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

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Abstract Vemurafenib improves survival in patients with melanoma bearing the ^{V600E}BRAF mutation, but it did not show any benefit in clinical trials focusing on wild type tumours while it may well inhibit ^{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, ^{WT}BRAF/^{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 ^{WT}BRAF/^{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, ^{V600E}BRAF, BRAF or Mitogen-activated protein kinase kinase (MEK) inhibitors. Vemurafenib (PLX4032, RG7204) is a ^{V600E}BRAF kinase inhibitor which improved rates of both progression-free and overall survival compared to dacarbazine in patients with previously untreated ^{V600E}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 ^{C121S}MEK1 [9], dimerisation of aberrantly spliced ^{V600E}BRAF [10] or prevalence of wild type cells in the tumours [11,12]. A recent study reported that despite a heterogeneity of ^{V600E}BRAF protein expression found in 22% of metastatic melanoma patients, these did not correlate with clinical outcome when treated with BRAF inhibitors [13], suggesting that wild type cells should have been sensitive to the drugs. However, attempts to use vemurafenib in ^{WT}BRAF metastatic melanoma were unsuccessful. Indeed, previous studies reported (i) no evidence of tumour regression in such patients [14] as well as (ii) the occurrence of early ^{WT}BRAF primary melanomas in vemurafenib-treated patients who had a clinically significant response [15]. Thus, the effect of ^{V600E}BRAF inhibitors on ^{WT}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 ^{WT}BRAF/^{WT}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 ^{WT}BRAF/^{WT}NRAS 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 Tocris 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 °C. Sp-cAMP and Rp-cAMP (from Enzo Life Sciences, Lausen, Switzerland) were dissolved in water and stored at –20 °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, respectively, BRAF and NRAS were assessed as previously reported [23].

2.3. Cell culture conditions

All cell lines were cultured at 37 °C in a humidified 95% air and 5% CO_2 atmosphere. Cells were propagated in flasks containing HAM-F10 medium supplemented