Inactivation of the HIF-1α/PDK3 Signaling Axis Drives Melanoma toward Mitochondrial Oxidative Metabolism and Potentiates the Therapeutic Activity of Pro-Oxidants

Jérôme Kluza1, Paola Corazao-Rozas1, Yasmine Touil1, Manel Jendoubi1, Cyril Maire1, Pierre Guerreschi1, Aurélie Jonneaux1, Caroline Ballot1, Stéphane Balayssac4, Samuel Valable2,2, Aurélien Corroyer-Dulmont2,3, Myriam Bernaudin2,3, Myriam Malet-Martino4, Elisabeth Martin de Lassalle1, Patrice Maboudou5, Pierre Formstecher1, Renata Polakowska1, Laurent Mortier1, and Philippe Marchetti1,5

Abstract

Cancer cells can undergo a metabolic reprogramming from oxidative phosphorylation to glycolysis that allows them to adapt to nutrient-poor microenvironments, thereby imposing a selection for aggressive variants. However, the mechanisms underlying this reprogramming are not fully understood. Using complementary approaches in validated cell lines and freshly obtained human specimens, we report here that mitochondrial respiration and oxidative phosphorylation are slowed in metastatic melanomas, even under normoxic conditions due to the persistence of a high nuclear expression of hypoxia-inducible factor-1α (HIF-1α). Pharmacologic or genetic blockade of the HIF-1α pathway decreased glycolysis and promoted mitochondrial respiration via specific reduction in the expression of pyruvate dehydrogenase kinase-3 (PDK3). Inhibiting PDK3 activity by dichloroacetate (DCA) or siRNA-mediated attenuation was sufficient to increase pyruvate dehydrogenase activity, oxidative phosphorylation, and mitochondrial reactive oxygen species generation. Notably, DCA potentiated the antitumor effects of elesclomol, a pro-oxidant drug currently in clinical development, both by limiting cell proliferation and promoting cell death. Interestingly, this combination was also effective against BRAF V600E-mutant melanoma cells that were resistant to the BRAF inhibitor vemurafenib. Cotreatment of melanomas with DCA and elesclomol in vivo achieved a more durable response than single agent alone. Our findings offer a preclinical validation of the HIF-1/PDK3 bioenergetic pathway as a new target for therapeutic intervention in metastatic melanoma, opening the door to innovative combinations that might eradicate this disease. Cancer Res; 72(19); 5035–47. ©2012 AACR.

Introduction

Over the last decades, huge efforts devoted to comprehend melanoma biology led to the identification of new targets for antimalanoma therapy (1). Apart from targeting the oncogenic mutations present in many (but certainly not all) melanomas, another promising strategy is to exploit biochemical particularities of melanoma cells. One biochemical hallmark of melanoma is the generation of excessive reactive oxygen species (ROS; refs. 2, 3). It is generally postulated that the cellular effects of ROS depend on the level at which ROS are produced. Controlled production of ROS participates in the promotion and progression of melanoma (3), whereas higher ROS generation displays cell-damaging effects (4). Constitutive production of the ROS weakens melanoma cells that are closer to the point where cell death can occur. Hence, melanoma cells show increased sensitivity to ROS-induced death as compared with melanocytes and to other tumors (5). According to this view, elesclomol, a pro-oxidant molecule that targets the mitochondrial electron transport chain (ETC; ref. 6), has been evaluated in clinical trials for the treatment of metastatic melanomas and has shown encouraging clinical responses. Intriguingly, clinical favorable responses occurred in a subset of patients distinguished by low serum lactate dehydrogenase (LDH; ref. 7). Thus, serum LDH can be considered as the predictor of response to elesclomol. At a cellular level, elesclomol requires a functioning ETC to induce ROS-mediated melanoma cell death (6). In this context, unraveling the regulatory mechanisms essential for enhancing ROS production is fundamental for improving the efficiency of pro-oxidants in melanoma.

Approximately, 60% to 90% of cancers display a metabolic profile, the so-called Warburg phenotype, characterized by...
their dependence upon glycolysis as the major source of energy, irrespective of the oxygen level (8). According to the Warburg effect, pyruvate, the end product of glycolysis, is mainly converted into lactate by LDH-A that is upregulated in transformed cells, rather than oxidized in mitochondria.

It seems conceivable that melanoma cells mainly rely on glycolysis for energetic needs based on the following reasons: (i) the glycolytic phenotype of melanoma cell lines has been recently identified by metabolic profiling (9); (ii) metastatic melanomas are characterized by their particularly high avidity for 2-[18F]fluoro-2-deoxy-D-glucose ([18F]FDG) clinically detectable on positron emission tomography (PET; ref. 10); (iii) the isoenzyme LDH-5, the more effective in the conversion of pyruvate to lactate, is detected on histologic sections and in serum from patients with highly metastatic melanoma (for review; ref. 11); and (iv) hypoxia-inducible factor-1α (HIF-1α), a master regulator of metabolism required for the active adaptation of cancer cells to hypoxic conditions, is overexpressed in human melanoma (12, 13). In addition to hypoxia, HIF-1α has also been found stabilized in normoxic conditions in melanoma cells. For instance, melanoma antigen-11 regulates HIF-1α accumulation by inhibition of prolyl hydroxylase activity (14). Endothelin or the antiapoptotic protein, Bcl-2, induces HIF-1α accumulation (15, 16). Genetic alterations, such as Microphthalmia-Associated Transcription Factor (MITF) germline mutation (17) or the oncogenic V600E BRAF mutation (18) have also been found stabilized in normoxic conditions in melanoma cells. In fact, melanoma antigen-11 regulates HIF-1α accumulation by inhibition of prolyl hydroxylase activity (14).

