Prominent role of cyclic adenosine monophosphate signalling pathway in the sensitivity of $^{\text{WT}}$BRAF/$^{\text{WT}}$NRAS melanoma cells to vemurafenib

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Abstract Vemurafenib improves survival in patients with melanoma bearing the $^{\text{V600E}}$BRAF mutation, but it did not show any benefit in clinical trials focusing on wild type tumours while it may well inhibit $^{\text{WT}}$BRAF considering the dosage used and the bioavailability of the drug. As tumours may contain a mixture of mutant and wild type BRAF cells and this has been also put forward as a resistance mechanism, we aimed to evaluate the sensitivity/resistance of six, randomly selected, $^{\text{WT}}$BRAF/$^{\text{WT}}$NRAS lines to vemurafenib and found four sensitive. The sensitivity to the drug was accompanied by a potent inhibition of both phospho-ERK and phospho-AKT, and a significant induction of apoptosis while absent in lines with intrinsic or acquired resistance. Phospho-CRAF expression was low in all sensitive lines and high in resistant ones, and MEK inhibition can effectively potentiate the drug effect. A possible explanation for CRAF modulation is cyclic adenosine monophosphate (cAMP), a mediator of melanocortin receptor 1 (MC1R) signalling, since it can actually inhibit CRAF. Indeed, we measured cAMP and found that all four sensitive lines contained significantly higher constitutive cAMP levels than the resistant ones. Consequently, vemurafenib and cAMP stimulator combination resulted in a substantial synergistic effect in lines with both intrinsic and acquired resistance but only restricted to those where cAMP was effectively increased. The use of a cAMP agonist overcame such restriction. In conclusion, we report that $^{\text{WT}}$BRAF/$^{\text{WT}}$NRAS melanoma lines with low phospho-CRAF and high cAMP levels may be sensitive to vemurafenib and that CRAF inhibition through cAMP stimulation may overcome the resistance to the drug.

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1. Introduction

Melanoma is the fifth most common cancer for males and the sixth for females [1]. The survival rate for patients with early detection of melanoma is about 99%, while it falls to 15% for those with advanced disease [2]. Whereas the incidence of many common cancers is falling, the incidence of melanoma continues to rise [3].

Increased understanding of the molecular events involved in melanoma development has led to the identification of novel targets and to the development of new targeted agents. Gene alterations identified in melanoma pointed to distinct molecular subsets of tumours with direct implications in therapeutic strategies. Among these, activating v-raf murine sarcoma viral oncogenes homolog B1 (BRAF) mutations occurring in 50–60% of melanomas [4] (V600E substitution represents about 90% of BRAF mutations) and Neuroblastoma RAS viral [V-ras] oncogene homolog (NRAS) mutations in 15–25% of melanomas (mutually exclusive with BRAF mutation) opened new therapeutic perspectives targeting the MAPK (mitogen activated protein kinase) pathway with, among others, \( \text{V}_\text{600E}\text{BRAF} \), BRAF or Mitogen-activated protein kinase kinase (MEK) inhibitors. Vemurafenib (PLX4032, RG7204) is a \( \text{V}_\text{600E}\text{BRAF} \) kinase inhibitor which improved rates of both progression-free and overall survival compared to dacarbazine in patients with previously untreated \( \text{V}_\text{600E}\text{BRAF} \) melanoma [5]. Nevertheless, in spite of significant initial responses in about half of melanoma patients, resistant relapses are largely documented [6]. Recent studies reported that recurrences may be due to switches between pathways [5], activation/stabilisation of v-raf-1 murine leukemia viral oncogenes homolog 1 (CRAF) [7], COT/MAP3K8 activation [8], appearance of new activating mutations in C121SMEK1 [9], dimerisation of aberrantly spliced WTBRAF/WTNRAS lines to vemurafenib. We also examined the mechanism(s) of resistance to the drug in cell lines with intrinsic as well as acquired resistance. Finally, we assessed the role of cAMP signalling in the sensitivity of \( \text{V}_\text{600E}\text{BRAF} \) melanoma cells to vemurafenib.

2. Material and methods

2.1. Effectors

Vemurafenib (PLX4032) and forskolin (FSK) (both from Selleck Chemicals, Houston, TX, United States of America (USA)), U0126 (from Toeris Bioscience, Ellisville, MO, USA) and 3-isobutyl-1-methylxanthine (IBMX) (from Sigma–Aldrich, St. Louis, MO, USA) were dissolved in DMSO and stored at \(-20^\circ C\). Rp-cAMP (from Enzo Life Sciences, Lausen, Switzerland) was dissolved in water and stored at \(-20^\circ C\).

