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Intermittent *Versus* Continuous Dosing of MAPK Inhibitors in the Treatment of *BRAF*-Mutated Melanoma

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Abstract

The development of BRAF and MEK inhibitors (BRAFi/MEKi) has led to major advances in melanoma treatment. However, the emergence of resistance mechanisms limits the benefit duration and a complete response occurs in less than 20% of patients receiving BRAFi ± MEKi. In this study, we evaluated the impact of an intermittent versus continuous dosing schedule of BRAF/MEK inhibition in a melanoma model mildly sensitive to a BRAF inhibitor. The combination of a BRAFi with three different MEKi was studied with a continuous or intermittent dosing schedule *in vivo,* in a xenografted melanoma model and *ex vivo* using histoculture drug response assays (HDRAs) of patient-derived xenografts (PDX). To further understand the underlying molecular mechanisms of therapeutic efficacy, a biomarker pharmacodynamic readout was evaluated.

An equal impact on tumor growth was observed in monotherapy or bitherapy regimens whether we used continuous and intermittent dosing schedules, with no significant differences in biomarkers expression between the treatments. The antitumoral effect was mostly due to modulations of expression of cell cycle and apoptotic mediators. Moreover, *ex vivo* studies did not show significant differences between the dosing schedules.

In this context, our preclinical and pharmacodynamic results converged to show the similarity between intermittent and continuous treatments with either BRAFi or MEKi alone or with the combination of both.

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Introduction

The last decade has witnessed some spectacular breakthroughs in the treatment of advanced and metastatic melanoma. The uncovering of the *BRAF* mutations, which are found in at least 50% of all melanoma cases [1] and are responsible for the constitutive activation of the mitogen-activated protein kinase (MAPK) pathway, has led to the development of MAPK-targeted therapies [2–4], and the present standard of care uses the combination of a BRAF inhibitor and an MEK inhibitor [5,6]. This association has been proven to delay the

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onset of resistance compared with BRAF inhibitor alone, allowing a more durable response that is usually better tolerated [7–9].

However, even with combination treatments the emergence of resistance is still a major concern [10–14]. Numerous resistance mechanisms to BRAF and MEK inhibitors have been described [15–17], and a large number of them rely on the reactivation of the MAPK pathway.

Intermittent therapy has been suggested to prevent or delay resistance to BRAF inhibition. Das Thakur et al. developed primary human melanoma xenograft models highly sensitive to BRAF inhibition, in which vemurafenib resistance was achieved by continuous administration of the drug [18]. They demonstrated that proliferation of vemurafenib-resistant cells was dependent on the continuous presence of the drug and that intermittent dosing of vemurafenib delayed the onset of drug resistance. They further made the proof-of-concept of the prevention of drug resistance onset by applying a discontinuous vemurafenib regimen in a vemurafenib--sensitive xenograft model. Thus far, such a regimen has not been extended to the combination of BRAF and MEK inhibitors.

A few clinical observations have reported the use of intermittent or sequential regimen for targeted therapies [19–21]. Patients with progressive disease (PD) that were given BRAF inhibitors (as a monotherapy or in combination with an MEK inhibitor) were given immune check point inhibitors and on progression were rechallenged with targeted therapies, with some efficacy, suggesting that resistance to MAPK-targeted therapies could be reversible. This takes into account that complete response (CR) occurs in less than 20% of patients receiving BRAF ± MEK inhibitors [22].

In this work, we aimed to gain further insight into the benefits of an intermittent versus continuous schedule of MAPK inhibitor administration in a melanoma model that is mildly sensitive to a BRAF inhibitor. This issue was addressed using an *in vivo* human-derived cell xenograft model and an *ex vivo* histoculture-drug response assay (HDRA) of melanoma patients-derived xenografts (PDX) [23]. To further understand the underlying molecular mechanisms of the therapeutic efficacy and the onset of drug resistance when using continuous and intermittent dosing schedules, we performed biomarker analyses that uncovered the molecular pathways involved in BRAF and MEK inhibitor mechanisms.

Materials and Methods

Patients and Specimens

Primary or metastatic melanoma specimens were collected from 6 patients who underwent surgery between 2013 and 2017 in the Department of Dermatology of Saint-Louis Hospital. The research was approved by the INSERM Institutional Review Board and Ethics Committee in Paris, France (IRB N° 00006477). All patients provided informed consent for the use of their tissue specimens and clinical features for research. All cases diagnosed were reviewed by the Department of Pathology. Melanoma surgical excision and patient's follow-up were performed according to the 2009 AJCC recommendations. Clinical records were retrospectively reviewed in standardized forms. The clinical data of the cohort used here are described in Table S2.

Cell Culture

Human melanoma cells SKMEL28 ($BRAF^{V600E}$-mutated) (ATCC Cat# HTB-72, RRID:CVCL_0526, USA), which were established from patient primary nodular melanomas, were maintained in RPMI medium (Gibco, Invitrogen, Life technologies, USA) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin, and 100 mg/mL streptomycin (Gibco, Invitrogen, Life technologies, USA). The cells were passaged at 80%–90% confluence and were tested free from bacterial and fungal contamination.

In Vivo Tumorigenicity Assays

Animals were housed under controlled conditions in terms of temperature, humidity, and light cycle (12 hours/12 hours) and were maintained under pathogen-free conditions and handled under stringent sterile conditions. Immunodeficient nude mice (NMRI--Foxn1 nu/nu) are a common model used in xenograft procedures. Methods were performed in accordance with the relevant guidelines and regulations in conformance to Directive 2010/63/EU, and all protocols were approved by the Committee on the Ethics of Animal Experiments of the French Ministry of Agriculture (Permit Number: B75-10-2014). All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals. The experiment design was based on the rule of the replacement, refinement, and reduction (3R's) to reduce suffering of the animals and use the minimum number of animals.

Female five-week-old NMRI-Foxn1 nu/nu mice (16–20 g) (Janvier Labs, France) were injected subcutaneously with 4×10^6 SKMEL28 cells ($n = 15$ per experimentation). When tumors were visible, approximately 7–10 days after tumor cell inoculation, mice were randomized and divided in three groups ($n = 5$) and treated as previously described with either vehicle (phosphate-buffered saline [PBS] containing 5% dimethyl sulfoxide [DMSO]) or continuous or intermittent (four weeks on treatment followed by a two weeks off of treatment) administration of dabrafenib (Tafinlar, GSK, UK) (100 mg/kg po) [24], cobimetinib (Cotellic, Roche, Switzerland) (5 mg/kg po) [25], trametinib (Mekinist, Novartis, Switzerland) (3 mg/kg po) [26], pimasertib (Merck, Germany) (20 mg/kg po) [27], or combination of dabrafenib with one of these MEK inhibitors. Vehicle and BRAF and/or MEK inhibitors were administered daily 5 days out of 7 via oral gavage using a stomach tube. The tumor growth was measured independently by two technicians with a digital caliper who were blinded to treatment status, and the volume was calculated using the formula: Length x $\text{Width}^2/2$. Mice were euthanized by cervical dislocation when their tumor reached a volume of ~1.5 cm^3. Tumors were immediately frozen and stored at −80 °C before cryostat sectioning. Statistical analyses were performed using GraphPad Prism5 software.