Endothelin or the antiapoptotic protein, Bcl-2, induces HIF-1α accumulation (15, 16). Genetic alterations, such as Microphthalmia-Associated Transcription Factor (MITF) germline mutation (17) or the oncogenic V600E BRAF mutation (18) have also been found stabilized in normoxic conditions in melanoma cells. In fact, melanoma antigen-11 regulates HIF-1α accumulation by inhibition of prolyl hydroxylase activity (14).

However, recent studies indicate that melanomas do not adopt a bona fide Warburg phenotype, as glucose stimulates mitochondrial metabolism to favor melanoma growth (9, 19). It is well established that, under hypoxic conditions, HIF-1α activates the expression of glycolytic enzymes and glucose transporters and downregulates mitochondrial activity and ROS production through several distinct mechanisms in a context-specific manner (20). HIF-1α has also been involved in the “glutamine addiction” of cancer cells (21).

Our objective was to investigate the influence of metabolic pathways on mitochondrial ROS production, particularly in melanoma under normoxic conditions. We hypothesized that HIF-1α can also prevent the onset of oxidative stress via downregulation of mitochondrial respiration, and therefore can itself represent a factor of resistance to pro-oxidative drugs in melanomas.

Materials and Methods

Chemicals

All chemicals were purchased from Sigma–Aldrich except CM-H2DCFDA from Life Technologies and YC-1) from Cayman Chemical Company (Interchim). Elesclomol, provided by Synta Pharmaceuticals Corp., was prepared as described (6). The combined effect of dichloroacetate (DCA) and elesclomol was analyzed by the combination index isobologram (22).

Patients and tissue samples

Cutaneous metastases were obtained from 4 melanoma patients (see Supplementary Table S1) at the Clinique de Dermatologie, CHRU (Lille, France). Ethical approval for this study was obtained from the local Person’s Protection Committee, and all patients provided informed consent. The samples came from patients who had to be treated surgically for cutaneous melanoma metastases.

Cell lines

HL60, A375, and SKMel-28 were obtained from American Type Culture Collection over the past 2 years. The human melanoma cell lines HBL, LND, MM074, and MM079 were established in the Laboratory of Oncology and Experimental Surgery (Institut Bordet, Université libre de Bruxelles, Brussels, Belgium) from lymph node metastases of patients with melanoma and were kindly provided by G. Ghanem in 2009. Mel-4M was established in the laboratory by Dr. Polakowska in 2009. All cells were passaged within 6 months of thawing. The identity of cell lines was regularly examined by morphologic criteria and the presence of differentiation markers (last test in March 2012). They were expressing at least one of the microsatellite differentiation markers (TYR, TYRP1, TYRP2/DCT, Melan-A/MART-1, gp100/pmel17, S100B) as routinely assessed with vemurafenib to obtain the resistant subline (A375-R) as described in the Supplementary material.

HIF-1α shRNA

Three HIF-1α shRNA constructs (see Supplementary material for sequences) were obtained by limiting dilution. Activation of HIF-1α was quantified by a DNA-binding ELISA kit (TransAM Active Motif).

Determination of glucose, lactate, and ATP

Extracellular glucose and lactate levels were measured on a SYNCHRON LX20 Clinical system (Beckman Coulter). For assessment of intracellular ATP, the Celltiter Glow Assay Kit (Promega) was used.

