MEK1/2 Inhibition Decreases Lactate in BRAF-Driven Human Cancer Cells

Maria Falck Miniotis¹, Vaitha Arunan¹, Thomas R. Eykyn¹,²,³, Richard Marais⁴, Paul Workman², Martin O. Leach¹, and Mounia Beloueche-Babari¹

Abstract

The RAS/BRAF/MEK/ERK signaling pathway is a central driver in cancer with many BRAF and MEK inhibitors being evaluated in clinical trials. Identifying noninvasive biomarkers of early pharmacodynamic responses is important for development of these targeted drugs. As increased aerobic glycolysis is often observed in cancer, we hypothesized that MEK1/2 (MAP2K1/MAP2K2) inhibitors may reduce lactate levels as detected by magnetic resonance spectroscopy (MRS), as a metabolic biomarker for the pharmacodynamic response. MRS was used to monitor intracellular and extracellular levels of lactate in human cancer cells in vitro and in melanoma tumors ex vivo. In addition, we used ¹H MRS and a fluorescent glucose analog to evaluate the effect of MEK inhibition on glucose uptake. MEK1/2 signaling inhibition reduced extracellular lactate levels in BRAF-dependent cells but not BRAF-independent cells. The reduction in extracellular lactate in BRAF-driven melanoma cells was time-dependent and associated with reduced expression of hexokinase-II driven by c-Myc depletion. Taken together, these results reveal how MEK1/2 inhibition affects cancer cell metabolism in the context of BRAF oncogene addiction. Furthermore, they offer a preclinical proof-of-concept for the use of MRS to measure lactate as a noninvasive metabolic biomarker for pharmacodynamic response to MEK1/2 inhibition in BRAF-driven cancers. Cancer Res; 73(13); 4039–49. ©2013 AACR.

Introduction

Hyperactivated extracellular signal–regulated kinase (ERK) 1/2 signaling occurs in many human cancers due to mutation or overexpression of components of the RAS/RAF/MEK1/2/ERK1/2 pathway. For example, BRAF mutation, especially the V600E variant, has been reported in approximately 50% of malignant melanomas and 40% of thyroid cancers (www.sanger.ac.uk/genetics/CGP/cosmic/). Thus, inhibition of BRAF/MEK1/2 signaling constitutes a key strategy for molecularly targeted cancer treatment (1), and the recent regulatory approval of the BRAF inhibitor vemurafenib (PLX4032/BG7294) for BRAF-driven melanoma shows the effectiveness of this approach. Many MEK1/2-selective inhibitors have also been described including PD184352 (CI-1040), PD325901, selumetinib (AZD6244/ARRY-142866), and trametinib (GSK1120212). PD325901 and selumetinib have shown potent antitumor activity in preclinical tumor models (2), and selumetinib has recently undergone phase II clinical trials in non-small cell lung cancer and melanoma (3, 4). Trametinib has shown clinical activity in BRAF-mutant melanoma (5).

Compared with cytotoxic therapy, RAF/MEK1/2 inhibitors are predominantly, but not exclusively, cytostatic. In BRAF-mutant cells, MEK inhibition can elicit both apoptotic and cytostatic effects (6). Tumor shrinkage may therefore not always be apparent in the early stages of treatment, indicating the need to identify early, and preferably, noninvasive, response biomarkers to assess target modulation and treatment efficacy.

Magnetic resonance spectroscopy (MRS) provides a noninvasive approach for monitoring metabolism. MRS has shown different metabolic characteristics in tumors versus normal tissues (e.g., elevated signals from lipids, choline-containing metabolites, and glycolytic intermediates; ref. 7), which are altered with chemotherapy and various molecularly targeted anticancer agents (8, 9).

The increased glycolytic activity of cancer cells under normoxic conditions, known as the "Warburg effect" is characterized by increased glucose uptake and lactate production (10). This allows fast-growing cancer cells to shunt glycolytic intermediates into anabolic reactions while the acid produced aids tumor invasion and stroma destruction (11). Increased tumor lactate correlates with poor prognosis in some cases of brain, breast, lung, and liver cancers (12). Furthermore, in human cervical cancer, high tumor lactate concentration is
correlated with metastasis and decreased survival, whereas low tumor lactate is associated with increased survival (13). Glycolysis is also regulated by oncogenic signaling and glycolytic alterations occur following inhibition of BCR-ABL (14), platelet-derived growth factor (PDGF) receptor (15), and phosphoinositide 3-kinase [PI3K (16)]. RAS activation increases glycolysis in rat fibroblasts (17, 18) and the ERK1/2 transcriptional targets c-Myc and NF-κB (19) upregulate glycolytic enzyme expression (20) and increase glucose transporter expression (21), respectively. Moreover, human cancer cells harboring oncogenic BRAF exhibit increased c-Myc expression (22), glucose uptake, and lactate production (23).

Therefore, we hypothesized that inhibition of MEK1/2 could impact on the levels of lactate formation in human cancer cells that may be detected by MRS. Here, we test this hypothesis and (i) assess whether inhibition of MEK1/2 signaling in human cancer cells alters lactate levels as detected by MRS and (ii) explore the underlying mechanisms for our observations. Furthermore, as highly activating BRAF mutations confer sensitivity to MEK1/2 signaling inhibitors (6), we also assess the dependency of the observed effects on BRAF mutation status.

