *Mol Cancer Ther.* 2012;11:909–920.
24. Posch C, Moslehi H, Feeney L, et al. Combined targeting of MEK and PI3K/mTOR effector pathways is necessary to effectively inhibit NRAS mutant melanoma in vitro and in vivo. *Proc Natl Acad Sci U S A.* 2013;110:4015–4020.
25. Cao J, Heijkants RC, Jochemsen AG, et al. Targeting of the MAPK and AKT pathways in conjunctival melanoma shows potential synergy. *Oncotarget.* 2017;8:58021–58036.
26. Riechardt AI, Maier AK, Nonnenmacher A, et al. B-Raf inhibition in conjunctival melanoma cell lines with PLX 4720. *Br J Ophthalmol.* 2015;99:1739–1745.
27. Weber JL, Smalley KS, Sondak VK, Gibney GT. Conjunctival melanomas harbor BRAF and NRAS mutations—letter. *Clin Cancer Res.* 2013;19:6329–6330.
28. Griewank KG, Westekemper H, Schilling B, et al. Conjunctival melanomas harbor BRAF and NRAS mutations—response. *Clin Cancer Res.* 2013;19:6331–6332.
29. Pinto Torres S, Andre T, Gouveia E, Costa L, Passos MJ. Systemic treatment of metastatic conjunctival melanoma. *Case Rep Oncol Med.* 2017;2017:4623964.
30. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods.* 1983;65:55–63.
31. Mallet JD, Gendron SP, Drigeard Desgarnier MC, Rochette PJ. Implication of ultraviolet light in the etiology of uveal melanoma: a review. *Photochem Photobiol.* 2014;90:15–21.
32. Tumeh PC, Harview CL, Yearley JH, et al. PD-1 blockade induces responses by inhibiting adaptive immune resistance. *Nature.* 2014;515:568–571.
33. Goldenberg-Cohen N, Cohen Y, Rosenbaum E, et al. T1799A BRAF mutations in conjunctival melanocytic lesions. *Invest Ophthalmol Vis Sci.* 2005;46:3027–3030.
34. Bloethner S, Snellman E, Bermejo JL, et al. Differential gene expression in melanocytic nevi with the V600E BRAF mutation. *Genes Chromosomes Cancer.* 2007;46:1019–1027.
35. Poynter JN, Elder JT, Fullen DR, et al. BRAF and NRAS mutations in melanoma and melanocytic nevi. *Melanoma Res.* 2006;16:267–273.
36. Ichii-Nakato N, Takata M, Takayanagi S, et al. High frequency of BRAFV600E mutation in acquired nevi and small congenital nevi, but low frequency of mutation in medium-sized congenital nevi. *J Invest Dermatol.* 2006;126:2111–2118.
37. Wu J, Rosenbaum E, Begum S, Westra WH. Distribution of BRAF T1799A(V600E) mutations across various types of benign nevi: implications for melanocytic tumorigenesis. *Am J Dermatopathol.* 2007;29:534–537.
38. Fallahi-Sichani M, Becker V, Izar B, et al. Adaptive resistance of melanoma cells to RAF inhibition via reversible induction of a slowly dividing de-differentiated state. *Mol Syst Biol.* 2017;13:905.
39. Poulidakos PI, Zhang C, Bollag G, Shokat KM, Rosen N. RAF inhibitors transactivate RAF dimers and ERK signalling in cells with wild-type BRAF. *Nature.* 2010;464:427–430.
40. Thumar J, Shahbazian D, Aziz SA, Jilaveanu LB, Kluger HM. MEK targeting in N-RAS mutated metastatic melanoma. *Mol Cancer.* 2014;13:45.
41. Falchook GS, Lewis KD, Infante JR, et al. Activity of the oral MEK inhibitor trametinib in patients with advanced melanoma: a phase 1 dose-escalation trial. *Lancet Oncol.* 2012;13:782–789.
42. Yeh TC, Marsh V, Bernat BA, et al. Biological characterization of ARRY-142886 (AZD6244), a potent, highly selective mitogen-activated protein kinase 1/2 inhibitor. *Clin Cancer Res.* 2007;13:1576–1583.
43. Kirkwood JM, Bastholt L, Robert C, et al. Phase II, open-label, randomized trial of the MEK1/2 inhibitor selumetinib as monotherapy versus temozolomide in patients with advanced melanoma. *Clin Cancer Res.* 2012;18:555–567.