Cytotoxicity analysis

Cell viability and cell cycle were assessed after staining with propidium iodide (PI). The detection of ROS was determined using the CM-H2DCFDA or hydroethidium probe following current protocols (25) and conducted on a FACS Canto II cytometer (Beckton Dickinson).
Figure 1. A, colony-forming assays of cells in the presence of glucose or glutamine (mean ± SD; n = 3, t-test, **, P < 0.01). B, cell-cycle analysis of cell lines treated with 10 μg/mL oligomycin, 5 μM rotenone, or 75 μM bromopyruvate for 24 hours. Numbers represent the percentage of sub-G1 cells. C, cells were treated with oligomycin for 4 hours then collected for ATP measurement (mean ± SD, n = 3). D, growing, the rate of glucose and lactate released by cells (0.9 × 10^6 cells/mL) in RPMI medium was calculated by dividing the difference of amount of glucose/lactate present in the supernatant between the 2 consecutive time points (2–6 hours) by the number of hours elapsed (mean ± SD, n = 5). E, oxygen consumption of intact cells. Data are representative of 5 experiments. F, proportions of mitochondrial oxygen consumption due to proton leak (respiration not modified by oligomycin) and ATP turnover (the respiration inhibited by oligomycin). Oxygen consumption was determined as in E (mean ± SD, n = 3). G and H, respiration rate (G) and oxygen consumption (H) in human skin metastasis and peritumoral tissue samples from 4 different patients (-----, patient 1 and patient 2; ---, patient 4; and ---, patient 3). Horizontal lines represent median values (paired t-test, **, P < 0.001). I, coronal images of PET/CT scan patient (patient 2; left) show multiple foci of abnormal FDG uptake. Right, axial image of one metastasis with laterothoracic localization. After metastasis ablation, 2 samples were obtained from sites with different SUV values then prepared for oxygen consumption.
Figure 2. A, confocal immunofluorescence analysis of HIF-1α expression (green) in melanoma cell lines. Magnification, ×630. DAPI, 4', 6-diamidino-2-phenylindole. B, immunoblots for HIF-1α accumulation in nuclear fraction from: cells maintained in normoxia or treated with 500 μmol/L desferrioxamine (DFO) for 18 hours (right); cells treated with YC-1 for 18 hours (left); cells cultured in hypoxic conditions (1% O2) for 24 hours (middle). Densitometric values of proteins normalized on lamin expression are expressed. C, quantitative real-time PCR analysis of HIF-1α target genes (mean ± SD, n = 4). D, HK2 and LDH expression by immunoblot analysis. Densitometric values of proteins normalized on actin expression are expressed. E, lactate was measured in the supernatant (mean ± SD, n = 5). F, immunoblot analysis of PARP cleavage in cells treated with 10 mmol/L 2-DG for 48 hours and/or the pan-caspase inhibitor z-VADfl. G, flow cytometry analysis of cells treated with 10 mmol/L 2-DG for 48 hours. Numbers represent the percentage of sub-G1 cells. H, colony-forming assays of cells in the presence of glucose or glutamine and counted after trypan blue exclusion. I, respiration rates of HBL cells exposed to YC-1 for 18 hours and HIF-1α shRNA cells. Oxygen consumption was determined as in Fig. 1D. J, proportion of OXPHOS-related respiration was obtained by the difference between routine and oligomycin-inhibited respiration normalized with maximum mitochondrial respiratory capacity. ATP levels were also determined (mean ± SD, n = 5).
**PCR analysis**

The mRNA was quantified by quantitative real-time PCR using a protocol optimized for Lightcycler 480 detector (Roche Applied Science; ref. 25). The primer sequences are available upon request. The transcript level in each sample was normalized to that of 18S rRNA. The relative expression of target mRNAs was analyzed using the Pfaffl method (25).

**Metabolite profiles**

Metabolites were extracted (26), and profiles were obtained using the 1H-NMR method as previously described (25).

**Immunoblot analysis**

Immunoblotting was conducted as indicated (25). To detect ETC proteins, monoclonal antibodies (OXPHOS complexes kit, MitoSciences; 1:1,000) were used. Otherwise, the primary antibodies used are described in Supplementary materials.

**Chromatin immunoprecipitation**

Cells were cross-linked in 1% formaldehyde for 8 minutes and processed for chromatin immunoprecipitation (ChIP) assay using the HIF-1a ExactaChIP Chromatin IP Kit (R&D Systems). Capture of the DNA fragments was tested by PCR using PDK3 primers previously reported (27).

**Measurement of oxygen consumption**

Oxygen consumption was monitored with Oxygraph oxygen electrodes (Hansatech Instruments Ltd.; ref. 25). Cell viability was checked by PI staining. For melanoma specimens, oxygen consumption assays were conducted using the respirometry system Oxygraph-2k (Oroboros Instruments). Data were normalized to the dry weight of the specimens.

**PDH activity and phosphoPDH detection**

The determination of pyruvate dehydrogenase (PDH) activity and phosphorylation of PDH were conducted using the PDH Assay Kit (MSP18, Mitosciences) and the PhosphoPDH In-Cell ELISA Kit Colorimetric (MSP48, Mitosciences), respectively.

**Transmission electron microscopy**

Cells were prepared for transmission electron microscopy as described (25).

**Histology**

Tumor specimens were stained with H&E. For in situ determination of cell proliferation or apoptosis, sections were analyzed with an antibody to Ki-67 or terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL; In Situ Cell Death Detection Kit, Roche). As a marker of ROS-mediated protein oxidation, protein carbonyls were detected by the dinitrophenyl hydrazine (DNPH) method using Oxyblot Protein Oxidation Detection Kit (Millipore).

**In vivo study**

All procedures with animals were conducted according to the Institutional guidelines. Immunodeficient female severe combined immunodeficient (SCID) mice, 6- to 8-week-old, under isoflurane anesthesia were injected with 2 × 10⁶ HBL cells, mixed (1:1 volume) with BD Matrigel Basement Membrane Matrix. When tumors reached 400 μL, the mice were divided into 4 groups: control group (n = 4): mice were treated with saline with the same schedule as the treated animals; elesclomol group (n = 6): mice were treated with elesclomol (20 mg/kg, i.v. injection for 5 d/wk); DCA group (n = 6): mice were treated with DCA (75 mg/L added to the drinking water); DCA/elesclomol group (n = 6) mice were treated with the combination of DCA and elesclomol. For patients’ tumors implanted in mice, fresh tumor samples were minced into small pieces, mixed (1:1 volume) with Matrigel, and then injected into the flank of SCID mice as described earlier.