Drugs Quantification in Mice Plasma

Blood was collected from the submandibular in mice glands 3 hours after dabrafenib and cobimetinib gavage po in EDTA microtubes. The blood samples were centrifuged at $3000 \times g$ for 5 min in a microcentrifuge to isolate plasma, which was then stored at −21 °C until analysis. The dabrafenib and cobimetinib quantification in plasma was performed according to a previously published method using a ultra performance liquid chromatography (UPLC) system coupled with Tandem mass spectrometer (MS/MS) in a positive ionization mode [28].

DNA/mRNA Extraction and Reverse Transcription

DNA and mRNA extraction were performed on frozen tumor samples using Maxwell RSC Tissue DNA and Maxwell RSC

simplyRNA Tissue kits, respectively, (Promega, USA) according to the manufacturer's protocol. The quality and quantity of the DNA and mRNA were determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, NanoDrop Technologies, USA). First-strand cDNA were synthesized from 1 μg of total RNA using a High-Capacity cDNA Archive Kit (Applied-Biosystems, Life technologies, USA) according to the manufacturer's protocol. The obtained cDNA samples were diluted 6 times for quantitative polymerase chain reaction (qPCR) assay.

Copy Number and mRNA Expression Analysis

Signaling pathways transcript analyses were conducted, following MIQE guidelines. All the analyses were conducted in duplicates using a personalized Human qPCR SignArrays® 384 system (gene profiling analysis Human qPCR SignArrays® 384 kit for 30 genes of interest; and Perfect MasterMix SYBR Green® [AnyGenes, France]) on a LightCycler 480 (Roche, France) as described by the manufacturer (Table S1). Quality control of qPCR data for consequent analysis was based on positive and negative PCR controls. Briefly, a total volume of 20 μl of PCR mix, which included 10 μl of Perfect MasterMix SYBR Green®, 8 μl of PCR grade water and 2 μl of cDNA or DNA was loaded into each well of the qPCR array. PCR amplification was conducted at 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. Studied genes are involved in RAS-RAF-MAPK pathway, cell cycle, or apoptosis and were selected for their validated or suggested role in BRAF inhibitors resistance [29,30]. Overall, mRNA expression analysis was performed on 30 genes (*BRAF, RAF1, ARAF, PDGFRB, IGF1R, MET, HGF, KIT, EGFR, ERBB2, MAP3K8, MKI67, E2F2, RB1, CDK2, CDK4, CDK6, CCNA1, CCND1, RRM2, BCL2, BCL2L1, BCL2L11, BMF, MCL1, BAD, PTEN, CDKN1A, CDKN1B, CDKN2A*) and copy number analysis on 11 genes (*BRAF, NRAS, MET, CDKN2A, CDK4, CDK6, CCND1, CCND2, CCND3, RB1, CTNNB1*). The mRNA expression for each gene was expressed as the ratio "copy number of gene of interest/copy number of *PPIA*." CNV data analysis was conducted using AnyGenes Excel analysis tools in Windows via ΔΔCq method by calculating fold changes for each gene as the difference in gene expression between control, continuous and intermittent dosing schedules.

Gene copy number quantification was performed by comparison with *GAPDH*, using 2 sets of primers for each gene, as described previously [31]. Relative copy numbers were calculated using the ΔΔCq method, where Cq is the threshold cycle of amplification. For each sample, differences in the Cq of targeted gene and *GAPDH* used as an internal control were compared with those in a reference pool of normal genomic DNA prepared from 10 samples of benign tissue. Relative copy number was calculated using the formula $2(-\Delta\Delta Cq)$ and converted to absolute copy numbers by assigning a value of 2 (diploid) to the reference pool and multiplying the relative copy number of samples by a factor of 2. Threshold of 5 and 0.5 were set to define DNA amplification and DNA deletion, respectively [32,33]. Primer sequences are available upon request.

Mitosis Counting

Mice's tumors were fixed in 4% formalin and embedded in paraffin. Tissues sections (4 μm) were stained with hematoxylin (Sigma-Aldrich, USA) for 1 min and with eosin (Sigma-Aldrich, USA) for 30 s. The tissue sections were examined under a light microscope (Nikon Instruments Inc., USA) after mounting with Eukitt mounting media (Dutscher, France).

18F-FDG PET/CT

2-deoxy-2-[^{18}F]fluoro-D-glucose (18F-FDG) positron emission tomography and computed tomography (PET/CT) procedures were conducted on the UCK (Unité Claude Kellershohn) preclinical imaging platform at the IUH (Institut Universitaire d'Hématologie) at Saint Louis Hospital. Mice were anesthetized with isoflurane and injected intravenously with 18F-FDG with a mean activity of 12 (±0,2) MBq. CT acquisition was performed, followed by the acquisition of a 20 min emission frame 60 min after tracer injection on a dedicated Inveon micro PET/CT scanner (Siemens Medical Solutions, USA). Images were then reconstructed with a three-dimensional (3D) ordered-subset expectation-maximization (OSEM) followed by a maximum a posteriori (MAP) algorithm. Images were then analyzed with the IRW (Siemens) software and SUVmax of tumor was measured.

Next-Generation Sequencing Custom Panel and Data Analysis

Targeted sequencing was performed with a customized AmpliSeq™ panel (Thermo Fisher Scientific, USA) that was specifically designed with the Ion AmpliSeq™ designer software (Life Technologies, USA) to target 74 genes, including genes involved in the MAPK, PI3K/AKT, cell cycle, and RTK pathways. The panel comprised 35 kb and generated 328 amplicon with an average size of 120 bp and a mean coverage of 92.79%. The sequencing amplicons libraries were synthesized from 50 ng of genomic DNA isolated from frozen tissue samples using an Ion AmpliSeq™ Library Kit 2.0 (ThermoFisher Scientific, USA) and were indexed with an Ion Xpress Barcode Adapters Kit (Life Technologies, USA) following the manufacturer's instructions. Amplification quality was defined with a BioAnalyzer (Agilent 2100). The library amplicon pool was sequenced with the Ion PGM™ Sequencer (ThermoFisher Scientific, USA) using Ion PGM Hi-Q Chef chemistry (single-end reads of 120 bp) on a 318-V2-BC sequencing flow cell. Base calling, alignment to the hg19 human reference sequence, and variant calling were performed using the Torrent Suite Software v5.8.0 (Thermo-Fisher-Scientific, USA). ANNOVAR was used for annotation through the Galaxy-Curie interface. Integrative Genomics Viewer (IGV) was used to visualize the read alignment and confirm the variant calls.

The selection of the variants was performed using Alamut (Interactive Biosoftware) with the following criteria: (1) located in an exonic or splice region; (2) nonsynonymous coding variant; (3) not previously annotated in the 1000 genomes project database or with an allelic frequency <1%; 4) threshold of coverage above or equal to 500X; (5) variant allelic fraction (VAF) ≥5%; and (6) strand bias <95%.