44. Wallin JJ, Edgar KA, Guan J, et al. GDC-0980 is a novel class I PI3K/mTOR kinase inhibitor with robust activity in cancer models driven by the PI3K pathway. *Mol Cancer Ther.* 2011;10:2426–2436.
45. Hoeflich KP, Merchant M, Orr C, et al. Intermittent administration of MEK inhibitor GDC-0973 plus PI3K inhibitor GDC-0941 triggers robust apoptosis and tumor growth inhibition. *Cancer Res.* 2012;72:210–219.
46. Sarker D, Ang JE, Baird R, et al. First-in-human phase I study of pictilisib (GDC-0941), a potent pan-class I phosphatidylinosi-

- tol-3-kinase (PI3K) inhibitor, in patients with advanced solid tumors. *Clin Cancer Res.* 2015;21:77-86.
47. Marone R, Erhart D, Mertz AC, et al. Targeting melanoma with dual phosphoinositide 3-kinase/mammalian target of rapamycin inhibitors. *Mol Cancer Res.* 2009;7:601-613.
 48. Bendell JC, Kurkjian C, Infante JR, et al. A phase 1 study of the sachet formulation of the oral dual PI3K/mTOR inhibitor BEZ235 given twice daily (BID) in patients with advanced solid tumors. *Invest New Drugs.* 2015;33:463-471.
 49. Wan PT, Garnett MJ, Roe SM, et al. Mechanism of activation of the RAF-ERK signaling pathway by oncogenic mutations of B-RAF. *Cell.* 2004;116:855-867.
 50. Haling JR, Sudhamsu J, Yen I, et al. Structure of the BRAF-MEK complex reveals a kinase activity independent role for BRAF in MAPK signaling. *Cancer Cell.* 2014;26:402-413.
 51. Yao Z, Yaeger R, Rodrik-Outmezguine VS, et al. Tumours with class 3 BRAF mutants are sensitive to the inhibition of activated RAS. *Nature.* 2017;548:234-238.
 52. Garnett MJ, Rana S, Paterson H, Barford D, Marais R. Wild-type and mutant B-RAF activate C-RAF through distinct mechanisms involving heterodimerization. *Mol Cell.* 2005;20:963-969.
 53. Smalley KS, Xiao M, Villanueva J, et al. CRAF inhibition induces apoptosis in melanoma cells with non-V600E BRAF mutations. *Oncogene.* 2009;28:85-94.
 54. Zuo Q, Liu J, Huang L, et al. AXL/AKT axis mediated-resistance to BRAF inhibitor depends on PTEN status in melanoma. *Oncogene.* 2018;37:3275-3289.
 55. Smalley KS, Haass NK, Brafford PA, Lioni M, Flaherty KT, Herlyn M. Multiple signaling pathways must be targeted to overcome drug resistance in cell lines derived from melanoma metastases. *Mol Cancer Ther.* 2006;5:1136-1144.
 56. Jokinen E, Koivunen JP. MEK and PI3K inhibition in solid tumors: rationale and evidence to date. *Ther Adv Med Oncol.* 2015;7:170-180.
 57. Hodi FS, O'Day SJ, McDermott DF, et al. Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med.* 2010;363:711-723.
 58. Schadendorf D, Hodi FS, Robert C, et al. Pooled analysis of long-term survival data from phase ii and phase iii trials of ipilimumab in unresectable or metastatic melanoma. *J Clin Oncol.* 2015;33:1889-1894.
 59. Robert C, Schachter J, Long GV, et al. Pembrolizumab versus ipilimumab in Advanced Melanoma. *N Engl J Med.* 2015;372:2521-2532.
 60. Weber JS, D'Angelo SP, Minor D, et al. Nivolumab versus chemotherapy in patients with advanced melanoma who progressed after anti-CTLA-4 treatment (CheckMate 037): a randomised, controlled, open-label, phase 3 trial. *Lancet Oncol.* 2015;16:375-384.
 61. Wolchok JD, Chiarion-Sileni V, Gonzalez R, et al. Overall survival with combined nivolumab and ipilimumab in advanced melanoma. *N Engl J Med.* 2017;377:1345-1356.
 62. Larkin J, Chiarion-Sileni V, Gonzalez R, et al. Combined nivolumab and ipilimumab or monotherapy in untreated melanoma. *N Engl J Med.* 2015;373:23-34.
 63. Cao J, Brouwer NJ, Richards KE, et al. PD-L1/PD-1 expression and tumor-infiltrating lymphocytes in conjunctival melanoma. *Oncotarget.* 2017;8:54722-54734.