**Imaging microPET scan analysis**

The PET experiments (authorization N° 14–55) were carried out under anesthesia. Images were acquired on a microPET Siemens Inveon Preclinical system. The emission scan lasting 1 hour was initiated after injection of ¹⁸FDG (~370 MBq/kg) through the tail vein. To quantify the ¹⁸F-FDG uptake on the last frame (corresponding to 40–60 min), the measured tissue activity concentration [counts (kBq)/mL] was divided by the injected activity in kBq per gram of body weight (kBq/g) to give a standardized uptake value (SUV).

**Statistical analysis**

Results were analyzed using GraphPad Prism version 5.00 (GraphPad Software). The Student t test was used to compare data sets. Statistical significance was set at P < 0.05.

---

**Table 1.** Quantified levels of mitochondrial TCA cycle-related metabolites, glutamine, and lactate in HBL scrambled vs. HBL HIF-1 shRNA

<table>
<thead>
<tr>
<th>Protein (nmol/mg)</th>
<th>Citrate</th>
<th>Succinate</th>
<th>Fumarate</th>
<th>Malate</th>
<th>Glutamine</th>
<th>Lactate</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBL scrambled</td>
<td>2.2 ± 0.7</td>
<td>5.4 ± 0.7</td>
<td>0.7 ± 0.2</td>
<td>13.7 ± 1.9</td>
<td>8.1 ± 3.0</td>
<td>332 ± 16</td>
</tr>
<tr>
<td>HBL HIF-1kd1</td>
<td>4.1 ± 0.7*</td>
<td>7.2 ± 0.4*</td>
<td>1.4 ± 0.2*</td>
<td>21.7 ± 2.2*</td>
<td>14.3 ± 3.8*</td>
<td>184 ± 18*</td>
</tr>
<tr>
<td>HBL HIF-1kd2</td>
<td>5.8 ± 1.0*</td>
<td>5.7 ± 0.3</td>
<td>1.0 ± 0.2*</td>
<td>16.8 ± 1.9*</td>
<td>17.7 ± 3.4*</td>
<td>172 ± 19*</td>
</tr>
<tr>
<td>HBL HIF-1kd3</td>
<td>6.6 ± 0.8*</td>
<td>6.1 ± 0.4*</td>
<td>1.6 ± 0.3*</td>
<td>19.3 ± 1.6*</td>
<td>23.1 ± 3.0*</td>
<td>185 ± 13*</td>
</tr>
</tbody>
</table>

*NOTE:* ¹H NMR spectra of the metabolome for each of the individual cell cultures when cells reached a subconfluent state of 70% to 80%. n = 10, results are mean ± SD.

*P < 0.05 versus scrambled.
**Figure 3.** A, schematic overview of pyruvate oxidation in mitochondria with inhibitors used in this study. B, mRNA expression levels of PDK1-4 isoforms. C, protein expression levels of PDK3. D, ChIP assay in HBL cells kept in normoxia or treated with 500 μmol/L DFO for 18 hours. HBL kd2 served as control. Reaction controls used nonspecific IgG antibodies and PCR conducted by using whole genomic DNA (input). Captured DNA was analyzed by PCR using Kluza et al. Cancer Res; 72(19) October 1, 2012

Cancer Research

Downloaded from cancerres.aacrjournals.org on July 21, 2017. © 2012 American Association for Cancer Research.
Results

Metabolic signature of metastatic melanoma cells

We first examined the effects of glucose and glutamine shortage on cell growth. In low glucose medium, HBL cells dramatically reduced their ability to proliferate. The absence of glutamine also limited HBL cell growth (Fig. 1A). Moreover, melanoma cells were partially resistant to cell death induced by the mitochondrial inhibitors (rotenone or oligomycin), whereas maximum cell death was achieved when cells were incubated with bromopyruvate to inhibit glycolysis. Conversely, the promyelocytic cell line, HL60, was critically dependent on OXPHOS for survival [Fig. 1B and (28, 29)]. All melanoma cell lines studied had a cell death profile similar to that of mitochondrial DNA–depleted melanoma cells (HBLp0), which lack functional ETC (24). Inhibition of ATP synthase with oligomycin had almost no impact on ATP in melanoma cells, whereas in HL60, ATP level was highly sensitive to inhibition by oligomycin (Fig. 1C). Thus, metastatic melanoma cells, such as respiratory deficient p0 cells, generate almost all ATP via glycolysis despite abundant oxygen, a situation known as the Warburg effect (30). Confirming this phenotype, a high glycolytic activity as measured by glucose consumption and lactate production characterized melanoma cells (Fig. 1D). Melanoma cell lines also exhibited a significantly lower oxygen consumption rate than HL60 (Fig. 1E). Moreover, the proportion of melanoma respiration used to produce ATP (ATP turnover) was far less important than that of HL60 (Fig. 1F). These results were confirmed in metastatic melanoma specimens from patients (see Supplementary Table S1 and Supplementary Fig. S1A) compared with the surrounding tissues (Fig. 1G and H). Finally, we evaluated oxygen consumption in melanoma cells isolated from cutaneous metastasis (patient 2) according to the intensity of 18FDG uptake, expressed as SUVs (Fig. 1I).