Ex Vivo Histoculture Drug Response Assays

Female 5-week-old NMRI-Foxn1 nu/nu mice (16−20 g) (Janvier Labs, France) were injected subcutaneously with 4×10^6 of SKMEL28 cells or, after Ketamine/Xylazine anesthesia, were xenografted with a patient-derived *BRAF V600E*-mutated tumor tissue in the brown fat. Mice were euthanized by cervical dislocation when their tumor reached a volume of approximately 1-1.5 cm^3. Tumors were excised and a representative tumor piece was washed with PBS and sliced. Tumor sections were then placed in wells coated with gelatin (Sigma-Aldrich, USA) in 24-well plates in quadruplicates. The tumor sections were incubated for 8 days at 37 °C in

250 µL complete media supplemented with 10 µM dabrafenib (GSK2118436, Selleckchem), 1 µM cobimetinib (GDC-0973, RG7420, Selleckchem), or a combination of both inhibitors dissolved in DMSO. As controls, some wells received an equivalent volume of medium containing 0.5% DMSO. The medium was replenished every two days, followed by a day off of treatment for the intermittent dosing schedule. At the end of the incubation period, cell proliferation was measured using the CellTiter 96® nonradioactive cell proliferation assay (MTS) (Promega, France), according to the manufacturer's instructions. HDRAs were conducted in 3 independent experiments.

Statistical Analyses

Data are expressed as mean ± standard error of mean (SEM). The statistical significance of a difference between two groups was determined by an unpaired Student *t*-test. One-way analysis of variance (ANOVA) with Bonferroni *post hoc* test was used to compare differences among various groups in HDRA analysis. Differences were considered significant when $P < 0.05$. GraphPad Prism 5 was used for the statistical analyses (GraphPad Software, USA).

Results

Ex Vivo Intermittent and Continuous Dosing Schedules with BRAF and MEK Inhibitors in a Melanoma Model Mildly Sensitive to BRAF Inhibitor

Ex vivo HDRA was conducted on *BRAF*-mutated SKMEL28 xenografts obtained in nude mice after subcutaneous injection of culture cells [23]. We tested continuous (drugs administered from day 0 to day 8) and intermittent (drugs administered for 2 days, followed by a day off of treatment) dosing schedules and determined the percentage of cellular proliferation inhibition compared with control.

SKMEL28 histocultures were mildly responsive to dabrafenib and showed a proliferation decrease of 38% ± 1.8% and 35% ± 4.4% at 10 µM (Figure 1*A*). However, SKMEL28 cells were highly responsive to cobimetinib and showed inhibition rates of 76% ± 4.3% and 79% ± 5% at 1 µM, for continuous and intermittent treatment, respectively. In addition, combination of both inhibitors also decreased cellular proliferation by 89% ± 1.8% for continuous and 94% ± 4.3% for intermittent dosing schedules. Importantly, no difference between continuous and intermittent dosing was observed for each treatment condition.

Intermittent Dosing of BRAF or MEK Inhibitor Monotherapy is Not Superior to Continuous Treatment In Vivo in a Mildly Responsive Human-Derived Cell Xenograft Model

In vivo studies evaluated dabrafenib (100 mg/kg per os once daily) or cobimetinib (5 mg/kg per os once daily) inhibitors in monotherapy in a SKMEL28 xenograft model in nude mice with either a continuous or an intermittent dosing schedules (four weeks on treatment followed by a two weeks off of treatment as previously describes by Das Thakur, Salangsang, Landman, Sellers, Pryer, Levesque, Dummer, Mcmahon, et al., 2013). Plasma samples from treated mice were collected and analyzed during dabrafenib and cobimetinib treatments. The median Cmax plasma concentrations of dabrafenib and cobimetinib at 3 hours reached 1764.2 ng/mL and 18 ng/mL, respectively (Supplementary Figure 1*A* and 1*B*), and were similar to the therapeutic levels achieved in previous studies (EMEA/H/C/002604/0000 [34] and EMEA/H/C/003960/0000 [35], respectively).

Dabrafenib continuous therapy showed a significant reduction of tumor proliferation (Figure 1*B*). Indeed, the significant tumor growth inhibition (TGI) calculated in reference to placebo-treated tumors (CTRL), was 63% ± 10% at day 134 ($P = 0.0304$). Intermittent therapy was slightly less effective in reducing tumor proliferation and achieved a TGI of 52% ± 9% at day 134, although there was no significant statistical difference between both dosing schedules.

Pharmacodynamic analysis of biomarkers involved in MAPK, cell cycle, apoptosis, and tyrosine kinase receptors pathways (studied genes are described in the Materials and Methods section) was performed on resected tumors (at the end of treatment/sacrifice). We were able to show an increase in *BAD* and *BMF* proapoptotic factors and a decrease in the cell proliferation factor *CCND1* in the BRAFi-treated groups versus the CTRL group. These results corroborate the tumor proliferation decrease observed *in vivo*. However, this moderate antitumor effect is also associated with a significant increase, for continuous dosing, in the antiapoptotic factors *BCL2L1* and *MCL1* and the tyrosine kinase receptor *MET*, enhancing cellular survival and proliferation through the activation of the RAS-ERK or PI3K-AKT pathways (Figure 1*D*). No other significant changes were observed in MAPK or in cell cycle pathways factors between CTRL and continuous or intermittent BRAFi-treated groups.