2.2. Melanoma cell lines

Human melanoma cell lines used in this study were all established in our laboratory from lymph node or skin metastases. V600E and G61R mutations in melanomas in vemurafenib-treated patients who had a clinically significant response [15]. Thus, the effect of \( \text{V}_\text{600E}\text{BRAF} \) inhibitors on \( \text{V}_\text{600E}\text{BRAF} \) melanoma remains a crucial unsolved issue [10].

The two major signalling pathways that are simultaneously activated in melanocytes are the cyclic adenosine monophosphate (cAMP) and the MAPK pathways and interactions between these pathways are essential for regulating melanocyte fate [16]. Cyclic AMP is a second messenger produced after the activation of G-protein-coupled receptors (GPCR). Through activation of the cAMP-dependent protein kinase A (PKA), cAMP stimulates both phosphorylation and activation of the cAMP responsive element-binding protein (CREB) transcription factor, which in return stimulates transcription of the microphthalmia-associated transcription factor (MITF) [17]. Melanocortin receptor 1 (MC1R) belongs to the latter class of receptors and is overexpressed in melanoma cells compared to melanocytes. Of note, MC1R mutations were found associated with BRAF mutations and confer high risk for melanoma [18]. Cyclic AMP is regulated in both a spatial and a temporal manner by cAMP phosphodiesterases (PDEs), which provide the sole route for degradation of cAMP in cells [19]. Cyclic AMP signalling can also regulate the MAPK kinase pathway and RAF isoform switching by inhibiting CRAF and activating BRAF [20–24], suggesting that it may be involved in resistance of melanoma cells to BRAF inhibitor.

Because of mutant/wild type BRAF heterogeneity in melanoma and its association with drug resistance, we aimed to evaluate the sensitivity of a panel of six \( \text{V}_\text{600E}\text{BRAF} \)/\( \text{V}_\text{WT}\text{NRAS} \) lines to vemurafenib. We also examined the mechanism(s) of resistance to the drug in cell lines with intrinsic as well as acquired resistance. Finally, we assessed the role of cAMP signalling in the sensitivity of \( \text{V}_\text{WT}\text{BRAF} \)/\( \text{V}_\text{WT}\text{NRAS} \) melanoma cells to vemurafenib.
with 5% heat-inactivated foetal calf serum, 5% heat-inactivated newborn calf serum and with L-glutamine, penicillin and streptomycin at standard concentrations (all from Gibco, Invitrogen, United Kingdom (UK)). Cells were harvested by trypsinisation (0.05% trypsin–EDTA) (Gibco) and subcultured twice weekly. Cell count was evaluated using a TC10 Automated Cell Counter (Bio-Rad, Hercules, CA, USA). Cell lines are regularly checked for mycoplasma contamination using MycoAlert® Mycoplasma Detection Kit (Lonza, Rockland, ME, USA).

2.4. Proliferation assays

Cell proliferation was assessed by crystal violet assay. All cells were seeded in 96-well plates (8 × 10^3 cells/well) in complete medium (day 1). One day after plating (day 0), the culture medium was replaced by fresh medium containing or not effectors depending on experimental conditions, and cells were cultured for 1, 2 or 3 additional days. Then, culture medium was removed and cells were gently rinsed with phosphate-buffered saline (PBS), fixed with 1% glutaraldehyde/PBS for 15 min and stained with 0.1% (v/v in water) crystal violet for 30 min. Cells were destained under running tap water and subsequently lysed with 0.2% (v/v in water) Triton X-100 for 90 min. The absorbance was measured at 570 nm using a Multiskan EX Microplate Photometer (Thermo Scientific, Courtaboeuf Cedex, France).

2.5. Apoptosis determination

Apoptotic cells were detected by Annexin-V: PE Apoptosis Detection Kit I (BD Pharmingen, Erembodegem-Dorp, Belgium), according to the manufacturer’s recommendations and analysed in a flowcytometer (FACS Calibur, Becton Dickinson, Franklin Lakes, NJ, USA).