As HIF-1 is a critical regulator of metabolism in cells under hypoxic conditions (31), we studied the role of HIF-1α in melanoma cells maintained in normoxia. Confirming previous data (12, 32), HIF-1α was expressed under normoxia in nuclei of melanoma cells (Fig 2A and B) and of metastatic melanomas from patients (Supplementary Fig. S1A). The influence of hypoxia on selective HIF-1α expression was moderate [Fig. 2A and ref. (32)]. We then used shRNA targeting HIF-1α mRNA for knockdown of gene expression (Supplementary Fig. S1C and S1D and Fig. 2B) in the HBL cell line selected for its elevated level of HIF-1α (Fig. 2A and B). HIF-1α knockdown cells resulted in significant decreased expression of key glycolytic genes and corresponding proteins, such as hexokinase II, the first step rate-limiting enzyme of glycolysis and LDH-A, which converts pyruvate into lactate, the final product of glycolysis (Fig. 2C and D). In addition, a prominent decrease in lactate release was observed in shRNA cells (Fig. 2E), and HIF-1α knockdown cells were relatively resistant to death induced by the glycolytic inhibitor, 2-DG (Fig. 2F).

Comparison of 18FDG accumulation in tumor xenografts showed a sharply higher activity in control cells compared with HIF-1α knockdown cells supporting the crucial role of HIF-1α in the glucose addiction of melanoma (Fig. 2G). Moreover, HIF-1α knockdown cells were resistant to the antiproliferative effect of glutamine shortage (Fig. 2H) suggesting that HIF-1α also contributes to the dependence of melanoma cells on glutamine. In comparison with control cells, HBL cells lacking HIF-1α showed a 2-fold increase in mitochondrial oxygen consumption (Fig. 2I and Supplementary Fig. S2A). Similar mitochondrial effects were observed when cells were incubated with YC-1 (Fig. 2I), an established HIF-1α inhibitor (ref. 33 and Fig. 2B). Relative to HBL scrambled cells, HIF-1α shRNA cells presented significantly higher levels of tricarboxylic acid (TCA) intermediates, accumulation of glutamine, and lower contents of intracellular lactate as quantified by 1H-NMR (Table 1). Derepression of mitochondrial respiration in cells lacking HIF-1α significantly enhanced the fraction of respiration that is used for ATP synthesis, and therefore they produced more ATP than scrambled shRNA cells (Fig. 2I).

By accomplishing these major effects on glycolysis, glutamine metabolism, and mitochondrial respiration, HIF-1α drives the main metabolic changes observed in melanoma.

A HIF-1α–dependent PDK3 pathway controls mitochondrial respiration

We then evaluated the mechanisms by which HIF-1α downregulates mitochondrial OXPHOS. We found no change in the shape and number of mitochondria (Supplementary Fig. S2B–S2D). Unlike previous description (34), the respiratory chain...
complex IV and the expression of ETC proteins were not affected by HIF-1α expression (Supplementary Fig. S2E and S2F). Four different PDH kinases (PDK1–4) are known to phosphorylate the E1 moiety of the PDH complex (PDH) at 3 serine sites, thereby decreasing PDH activity and preventing pyruvate from undergoing mitochondrial oxidation [Fig. 3A and ref. (35)]. HIF-1α knockdown correlated with the reduced expression of PDK3 mRNA without significantly affecting the related isoforms (Fig. 3B). Results were confirmed at the protein level (Fig. 3C and Supplementary Fig. S1B). Chromatin immunoprecipitation assay showed the direct binding of HIF-1α to the PDK3 promoter in living cells (Fig. 3D). In agreement with the aforementioned data on PDK3 downregulation, PDH activity was enhanced in HIF-1α knockdown cells or in melanoma cells incubated in the presence of YC-1 (Fig. 3E and Supplementary Fig. S3). Increased PDH activity was also observed in cells transfected with siRNA directed against PDK3 or in the presence of the pyruvate dehydrogenase kinase (PDK) inhibitor, DCA (Fig. 3E and Supplementary Fig. S3). Dephosphorylation of PDH in HIF-1α knockdown cells or in HBL cells treated with DCA (Fig. 3F) correlated with the activation of PDH.

Accordingly, exposure of HBL cells (Fig. 3G), HBL tumors from SCID mice (Fig. 3H), or freshly resected cutaneous metastases (Fig. 3I) to DCA resulted in a dose-dependent increase of mitochondrial respiration regardless of the presence or absence of glutamine (Fig. 3G). No such effects were observed in HBL p53 cells or in the OXPHOS-dependent cell line HL60 (Fig. 3G, top left). DCA-induced increase in respiration was abolished by overexpression of PDK3 (Fig. 3G, bottom) suggesting that PDK3 is an important target for DCA. Moreover, DCA decreased the nuclear level of HIF-1α as well as its target LDH-A (Fig. 3K). There was a similar trend toward increased mitochondrial respiration in melanoma cells transfected with PDK3 siRNA (Fig. 3G, top right), comparable with that observed for cells lacking HIF-1α (Fig. 2I). We noted a significant elevation in several TCA cycle intermediates, glutamine, and a low lactate content in DCA-treated cells (Fig. 3J) mirroring the phenotype observed in HIF-1α kd cells (Table 1). Thus, a HIF-1α/PDK3 axis contributes to the control of mitochondrial respiration observed in melanoma.