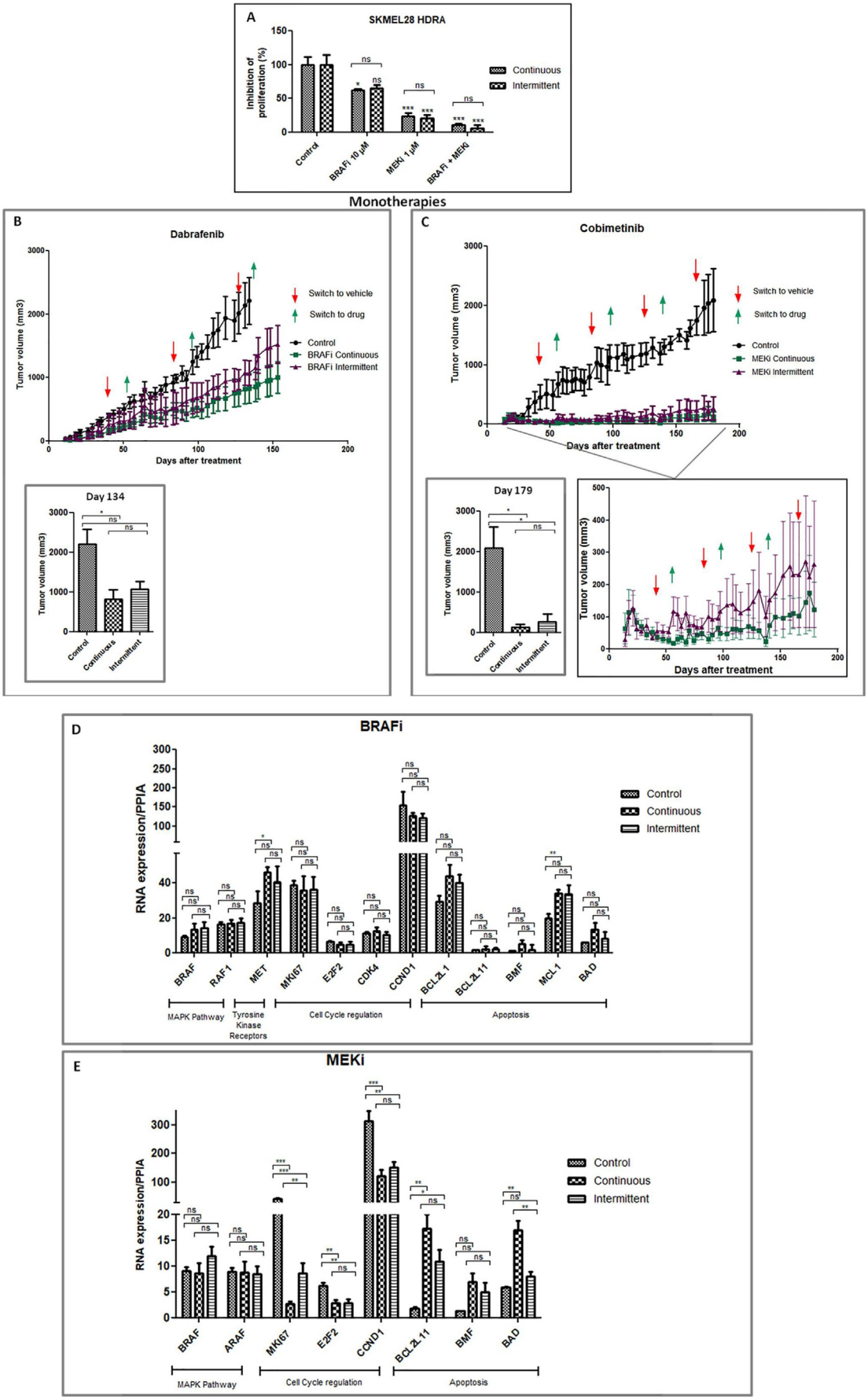
Cobimetinib treatment led to significant tumor regression and disease control using both continuous and intermittent dosing schedules ($P = 0.0107$ and $P = 0.0181$, respectively). Indeed, at day 179, the TGI values were 94% ± 4% and 87% ± 9%, respectively (Figure 1*C*). Biomarker analysis revealed a significant decrease in biotherapy-treated tumors (continuous or intermittent) compared with CTRL tumor in terms of cell cycle pathway factors, including *MKI67* ($P = 0.0001$ and $P = 0.0001$, respectively), *E2F2* ($P = 0.0096$ and $P = 0.0070$, respectively), and *CCND1* ($P = 0.0007$ and $P = 0.0023$, respectively) (Figure 1*E*). In addition, a significant increase of proapoptotic factors was observed in bitherapy-treated tumors versus CTRL, including *BCL2L11* ($P = 0.0023$ and $P = 0.0141$, respectively) and *BAD* ($P = 0.0018$ and ns, respectively). These results are consistent with the highly effective antitumor effects observed.

No significant difference was observed between continuous and intermittent treatments, although intermittently treated tumors tended to be larger than continuous treated tumors at each treatment interruption. These observations are associated with a significant difference of *MKI67* ($P = 0.0049$) and *BAD* ($P = 0.0019$) expression observed between continuous and intermittent groups (Figure 1*E*). Some xenografts resulted in tumor control throughout the experiment. One animal had a progressive tumor in intermittent arm that resulted in the onset of resistance. One case of life-threatening toxicity occurred in the continuous group, and two cases occurred in the intermittent group, and the affected animals had to be sacrificed.

To date, our data on the SKMEL28 melanoma model, which is mildly sensitive to BRAF inhibition, do not show significant benefit of intermittent treatment compared to continuous treatment. This cell line was highly responsive to MEK inhibitor (cobimetinib) during both the continuous and intermittent dosing schedules.

Intermittent Dosing With a Combination of BRAF and MEK Inhibitors is Not Superior to In Vivo Continuous Treatment in a Mildly Responsive Human-Derived Cell Xenograft Model

We then evaluated dabrafenib (100 mg/kg po once daily) in combination with either cobimetinib (5 mg/kg po once daily), trametinib (3 mg/kg po once daily), or pimasertib (20 mg/kg po once


A
SKMEL28 HDRA
Inhibition of proliferation (%)
Continuous
Intermittent
Control
BRAFi 10 μM
MEKi 1 μM
BRAFi + MEKi
Monotherapies
B
Dabrafenib
Tumor volume (mm3)
Days after treatment
Switch to vehicle
Switch to drug
Control
BRAFi Continuous
BRAFi Intermittent
Day 134
C
Cobimetinib
MEKi Continuous
MEKi Intermittent
Day 179
D
BRAFi
RNA expression/PPIA
BRAF
RAF1
MET
MKI67
E2F2
CDK4
CCND1
BCL2L1
BCL2L11
BMF
MCL1
BAD
MAPK Pathway
Tyrosine Kinase Receptors
Cell Cycle regulation
Apoptosis
E
MEKi
ARAF

daily) inhibitor in an SKMEL28 xenograft model in nude mice with a continuous or intermittent dosing schedule for both drugs (four weeks on treatment followed by a two weeks off of treatment).

The combination of BRAF inhibitor with cobimetinib treatment led to significant tumor regression and disease control when using both the continuous and intermittent dosing schedules ($P = 0.0080$ and $P = 0.0107$, respectively) (Figure 2*A*). Indeed, at day 179, the TGI values were 99% ± 0.4% and 94% ± 4%, respectively. No significant difference was observed between continuous and intermittent treatments; however, at each treatment interruption, tumors showed some regrowth that could not be completely rescued by restoration of treatment. Over time, tumors in this group tended to be larger than those in the continuous group. The antitumor effect was associated with a significant decreased in *BRAF* ($P = 0.0008$ for continuous treatment) and *ARAF* ($P = 0.0001$ for both treatments) expression in both treated groups compared with that in CTRL group (Figure 2*D*). Moreover, a significant increase in proapoptotic factors, such as *BCL2L11* ($P = 0.0388$ and ns, respectively), *BMF* ($P = 0.0401$ and $P = 0.0055$, respectively), and *BAD* ($P = 0.0005$ and $P = 0.0442$, respectively) in both treated groups was also observed, and there were no significant differences between continuous and intermittent dosing schedules, except for *BCL2L11* expression ($P = 0.0458$). Furthermore, an important decrease in *CCND1* expression was observed in both treated groups ($P = 0.0054$ and $P = 0.0117$ in continuous and intermittent tumors, respectively) with no significant differences between continuous and intermittent treatment groups (2-fold decrease for both). Thus, the antitumor effect observed in dabrafenib-/cobimetinib-treated groups may be due in part to the inhibition of cell survival and proliferation. However, some mice developed toxicity, with loss of weight, and abdominal swelling, and they had to be euthanized.