2.6. Western blot analysis

Western blot analysis was performed as previously described [25]. Immunodetections used antibodies raised against phospho-BRAF (Ser445) (1/1000), CRAF (1/1000), phospho-CRAF (Ser 338) (1/1000), v-akt murine thymoma viral oncogene homolog (AKT) (1/1000), phospho-AKT (Ser 473) (1/500) (both from Cell Signaling Technology), extracellular signal-regulated kinase (ERK) 2 (1/500), phospho-ERK (Tyr 204) (1/500), BAX (1/1000), p21 (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA) and β-actin (1/500) (both from GE Healthcare Europe GmbH, Diegem, Belgium) were used as secondary reagents to detect corresponding primary antibodies.

2.7. cAMP measurement

Intracellular cAMP was measured with the Cayman cAMP ACE™ competitive EIAs (Cayman Chemical Company, Ann Arbor, MI, USA) according to the manufacturer’s protocol. Briefly, cells were seeded in Petri dishes (3 × 10^6 cells/dish) in culture medium. One day after plating, the culture medium was replaced by a fresh one and further left for 2 days. Then, cells were exposed or not to IBMX or FSK for 30 min and absorbance was measured at 405 nm in a Multiskan EX Microplate Photometer (Thermo Scientific).

2.8. Total protein evaluation

Protein concentrations in total cell lysates obtained by detergent (for Western blotting) or acid (for cAMP measurement) extraction were determined by the BCA Protein Assay (Pierce, Rockford, IL, USA) using bovine serum albumin as standard.

2.9. Statistical analysis

IC50 were calculated using GraphPad Prism software (GraphPad Software, La Jolla, CA, USA). Data are expressed as means ± SD of at least three independent experiments. Significance was calculated by Student’s t-test.

3. Results

3.1. Comparison of vemurafenib IC50 with constitutive status of relevant signalling pathways in a panel of representative melanoma cell lines

We first assessed the relative sensitivity to vemurafenib of six randomly selected WT BRAF/WT NRAS melanoma lines (Fig 1A). We used two positive (V600E BRAF) and two negative (Q61R NRAS) lines as controls. Surprisingly, four out of six lines with WT BRAF/WT NRAS were sensitive to vemurafenib with IC50 ranging between 0.8 and 3.0 μM, while two lines were resistant lines with IC50 above 10 μM. As expected, the V600E BRAF lines were sensitive to vemurafenib while the Q61R NRAS mutated ones were resistant. To investigate the mechanism(s) underlying this sensitivity, we compared the expression of key effectors of the MAPK and AKT signalling pathways by Western blot (Fig 1B). Phospho-ERK was significantly inhibited by 1 μM vemurafenib in sensitive lines, while it was clearly increased in the resistant ones. Importantly, phospho-CRAF was significantly higher in resistant lines. Phospho-BRAF was not affected by vemurafenib, while phospho-AKT was inhibited in all lines.
3.2. Compared effect of vemurafenib on proliferation and apoptosis in vemurafenib sensitive and resistant wild type BRAF/NRAS lines

To further explore the sensitivity/resistance of wild type BRAF/NRAS lines to vemurafenib, we compared two representative sensitive (HBL) and resistant (LND1) lines knowing that vemurafenib decreased phospho-ERK in a concentration-dependent manner (from 0.01 to 10 μM) in the former one, while it increased phospho-ERK at 1 and 3 μM in the latter (Fig. 2A). Consequently, cell proliferation was completely inhibited by 1 μM vemurafenib in the sensitive line, while it was not affected or only partially inhibited by 10 μM vemurafenib after 3 days of exposure in the resistant one (Fig. 2B). As expected, vemurafenib significantly induced apoptosis after 2 days of treatment only in the sensitive line as assessed by Annexin-V binding and flow cytometry (Fig. 2C).

3.3. Breaking intrinsic resistance to vemurafenib by combining BRAF to MEK inhibition in wild type BRAF/NRAS cells

As resistance to vemurafenib was associated with an enhancement of ERK phosphorylation in cells with intrinsic resistance (LND1), we assessed the effect of BRAF and MEK inhibitor combination on cell proliferation and apoptosis. We found that the MEK inhibitor U0126 broke the resistance to vemurafenib in a concentration-dependent manner with up to 10-fold decrease of IC50 with 5 μM U0126 (Fig. 3A). This was
accompanied by a significant increase of apoptosis (Fig. 3B). In contrast, this combination was ineffective in sensitive cells as phospho-ERK was already completely inhibited by vemurafenib alone.