**Inhibition of the HIF-1α-dependent PDK3 pathway enhances mitochondrial ROS generation**

We next decided to investigate whether the enhanced mitochondrial respiration would lead to increased mitochondrial ROS generation. We observed a significant increase in the ROS levels when PDK3 activity was inhibited by siRNA or DCA (Fig. 4A and Supplementary Fig. S4). Incubating PDK3 siRNA- or DCA-treated HBL cells with distinct ROS inducers further increased ROS levels. The pro-oxidative effect of DCA was maintained after glutamine withdrawal (Fig. 4A). Remarkably, DCA had no additional effect on ROS production in PDK3 siRNA knockdown cells, and PDK3 overexpression impeded DCA-induced increase in ROS generation confirming PDK3 as a potential target for DCA (Fig. 4B). Consistently, exposure of cells lacking HIF-1α to the ROS inducers also generated more ROS than cells expressing HIF-1α (Fig. 4C and Supplementary Fig. S4). In the p5 clone, due to its lack of basal (Fig. 1E) and DCA-induced respiration (Fig. 3G), the pro-oxidant capacities of DCA were markedly diminished compared with HBL parental cells (Fig. 4D). The complex I inhibitor, rotenone, dramatically decreased DCA-induced ROS generation in the same manner as the classic antioxidants, vitamin C and N-acetyl cysteine (NAC; Fig. 4E). It must be noted that apocynin, a classic inhibitor of NADPH oxidase, which has also predominant antioxidant capacities (36), reduced DCA-stimulated ROS production with about 40% ROS inhibition. The inhibitor of complex III, antimycin A, also reduced ROS production when combined with DCA, whereas the complex IV and V inhibitors had no noticeable effects (Fig. 4E). This would indicate that mitochondrial ROS promoted by DCA originated from complex I and/or III. Altogether, our results suggest that the HIF-1α/PDK3 axis protects melanoma from mitochondrial ROS production.

**Inhibition of the PDK3-dependent HIF-1α pathway promotes antimelanoma activity of elesclomol**

Finally, we investigated whether mitochondrial ROS generation promoted by DCA and elesclomol had potent antimelanoma activities. The combination of DCA and elesclomol inhibited cell viability more than each treatment did alone (Fig. 5A–C and Supplementary Fig. S5). In congruence with these findings, PDK3 inhibition rendered HBL cells highly sensitive to elesclomol-induced cell death (Supplementary Fig. S5). Selected concentration curves indicate a synergistic effect for HBL cells when DCA was combined with elesclomol (Fig. 5A). Interestingly, combination of DCA and elesclomol was sufficient to induce oxidative cell death in A375 melanoma cells with acquired resistance to vemurafenib (Fig. 5D).

To explore the therapeutic usefulness of pharmacologic combination, we sought to determine whether DCA treatment sensitized HBL xenografts to elesclomol in vivo. DCA or elesclomol alone reduced growth of HBL xenografts (Fig. 5E). In comparison, the combination of DCA and elesclomol was more effective in delaying tumor growth than single agent alone. Very similar results were obtained in a preclinical model consisting to evaluate the response of human tumor fragments grafted into SCID mice to drug combination (Fig. 5F). Treatment of mice with the combination of DCA and elesclomol was more effective in accumulating oxidatively modified proteins, reducing cell proliferation, and inducing apoptosis of HBL xenografts than single agent alone (Fig. 5G). Altogether, these data suggest that the antimelanoma activity of elesclomol, although effective alone, can be significantly enhanced by DCA.