Dabrafenib and trametinib combination led to a marked difference between control tumors and tumors from both continuous and intermittent treated groups ($P = 0.0218$ and $P = 0.0303$, respectively) (Figure 2*B*). Indeed, at day 103 of treatment, the TGI values were 84% ± 5% and 66% ± 7%, respectively. At this time, no significant differences were observed between tumors volumes in treated groups. In addition, biomarker analysis revealed no significant changes in the expression of proapoptotic factors in both treated groups, whereas a decrease was observed in antiapoptotic *BCL2* factor during continuous treatment ($P = 0.0491$) (Figure 2*E*). Moreover, *CCND1* ($P = 0.0089$), *MKI67* ($P = 0.0295$), and *E2F2* ($P = 0.0474$) expression was significantly decreased in continuous dosing group, although a nonsignificant decrease in these factors expression were observed in intermittent group compared with control. Nevertheless, the limited antitumor response of this combination in both treated groups may be explained by the increase in the expression of tyrosine kinase receptor *MET,* involved in cellular survival and the absence of expression variation of the MAPK pathway factors (Figure 2*E*).

Continuous and intermittent dosing schedules were also evaluated using dabrafenib combined with a third MEK inhibitor, pimasertib, in SKMEL28-xenografted melanoma cells. Both dosing schedules showed a very weak reduction of tumor growth compared with control, and no significant differences were observed between bitherapy-treated groups. However, maximum tumor regression was observed at day 84 (TGI was 57% ± 7% ($P = 0.0007$) and 47% ± 11% ($P = 0.0125$) for continuous and intermittent groups, respectively) (Figure 2*C*). When comparing MAPK inhibitor- and CTRL-treated tumors, we observed, in both continuous and intermittent treated groups, an increase in the proapoptotic *BCL2L11* factor and a decrease in the cell cycle progression factors, *CCND1* ($P = 0.0404$ and $P = 0.0243$, respectively), *E2F2* ($P = 0.0295$ and $P = 0.0293$, respectively), and *CDK6* ($P = 0.0007$ and $P = 0.0012$, respectively) (Figure 2*F*). Nevertheless, this moderated antitumor response to treatment combination may also be due to an increase in the tyrosine kinase receptor *MET* ($P = 0.0009$ and $P = 0.0023$, respectively). Again, we did not observe any significant difference in biomarker variations between continuous and intermittent dosing schedules (Figure 2*F*).

To further investigate these data, a histological analysis of the mitotic rate of xenografts was performed, revealing a significant difference between treated and CTRL tumors 1.9 ± 0.28 mitoses per microscopic field in CTRL group, and 0.92 ± 0.33 ($P = 0.002$) and 0.9 ± 0.19 ($P = 0.01$) mitoses per field in continuous and intermittent groups, respectively (Supplementary Figure 2*A*). A follow-up test of glucose metabolism was performed in these xenografts with FDG micro PET/CT (Supplementary Figure 2*B*). In the first PET, performed within the first week of treatment, FDG uptake was significantly higher in the control group (SUVmax = 1.81 ± 0.19) compared with both continuous (SUVmax = 1.1 ± 0.14, $P = 0.001$) and intermittent group (SUVmax = 1.02 ± 0.21, $P = 0.027$), suggesting a rapid effect of treatment on tumor metabolism. At day 68 of treatment, FDG uptake in untreated tumors remained higher (SUVmax = 2.7 ± 0.65) than in continuous (SUVmax = 1.7 ± 0.17) and intermittent (SUVmax = 1.69 ± 0.18) tumors ($P = 0.031$ and $P = 0.037$, respectively). Overall, the histological and imaging data corroborate the effect of treatment, which slowed tumor growth, but did not completely stop tumor proliferation.

Overall, these *in vivo* and pharmacodynamic studies clearly showed that in the mildly BRAFi-responsive SKMEL28 melanoma model,

Figure 1. Antitumoral effect of BRAFi/MEKi in *BRAF*-mutated SKMEL28 xenografts treated with continuous/intermittent dosing schedules. (A): *Ex vivo* SKMEL28 HDRA. Percentage of cellular proliferation inhibition with continuous and intermittent dosing schedules during treatment with dabrafenib at 10 μM (BRAFi), cobimetinib at 1 μM (MEKi), or combination of both (BRAFi + MEKi) compared with control in SKMEL28 xenograft-derived histocultures, as determined by MTS assay. The results are representative of 3 independent experiments conducted in quadruplicate. (Mean ± SEM; #, $P < 0.05$ for continuous *versus* intermittent treatments; *$P < 0.05$, **$P < 0.01$, ***$P < 0.0001$ for continuous or intermittent treatment *versus* corresponding control; ns, non significant.) (B): *In vivo* SKMEL28 xenograft growth in nude mice treated with dabrafenib (100 mg/kg po once daily) or (C): with cobimetinib (5 mg/kg po once daily) with continuous and intermittent monotherapy dosing schedules compared with vehicle. (Mean ± SEM; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, non significant). Controls were treated with PBS-DMSO 5%. Five mice were tested in each treatment and control group. (D): Transcript levels analysis for BRAF or MEK inhibitor monotherapies. mRNA analysis performed on CTRL and continuous or intermittent dabrafenib or (E): cobimetinib-treated tumors by qRT-PCR on 30 genes involved in MAPK, cell cycle, or apoptosis pathways compared with the reference gene *PPIA*.(Mean ± SEM; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, non significant). The results are representative of 3 independent experiments conducted in triplicate.