3.4. Breaking acquired resistance to vemurafenib by combining BRAF to MEK inhibition in wild type BRAF\textsuperscript{NRAS} cells

Firstly, we generated a melanoma line, termed HBL-R, with acquired resistance to vemurafenib by chronic exposure of the sensitive HBL line to increasing concentrations of the drug (from 0.01 to 2 μM) over a period of 12 weeks (Fig. 4A). Secondly, we examined the changes in relevant signalling pathways comparing the resistant line, in the presence or after washing out the drug, with the parental one (Fig. 4B). Both phospho-ERK and phospho-CRAF expression were higher in the resistant line, with no significant changes in phospho-BRAF or phospho-AKT. Like it was the case above for cells with intrinsic resistance (LND1), vemurafenib did not completely inhibit phospho-ERK but stimulated phospho-CRAF, thus leading to a 100-fold increase in IC50 shifting the cells from sensitive to resistant (Fig. 4C). Again, combining vemurafenib to MEK inhibitor, we found a very significant inhibition on cell proliferation (IC50 ~10-fold lower) (Fig. 4C) as well as a significant increase in cell apoptosis (Fig. 4D).

3.5. cAMP intracellular levels in wild type BRAF\textsuperscript{NRAS} vemurafenib sensitive or resistant melanoma cells

Because of known cAMP/CRAF pathway, we investigated whether high CRAF activity-mediated phospho-
ERK rebound is due to low levels of cAMP. Indeed, we found that the four vemurafenib sensitive lines contained higher constitutive amounts of cAMP than the two intrinsically resistant ones (Fig. 5A). Furthermore, the line with acquired resistance (HBL-R) also showed a lower cAMP level in comparison with its parental line (HBL) (Fig. 5B). We also checked whether cAMP could be stimulated in resistant cells and found that phosphodiesterase inhibitor (IBMX) or the adenyl cyclase stimulator (FSK) caused a significant increase in all lines except in one, MM098, suggesting a defective cAMP signalling in the latter line (Fig. 5B).

3.6. Breaking resistance to vemurafenib by cAMP stimulation

Stimulating cAMP by IBMX or FSK in cells with intrinsic (LND1) or acquired (HBL-R) resistance causes an inhibition of phospho-ERK rebound after vemurafenib exposure, associated with a dramatic inhibition of phospho-CRAF (Fig. 6A). As expected, such effect could not be achieved in the resistant MM098 cells as they have a defective cAMP signalling. The use of a cAMP agonist (Sp-cAMP) but not its antagonist (Rp-cAMP) was effective not only in the latter line but in all resistant lines (Fig. 6A), confirming that cAMP may impact CRAF phosphorylation and subsequently ERK activity. This also translated into a very significant synergistic effect on proliferation of lines with intrinsic and acquired resistance whatever was the mean used to elevate cAMP intracellular levels (Fig. 6B and C).

4. Discussion

A decade after the discovery of BRAF mutations in melanoma [4], vemurafenib, an orally available and well tolerated V600E BRAF inhibitor, ushered in a new era of molecular treatments for advanced melanoma [26]. Unfortunately, patients’ disease is more likely to progress following initial response to the drug. Heterogeneity of the BRAF mutation (V600E) within and among lesions of the same patient has been recently suggested to be relevant for the variable clinical responses to vemurafenib both in case of intrinsic or acquired resistance [11,12].

Several arguments are in favour of a possible inhibition of WT BRAF by vemurafenib in patients. A recent ex-vivo study on tumour biopsies reported that a few
WTBRAF melanomas showed kinase inhibition profiles similar to mutated BRAF tumours [27]. Another recent study put forward that heterogeneity of \(^{\text{V600E}}\)BRAF protein expression in patients treated with mutated BRAF inhibitors did not correlate with clinical outcome [13]. In addition, a previous study showed that vemurafenib had an effective IC50 of 100 nM to inhibit \(^{\text{WT}}\)BRAF activity compared to 31 nM for \(^{\text{V600E}}\)BRAF [28].

In keeping with this view, at the recommended phase II dose of 960 mg twice daily, the mean maximum vemurafenib concentration at steady state was 86 ± 32 µM, the mean area under the plasma concentration–time curve over a 24-h period (\(\text{AUC}_{0-24}\)) was 1741 ± 639 µM × h, and the mean half-life was estimated to be 50 h (range 30–80) [14], suggesting that such levels can be sufficient to affect both mutant and wild type BRAF melanoma cells.