**Discussion**

Our results show that melanoma cells use mainly glycolysis, and not mitochondria, for energy production and cell proliferation. In agreement with other reports (9, 19), we observed that glutamine also supports melanoma cell growth. Glucose and glutamine metabolic pathways are able to compensate from one another to promote cancer cell survival (37, 38). However, unlike the model originally stipulated by Warburg (39), melanoma cells maintain functional mitochondria. Thus,
Figure 4. A, cells were treated with 10 μmol/L menadione, 5 μmol/L N-EM, or 300 nmol/L elesclomol for 8 hours and PDK3 was inhibited by siRNA (left) or by DCA (0.5 mmol/L for 8 hours). When indicated, HBL cells were cultured in the presence or absence of glutamine before ROS determination (mean ± SD, n = 4; t-test, *, P < 0.05 and **, P < 0.01). B, left, cells were treated as before ROS determination; right, HBL cells were transiently transfected with empty vector (Mock) or full-length human PDK3 cDNA (PDK3) and ROS production was assessed in cells treated with 5 mmol/L DCA for 12 hours (mean ± SD, n = 4; t-test, *, P < 0.05 and **, P < 0.01). C, cells were treated as in A and HIF-1 was inhibited by 100 μmol/L YC-1 or by shRNA (right), then ROS generation was measured by flow cytometry (mean ± SD, n = 3; t-test, *, P < 0.05 and **, P < 0.01). D, HBL and HBL ρo cells were incubated with 5 mmol/L DCA and/or 10 mmol/L NAC for 12 hours before cyttofluorometric analysis. The mean fluorescence intensity (MFI) values are shown. E, cells were preincubated for 30 minutes with 1 μmol/L rotenone, 1 μmol/L antimycin A (AA), sodium azide (NaN3), 2 μg/mL oligomycin, 100 μmol/L apocynin, or vitamin C (100 μmol/L) and NAC (10 mmol/L) then incubated for 8 hours with 0.5 mmol/L DCA. Results are percentages of ROS inhibition calculated in comparison with DCA-stressed cells (mean ± SD, n = 3).
Figure 5. A, left, combination effect of DCA plus elesclomol on HBL cells incubated for 8 hours before assessment of cell viability. Cells were pretreated with 10 mmol/L NAC for 1 hour (mean ± SD, n = 3). Right, IC50 isobologram of the combination treatments. A plot under the line indicates a synergistic combination. B, colony-forming ability of HBL cells treated with 0.5 mmol/L DCA and/or 300 nmol/L elesclomol. Two weeks later crystal violet–stained colonies were counted. C, HBL spheroids were grown for 15 days then treated as in B. # or ##, P < 0.05 and ## or ###, P < 0.01. D, effects of the combination DCA + elesclomol on vemurafenib-resistant melanoma cells. The A375 parental cell line and the A375-R vemurafenib-resistant cells were exposed to 1 mmol/L DCA and/or 300 nmol/L elesclomol for 8 hours or 300 nmol/L vemurafenib for 48 hours and submitted to CM-H2DCFDA staining. The gray peak represents cells not exposed to drugs. Numbers refer to the percentage of cell death determined by PI staining cells from parallel culture. E, the tumor-bearing mice were divided into 4 treatment groups: control, DCA alone, elesclomol alone, or the combination of elesclomol and DCA (mean ± SD, n = 4 to 6). * or **, P < 0.05. F, xenografted tumor fragments from patient 1 were treated (indicated by arrow) following the protocol described in E. G, sections from the tumors of HBL-injected mice were stained with an antidiphenylhydrazine antibody for detection of carbonylated proteins as a marker of irreversible oxidative damage, with an anti-Ki67 antibody to assess proliferation and by TUNEL assay to detect apoptotic cell death. Insert corresponds to positive control for TUNEL (mean ± SD, n = 4, #, P < 0.05 vs. control).
we showed that inhibition of the HIF-1α/PDK3 axis is able to restore respiration in mitochondria of melanoma cells. These findings confirm that cancer cells possess a highly adaptable metabolism. Reduction in LDH-A activity (40) causes derepression of mitochondrial respiration (25). Inhibition of PDK favors mitochondrial oxidation and ROS production in lung carcinoma (41). Forced oxidative metabolism interferes with tumor cells proliferation and survival (25, 40, 41). Thereby, the metabolic flexibility of cancer cells creates a new perspective for the development of therapeutic approaches.

HIF-1α has been found highly expressed in melanoma cells kept under normoxic conditions (12, 32). The elevated expression of HIF-1α is correlated with the advanced stage of melanoma (12). Our results are consistent with the view that melanoma progression is associated with the apparition of changes in mitochondrial metabolism in a HIF-1α dependent manner. Conversely, nonmetastatic primary melanomas upregulate genes involved in mitochondrial oxidative phosphorylation (42).

Our study defines the HIF-1α/PDK3 axis as a sensor for metabolic stress that regulates mitochondrial ROS level under normoxia. Overexpression of HIF-1α reduces the basal level of ROS in hypoxic cancer cells (43). The isozyme PDK3 is expressed in several cancer cells, such as neuroblastoma, colon cancer (27), or leukemia (25). Hypoxic activation of the PDK3-dependent HIF-1α pathway has been previously involved in tumor progression and drug resistance (27). In agreement with our findings, the transcription profiling of human cancer cell lines confirmed the high expression of PDK3 in melanomas (deposited in Array Express database, accession number E-MTAB-37). PDK3 is the only isoform reported to be not inhibited by a high concentration of pyruvate (44). Interestingly, high pyruvate and lactate concentrations result in nonhypoxic HIF-1α stabilization (45). Thus, as suggested (45), HIF-1α-mediated PDK3 expression may favor HIF-1α stabilization independently of hypoxia in a self-amplifying loop that contributes to locking the melanoma cell metabolism into aerobic glycolysis.

Our findings provide an attractive explanation for the predictive value of LDH in patients treated with elesclomol (7). Because LDH-A is transcriptionally regulated by HIF-1α (Fig. 2C and D), serum LDH may be considered as an indirect marker of the HIF-1α-mediated defective mitochondrial activity. In that way, we propose that tumors from patients with high LDH levels have also inactive mitochondria, a situation that we found associated with resistance to elesclomol-induced cell death in vitro (6). Under such conditions, inhibition of HIF-1α and LDH expression by DCA (Fig. 3K) can contribute to restore sensitivity to elesclomol. Finally, we have shown that combined inhibitors of the HIF-1α/PDK3 axis along with pro-oxidant drugs have potential antimalanoma capacities. The hypoxia-independent activation of HIF-1α, found in certain metastatic melanomas (such as those with activation of the MTF7 pathway or harboring BRAF mutation), would confer particular sensitivity to this drug combination. Thus, in line with our results, this new therapeutic approach would be likely beneficial to patients progressing on BRAF inhibitors.