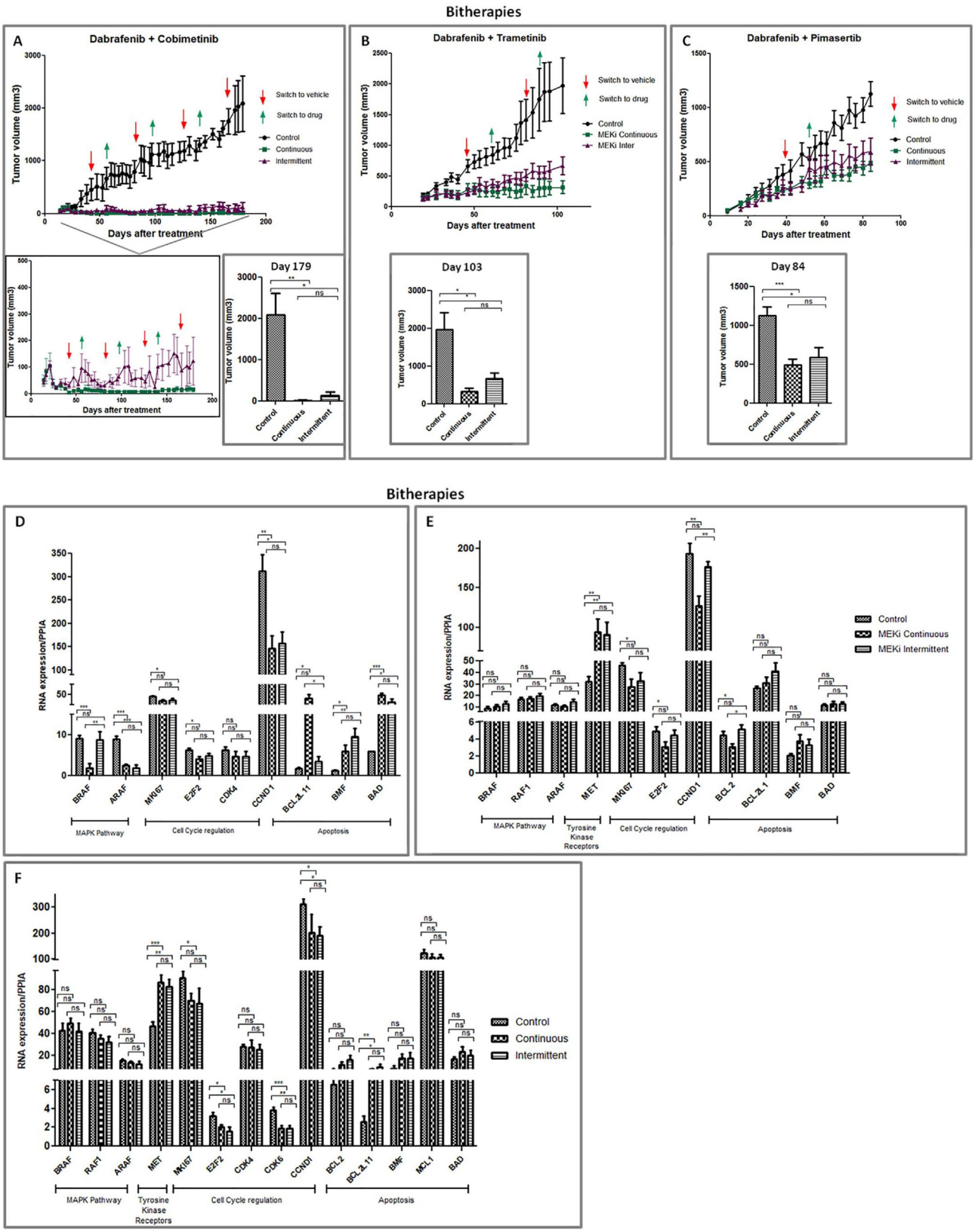


Figure 2. Antitumoral effect of BRAFi combined with MEKi in *BRAF*-mutated SKMEL28 xenografts treated with continuous/intermittent dosing schedules. (A): *In vivo* SKMEL28 xenograft growth in nude mice treated with dabrafenib (100 mg/kg po once daily) combined with cobimetinib (5 mg/kg po once daily), (B): with trametinib (3 mg/kg po once daily) or (C): with pimasertib (20 mg/kg po once daily) in continuous and intermittent bitherapy dosing schedules compared with vehicle. (Mean ± SEM; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, non significant). Controls were treated with PBS-DMSO 5%. Five mice were tested in each treatment and control group. (D): Transcript levels analysis of BRAFi combined with different MEK inhibitors on bitherapies. mRNA analysis was performed for CTRL tumors and for tumors treated with continuous or intermittent dabrafenib combined with cobimetinib, (E): trametinib, or (F): pimasertib by qRT-PCR on 30 genes involved in MAPK, cell cycle, or apoptosis pathways compared to the reference gene *PPIA*. (Mean ± SEM; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, non significant). The results are representative of 3 independent experiments conducted in triplicate.

dabrafenib and MEK inhibitor combinations did not reveal the superiority of an intermittent dosing schedule compared with continuous treatment, and this was observed with the three MEK inhibitors, cobimetinib, trametinib, and pimasertib.

Intermittent Dosing of BRAF and/or MEK Inhibitors is Not Superior to Continuous Treatment in Patient Tumors According to Histoculture Drug Response Assays

To evaluate the potential efficacy of continuous and intermittent dosing of BRAF and MEK inhibitors in the treatment of human melanoma, *ex vivo* HDRA were conducted on 6 $BRAF^{V600E}$-mutated PDX and the percentage of cellular proliferation inhibition compared with control was determined. Patients' main clinical data are summarized in Table S2. Of the 6 $BRAF^{V600E}$ tumors collected, 5 represented baseline lesions (before BRAFi therapy) and 1 was a relapsed tumor in response to BRAFi treatment as first line. The clinical follow-up revealed that 5 out of 6 patients received an MAPK inhibitor therapy (BRAF or BRAF combined with MEK inhibitors) and 3 patients were partial responders and the 3 others were complete responders (response duration 4−48 months). One patient developed high-grade toxicity early after therapy initiation drug reaction with eosinophilia and systemic symptoms (DRESS), leading to treatment interruption and a switch to dabrafenib combined with trametinib (Figure 3*A*).

For the 6 analyzed PDX, HDRA revealed a significant sensitivity to continuous and intermittent treatment with BRAFi (dabrafenib) or MEKi (cobimetinib) alone and in combination (mean decrease of proliferation rate of 58% and 63%, for BRAFi, 59% and 63% for MEKi and 81% and 81% for combination, respectively) (Figure 3*B*). Overall, in this HDRA study, two melanoma responsive profiles were observed: a mild and a highly sensitive profile with a proliferation decrease between 20 and 50% and another with a decrease of more than 50%. Indeed, HDRA of PDX from patients 1, 3, and 5 were mildly responsive to continuous and intermittent BRAF inhibitor treatment and showed decreases in proliferation of 44 ± 5% and 56 ± 5%, 28 ± 7% and 25 ± 6%, and 20 ± 5% and 35 ± 5%, respectively. Moreover, these HDRAs also showed mild sensitivity to continuous and intermittent MEK inhibitor treatment with decreases in proliferation of 47 ± 6% and 50 ± 18%, 39 ± 7% and 35 ± 5%, and 43 ± 6% and 38 ± 3%, respectively. Additionally, these three HDRAs showed high sensitivity to continuous and intermittent BRAF and MEK inhibitors combination with proliferation decreases of 84 ± 8% and 80 ± 6%, 57 ± 8% and 59 ± 5%, and 79 ± 3% and 67 ± 6%, respectively (Figure 3*C*).

The second HDRA profile includes PDX from patients 2, 4, and 6, which were highly responsive to continuous and intermittent BRAF inhibitor treatment, with decreases of 77 ± 3% and 81 ± 2%, 86 ± 2% and 90 ± 2%, and 92 ± 2% and 93 ± 5%, respectively, and also highly sensitive to continuous and intermittent MEK inhibitor treatment, with decreases in cell proliferation of 73 ± 3% and 86 ± 1%, 76 ± 4% and 89 ± 5%, and 74 ± 5% and 81 ± 8%, respectively. The tumors were, as expected, highly responsive to continuous and intermittent treatment with a combination of dabrafenib and cobimetinib, with decreases of proliferation of 87 ± 1% and 92 ± 2%, 89 ± 2% and 94 ± 4%, and 91 ± 4% and 96 ± 2%, respectively. Both HDRA profiles did not show a significant difference in the dosing schedules for the BRAF and MEK inhibitors (Figure 3*C*).