Unlike a few \textit{in vitro} studies that evaluated the response of \(^{\text{WT}}\)BRAF/\(^{\text{WT}}\)NRAS melanoma lines to \(^{\text{V600E}}\)BRAF inhibitors and have invariably reported that such lines were resistant to the drugs (IC50 > 10 µM) [10,29–31], and by carrying out a wide drug screening in a large panel of melanoma lines, we unexpectedly and repeatedly found four out of six tested \(^{\text{WT}}\)BRAF/\(^{\text{WT}}\)NRAS lines sensitive to vemurafenib (IC50 < 3 µM) through a strong inhibition of ERK phosphorylation, but without changes in BRAF expression or phosphorylation as previously reported [29].
These findings prompted us to further explore the underlying mechanisms.

We first explored the mechanism(s) of cell resistance to the drug and found that resistant wild type lines exhibited high level of CRAF phosphorylation, even more after treatment, and that vemurafenib increased ERK phosphorylation as well. Accordingly, previous data showed that, while vemurafenib can suppress ERK phosphorylation and induce cell cycle arrest and apoptosis in V600E BRAF lines [32], it can paradoxically activate the MAPK pathway, enhancing ERK phosphorylation and promoting cell proliferation via a CRAF-mediated mechanism [33] involving the formation of BRAF/CRAF heterodimers or CRAF/CRAF homodimers in oncogenic RAS lines [7] as well as in WT BRAF ones [10,29–31]. Thus, resistance to vemurafenib may be associated with CRAF activation in mutant BRAF lines as well as in wild type BRAF ones with both intrinsic and acquired resistance to the drug.

Therefore, to break the resistance to vemurafenib in wild type BRAF/NRAS melanoma cells and based on a recent study showing a high sensitivity of some lines with WT BRAF status to the MEK inhibitor trametinib [34], we combined vemurafenib to a MEK inhibitor and obtained a significant synergistic effect on cell proliferation and apoptosis. Up to our knowledge, no such data have been previously reported, confirming that the rebound in ERK activity following treatment with vemurafenib is dependent on CRAF which can be downstream inhibited by MEK inhibitors.

Another way to moderate CRAF is acting through cAMP signalling pathway as it is involved in a unique feature of the melanocyte that is melanogenesis. Indeed, cAMP is a second messenger which affects MAPK

Fig. 5. Measurement of cyclic adenosine monophosphate (cAMP) in sensitive and resistant WT BRAF lines. (A) Intracellular cAMP levels in vemurafenib sensitive and resistant WT BRAF lines. Data are presented as means ± SD (n = 3). (B) Effect of 100 μM 3-isobutyl-1-methylxanthine (IBMX) and 10 μM forskolin (FSK) on cAMP in the sensitive HBL line, in HBL-R line with acquired resistance and in intrinsically resistant LND1 and MM098 lines exposed for 30 min to effectors. Data are presented as means ± SD (n = 3) compared to untreated cells. ***p < 0.001 (Student’s t-test).
pathway through indirect action on BRAF and CRAF via the PKA [21,24]. One of the hallmarks of cAMP is its ability to inhibit proliferation in many cell types, but to stimulate proliferation in others [22,23]. Therefore, we first measured cAMP in WTBRAF/WTNRAS melanoma lines and found that vemurafenib sensitive lines had a significantly higher cAMP content than the resistant ones. Accordingly, cAMP stimulation by either a phosphodiesterase inhibitor (IBMX) or an adenylyl cyclase activator (FSK) in resistant cells rendered them sensitive to the drug, through the inhibition of CRAF phosphorylation as monitored by Western blotting. Moreover, the crucial role of cAMP in sensitising resistant cells to vemurafenib was demonstrated with the MM098 line. Indeed, it has a defective cAMP signalling so that resistance could not be reversed under IBMX or FSK treatment but it was with cAMP agonist, as was also the case with all the other resistant lines.

In conclusion, we show that the ability of vemurafenib to inhibit WTBRAGWTRAS melanoma cell proliferation is dependent on cAMP pathway activity inhibiting CRAF. One of the possible consequences would be that cAMP may be potently stimulated by the zMSH/MC1-R signalling leading to the activation of melanogenesis in patients. In this context, we observed that the sensitive lines, which have high level of cAMP, have also high ability to pigment (data not shown). Accordingly and in WTBRAF/WTRAS melanoma, pigmentation may be proposed as a predictor of tumour sensitivity to vemurafenib. Conversely, low cAMP level...
in WT-BRAF/WT-NRAS melanoma tissue could explain the lack of vemurafenib efficacy in such tumours despite the relatively high drug dosage as reported in previous clinical trials [14,15].

Conflict of interest statement

None declared.

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