To best translate in vitro results into clinical application, we have chosen the combination of existing drugs (DCA and elesclomol) that are already in the clinic. Elesclomol is one of the first mitochondrial-targeted drugs that has recently entered the clinical trials (7). Elesclomol does not act by specific targeting of mitochondrial proteins but most likely by interfering with the electron flow to promote mitochondrial ROS production (6). Thus, we have shown that the DCA-dependent enhancement of the electron flow impaired by elesclomol results in huge production of ROS. DCA, previously used to treat mitochondrial diseases without major toxicity, is currently undergoing clinical evaluation for cancer (46). DCA irreversibly inhibits all PDK isoenzymes (47). We have shown that knocking down HIF-1α markedly reduced PDK3 expression but not other isoenzymes under normoxic conditions (Fig. 3B). However, both PDK1 and PDK3 were upregulated under hypoxic conditions (not shown), and it has been proven that PDK1 prevents the overproduction of mitochondrial ROS in response to hypoxia (48, 49). Because solid tumors consist of both normoxic and hypoxic regions, the use of a broad-spectrum inhibitor of all 4 PDK isoenzymes, such as DCA, is of particular interest in vivo to fully reactivate mitochondria. However, the main disadvantage of DCA, when used alone, is that its in vivo antitumor activity is only apparent at high concentrations (46). Our results suggest that DCA can be also used in combination regimens to boost the mitochondrial electron flux and then potentiate the anti-tumor activity of pro-oxidant drugs, such as elesclomol. Thus, our results offer proof-of-concept for using elesclomol combined with DCA against metastatic melanoma including those who have become resistant to BRAF inhibitors. In conclusion, inactivation of the HIF-1α/PDK3 axis presents new therapeutic opportunities against metastatic melanoma.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: J. Kluzà, P. Corazza-Rozas, M. Jendoubi, A. Jonneaux, C. Ballot, S. Valable, R. Polakowska, L. Mortier, P. Marchetti
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Kluzà, P. Corazza-Rozas, M. Jendoubi, C. Maire, A. Jonneaux, C. Ballot, S. Valable, L. Mortier
Writing, review, and/or revision of the manuscript: J. Kluzà, P. Guerreschi, S. Valable, M. Bernaudin, R. Polakowska, L. Mortier, P. Marchetti
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. Kluzà, P. Guerreschi, L. Mortier
Study supervision: J. Kluzà, P. Formstecher, R. Polakowska, L. Mortier, P. Marchetti

Acknowledgments
The authors thank Dr. Aubert (CHRU, Lille) for immunostaining, Pr. Hugo (CHRU, Lille) for PET-Scan analysis, Drs. Bates, Blackman, and Prins (Synta Pharmaceuticals Corp.) for providing elesclomol, Pr. Ghanem and Dr. Journe (Institut Jules Bordet, Bruxelles) for melanoma cell lines and Thomas Cruz for technical help.
References

11. Mills CN, Joshi SS, Niles RM. Expression and function of hypoxia inducible factor-1 alpha in human melanoma under non-hypoxic conditions. Mol Cancer 2009;8:104.

Grant Support

This work received a financial support from INSERM, UNIVERSITE DE LILLE II, Société Française de Dermatologie (P. Marchetti), Ligue contre le Cancer (Comité de l’Aisne; P. Marchetti and P. Formstecher), Société de Recherche Dermatologique (L. Mottier), BMS-Groupe de Cancérologie Cutanée (J. Kluza), and a special financial support from the Association pour l’Étude des Anomalies Congénitales Neurovégétives de Pr. B. Poupard (P. Guerreschi). J. Kluza received a fellowship from ABE and the Fondation de France. P. Coraza-Rozas is a recipient of a CHRU Lille-Région Nord-Pas de Calais fellowship.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received March 14, 2012; revised July 27, 2012; accepted July 27, 2012; published OnlineFirst August 3, 2012.
Inactivation of the HIF-1α/PDK3 Signaling Axis Drives Melanoma toward Mitochondrial Oxidative Metabolism and Potentiates the Therapeutic Activity of Pro-Oxidants

Jérome Kluza, Paola Corazao-Rozas, Yasmine Touil, et al.


Updated version  Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-12-0979

Supplementary Material  Access the most recent supplemental material at: http://cancerres.aacrjournals.org/content/suppl/2012/08/02/0008-5472.CAN-12-0979.DC1

Cited articles  This article cites 48 articles, 21 of which you can access for free at: http://cancerres.aacrjournals.org/content/72/19/5035.full#ref-list-1

Citing articles  This article has been cited by 5 HighWire-hosted articles. Access the articles at: http://cancerres.aacrjournals.org/content/72/19/5035.full#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.