In parallel with the HDRA, a comprehensive biomarker analysis was conducted to identify meaningful mutations (next-generation sequencing [NGS] and copy number analyses) and alterations in gene expression involved in signaling pathways targeted directly or indirectly by BRAF/MEK inhibitors. For this, PDX tumor slices obtained before treatment were subjected to genomic and transcriptomic analyses using the same gene panel presented above. DNA OncoPrint and mRNA heatmap revealed several gene alterations, including mutations, amplifications/deletions as well as transcripts overexpressions or downexpressions (Figure 3*D* and *E*). Indeed, both oncogenes and tumor suppressor genes were modulated in the two HDRA profiles, with no significant differences in tumor burden. DNA analysis allowed the detection in PDX samples of 2 mutated genes with predominance for *TP53* mutations in 2 out of 6 cases (Figure 3*D*). In addition, *MET* amplification and *CDKN2A* alteration (as analyzed by copy number variation, CNV) were the most frequently observed CNVs (2 out of 6 patients [29%] and 3 out of 6 patients [43%], respectively). Regarding the mRNA analysis, the mildly sensitive PDX profile showed an increase in 2 out of 3 PDX of genes that were mainly involved in the cell cycle, apoptosis and RTK pathways (*KI67*, *CCND1*, *E2F*, *CDK6*, *IGF1R*, *EGFR*, *ARAF*, *BCL2L11*, *MCL1*, and *BAD*). Indeed, an increase in the oncogenes *ARAF*, *IGF1R*, or *CDK6* (PDX 1 and 5) and a decrease in the suppressor genes *PTEN* (PDX 1, 5, and 3), *CDKN1A*, and *CDKN1B* (PDX 1 and 5) were observed.

Overall, our results further confirm that intermittent dosing with BRAF and/or MEK inhibitors is not superior to continuous treatment in melanoma PDX on the basis of *in vivo* and *ex vivo* evaluations, and these findings are corroborated by the NGS and mRNA biomarker analyses of the tumors.

Discussion

The use of intermittent versus continuous regimen of BRAF and/or MEK inhibitors is a current issue that warrants further investigation. In this study, we provide insights that demonstrate an equal impact *in vivo* and *ex vivo* of the intermittent and continuous regimens of BRAF and/or MEK inhibitor treatment on model that is mildly responsive to BRAF inhibitor in monotherapy. In addition, our data are strengthened by a DNA and RNA biomarker study that explains in a complementary manner the antitumor effects observed.

The SKMEL28 melanoma model studied was mildly responsive to BRAF inhibitor but highly sensitive to MEK inhibitor in monotherapies, as demonstrated by *in vivo* and *ex vivo* experiments.

In human melanoma vemurafenib-resistant xenograft models obtained by continuous administration of the drug, Das Thakur et al. demonstrated that proliferation of vemurafenib-resistant cells became dependent on the continuous presence of the drug and that intermittent dosing of vemurafenib delayed the onset of drug resistance, and they also showed that a discontinuous vemurafenib regimen could prevent such resistance [18].

In our mildly sensitive model, the *in vivo* effect of dabrafenib was associated not only with a small decrease in the cell cycle gene *CCND1* but also with increases in the apoptosis genes *BCL2L1* and *MCL1* and the tyrosine kinase receptor *MET*. In contrast, Das Thakur's study of BRAF resistance was mainly explained by the overexpression and/or amplification of $BRAF^{V600E}$, demonstrating that genomic differences in the studied models could explain the observed discrepancies.

Regarding MEK inhibitors, the high efficacy observed with continuous cobimetinib in monotherapy or cobimetinib in combination with dabrafenib may maintain a suppression of tumor growth for a prolonged period of time without onset of resistance. Nevertheless,

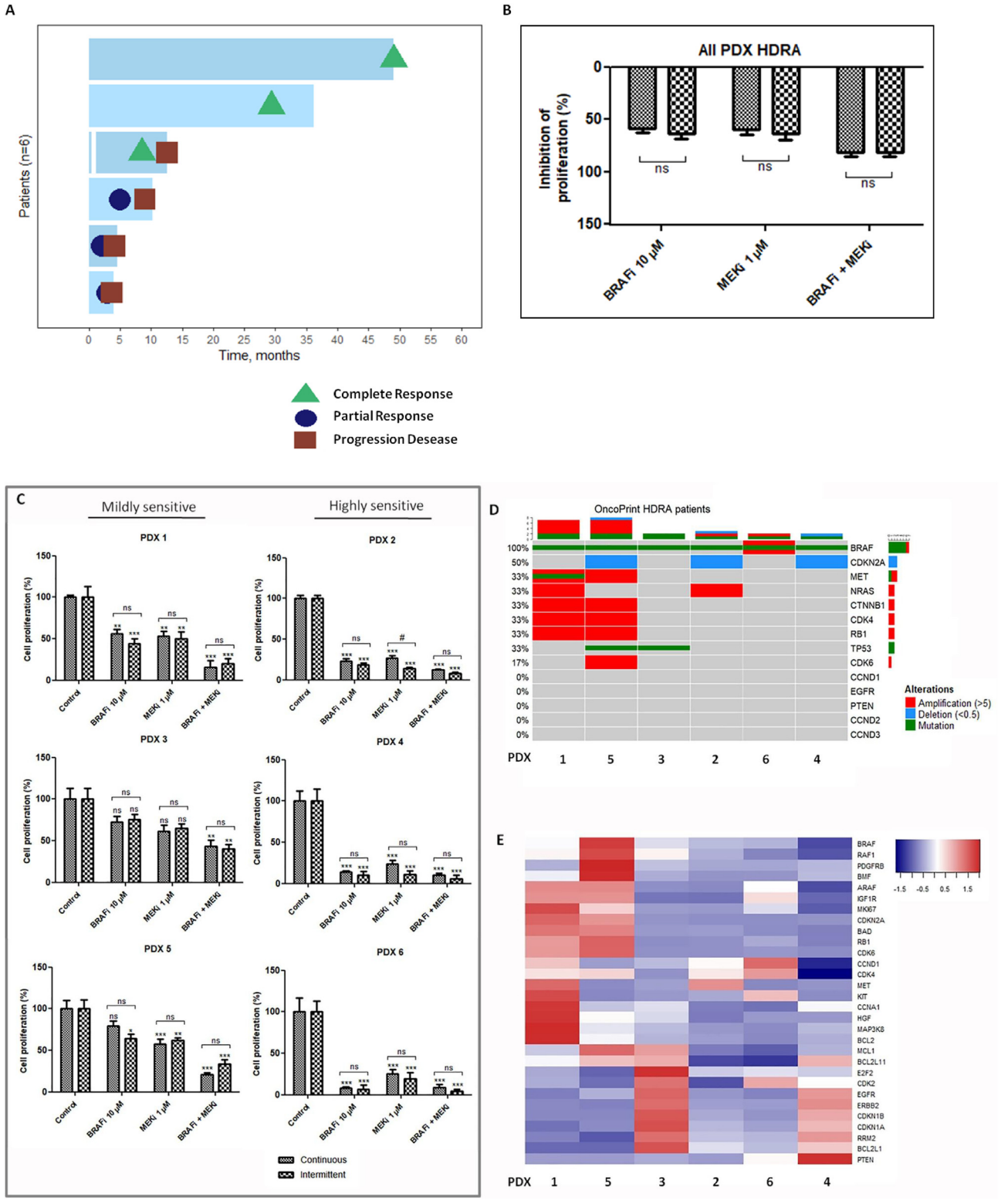


Figure 3. Antitumoral effect of dabrafenib and/or cobimetinib in patient-derived xenografts (PDX) measured by histoculture drug response assays. (A) Swimmer plot of the 6 included patients ranked according their overall survival. T_0 is the time of BRAF and/or MEK inhibitors initiation. (B) All and (C) separated *ex vivo* HDRA conducted on 6 BRAF-mutated PDX. Inhibition rates of dabrafenib at 10 µM (BRAFi), cobimetinib at 1 µM (MEKi) or combination of both (BRAFi + MEKi), as determined by MTS assay. The results are representative of 3 independent experiments conducted in quadruplicates. (Mean ± SEM; #, $P < 0.05$ for continuous *versus* intermittent treatments; *$P < 0.05$, **$P < 0.01$, ***$P < 0.0001$ for continuous or intermittent treatment *versus* corresponding control; ns, non significant.) DNA alterations and transcriptional changes in PDX used for HDRA. (D) OncoPrint showing the genomic profile of 6 tumors from PDX. Mutations are represented in green; amplifications and deletions are in red and blue, respectively. (E) Heatmap generated from mRNA analysis of the 30 genes in 6 melanoma tumors from PDX. Colors represent the relative expression of each gene in each sample, which is centered on the mean and scaled to the standard deviation. Blue shows low expression and red shows high expression.

cobimetinib intermittent dosing led to tumor regrowth after treatment interruptions that could not be compensated when resuming drug administration. These observations were associated with significant increases in *BCL2L11*, *BMF*, and *BAD* and significant decreases in *BRAF*, *ARAF*, and *CCND1*. It is interesting to note that the mild *in vivo* effects obtained with trametinib and pimasertib are also explained by a molecular analysis that revealed not only decreases in *CCND1* and *E2F2* expression but also an increase in *MET* expression to enhance cell proliferation and survival.

Xue et al. noted that sequential monotherapy is not recommended because it is responsible for selecting resistant clones. Indeed, with a comparison of RAFi, MEKi, or ERKi monotherapy treatment, the authors proposed that in subclonal populations, higher levels of *BRAF* amplification are needed to overcome the effect of the targeted therapy. Moreover, Xue et al. demonstrated that the triple association of RAF, MEK, and ERK inhibitors could achieve a long-lasting target inhibition, without selection of such resistance-associated alterations. Furthermore, Xue et al. confirmed the efficacy of several intermittent regimens that used either two weeks of treatment followed by two-weeks off of treatment, or a schedule in which three drugs were administered 3 days out of 7, performing equally with continuous dosing. Thus, an intermittent administration regimen allows a recovery time for the treated subject, potentially limiting toxicity and removing some of the selective pressure on the tumor [36].

However, some data are conflicting in the clinical setting. Indeed, Fisher et al. described a decrease in tumor growth, after cessation of MAPK inhibitors administration in some progressive patients, whereas an accelerated progression after cessation of tyrosine kinase inhibitors was also observed in the same study and represents a concern in a fraction of patients [37]. Moreover, the complexity of involved mechanism and the heterogeneity of tumor cells within the same patient may make it difficult to determine how a particular patient will react to different therapy schedules [38]. In addition, a recent analysis showed important heterogeneity in genomic alterations in patient tumors from complete and partial responders before vemurafenib ± cobimetinib treatment. Indeed, some differences in baseline tumors in *MITF, TP53*, or *NF1* expression levels were found between CR and PD patients, whereas mutational alterations were similar in patients with both types of response, suggesting that no mutations were identified as response drivers in the population [39]. Given the different behaviors of tumors, an approach allowing for prediction of antitumor efficacy of MAPK inhibitors and drug-resistance onset would be very valuable.

In our study, HDRAs of xenografted tumor cells and PDX correctly predicted drug sensitivity to monotherapy and the synergistic effects of combination therapy with a dose-effect trend. We determined if HDRA could also identify patients who may benefit from an intermittent schedule. Our *in vivo* results were validated by HDRA and did not reveal significant differences between continuous and intermittent schedules *ex vivo*. This could be due to the short time of HDRA (8 days), which is not sufficient to show a significant difference between treatment schedules, or to the fact that there might not be a difference in these particular cases.

In addition, two groups of PDX identified either mildly or highly responsive to MAPK inhibitors harbored similar tumor mutation burden, including alterations in oncogenes and suppressor genes. These results are consistent with previous proposed molecular signatures (that included both oncogenes and suppressive regulators), as relevant predictors of clinical response to BRAF inhibitors included both activating [40,41]. If confirmed in larger sample, an exhaustive study of molecular profiles of tumor and an *ex vivo* drug sensibility assay could further contribute to the tailoring of a therapeutic strategy for a given patient.

Currently, a randomized phase 2 clinical trial comparing intermittent and continuous dosing schedules of dabrafenib combined with trametinib is being investigated and will probably provide some valuable information on how this approach should best be used in treating advanced $BRAF^{V600E}$-mutant melanoma (SWOG/CTEP S1320, NCT02196181, [42]). Collection of biomarkers and molecular data from naïve and treated patients as much as possible to further understand treatment sensibility and resistance mechanisms is also of paramount importance.

Conclusions

These preliminary *in vivo* and *ex vivo* works, realized in xenografted melanoma cell models and PDX histocultures, converge to show the similarity between intermittent and continuous treatments with either BRAF or MEK inhibitors monotherapy or bitherapy. To confirm these data, larger studies that consider tumor heterogeneity, which is a determining factor in tumor treatment response, are mandatory.

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Conflict of Interest

CRdM, LV, FJ, BB, AS, BL, JD, KS, LG, PM have no conflicts of interest to declare. MB declares a consulting role for Histalim, BMS, and Innate Pharma. CL declares honoraria from Roche, advisory roles at Roche, GSK, Novartis, BMS, MSD, and Amgen and travel accommodation provided by Roche. SM declares a consulting role at Roche, Janssen, Biocartis and Novartis.

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Appendix B. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tranon.2019.10.003.

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