We show that MEK1/2 inhibition decreases lactate levels exclusively in BRAF-driven cancer cells. This effect, which is also observed in a BRAF-mutant human melanoma xenograft model, is associated with reduced glucose uptake and hexokinase-II expression, which is likely mediated by c-Myc depletion.

Materials and Methods

Cell lines

The cell lines used, their tissue type, and BRAF/RAS status are shown in Table 1. WM266.4, SKMEL-28, CHL-1, MDA-MB231, and HCT116 cells were purchased from American Type Culture Collection (ATCC; which uses short tandem repeat profiling for cell line authentication). A2058, DO4, and colo829 cells were donated by R. Kirk (Cancer Research UK Centre for Cell and Molecular Biology, Institute of Cancer Research (ICR; London, United Kingdom). HT29, HT55, DLD1, and RKO were purchased by Dr. P. Clarke, Cancer Research UK Centre for Cell and Molecular Biology. The donated cell lines were from ATCC and sequenced in-house for BRAF mutation status (24). The Ba/F3 murine hematopoietic cell lines, containing wild-type (WT) or V600E BRAF, were produced by Dr. S. Whittaker, Cancer Research UK Centre for Cell and Molecular Biology, ICR. Cells were preserved and passaged according to ATCC’s protocols for no longer than 3 months and screened monthly for mycoplasma.

Cell culture

All cell lines were grown and maintained as previously described (25) in Dulbecco’s Modified Eagle Medium (DMEM) except for DO4 and colo829, which were cultured in RPMI. BRAF WT-expressing Ba/F3 cells were grown in RPMI containing 10% heat-inactivated FBS and 1 ng/mL interleukin (IL)-3. BRAF V600E-expressing Ba/F3 cells were grown with (BRAF V600E-independent) or without interleukin (IL)-3 (BRAF V600E-dependent).

Cell culture materials were from Life Technologies. CI-1040 and PD325901 were purchased from the University of Dundee (Dundee, United Kingdom). Selumetinib was kindly provided by Dr. P.D. Smith, AstraZeneca.

Inhibition of cell proliferation

Inhibition of proliferation in adherent cells was assessed using the sulforhodamine B (SRB) assay (26). For Ba/F3 suspension cultures, the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation (MTS) assay was used according to suppliers’ instructions (Promega). GI50 values were calculated using GraphPad Prism (v5.01).

| Table 1. Cell lines used, their tissue of origin, and BRAF/RAS mutational status |
|---------------------------|---------------------------|---------------------------|
| **Cell line** | **Tissue type** | **BRAF mutation status** | **RAS mutation status** |
| Human | | | |
| DO4 | Melanoma | WT | NRAS Q61L |
| CHL-1 | Melanoma | WT | WT |
| HCT116 | Colorectal cancer | WT | KRAS G13D |
| HT55 | Colorectal cancer | WT | WT |
| DLD1 | Colorectal cancer | WT | KRAS G13D |
| MDA-MB231 | Breast cancer | G464V | KRAS G13D |
| WM266.4 | Melanoma | V600D | WT |
| SKMEL-28 | Melanoma | V600E | WT |
| A2058 | Melanoma | V600E | WT |
| Colo829 | Colorectal cancer | V600E | WT |
| HT29 | Colorectal cancer | V600E | WT |
| RKO | Colorectal cancer | V600E | WT |
| Murine | | | |
| Ba/F3 | Hematopoietic | V600E/WT | WT |
MEK1/2 inhibition

Equitopic concentrations of MEK1/2 inhibitors that achieved target modulation (reduced ERK1/2 phosphorylation) and approximately 20% to 30% reduction in cell counts were used. All cell lines were treated with CI-1040 and a selected subset also treated with PD325901 for comparison (Supplementary Materials and Methods). Cell counts and viability were monitored with Vi-CELL Cell Viability Analyzer (Beckman Coulter). Protein expression analysis was conducted by Western blotting as previously described (25). Primary and secondary antibody information is provided in Supplementary Materials and Methods.

Cell-cycle analysis

Flow cytometry was conducted to analyze cell cycle distributions as previously described (27).

1H MRS

Metabolite extraction was conducted by a dual-phase methanol–water–chloorform method (27). The lyophilized aqueous phase was resuspended in 540 μL D2O containing 0.015% v/v 3-(trimehylsilyl) propionic-2,2,3,3-d4 acid (TSP-internal standard). Media (0.8 mL) were collected from control and treated cells, lyophilized, and resuspended in D2O + TSP as above. 1H MRS data were acquired on a Bruker 500 MHz spectrometer at 25°C. Spectral processing and metabiote quantification were conducted as previously described (25).

Colorimetric lactate measurements

WM266.4, A2058, HT29, BKO, color289, DO4, HT55, and DLD1 cells were grown in 96-well plates and media lactate levels following a 24-hour treatment with 0.1% DMSO (control) or 1 μmol/L CI-1040 monitored with a colorimetric assay according to manufacturer’s protocol (Cambridge BioSciences). Readings were corrected for viable cell content measured with the MTS assay.

Glucose uptake

WM266.4 cells were incubated for 22 hours with CI-1040 followed by 2 hours in 1 μmol/L CI-1040 + 10 μmol/L 2-NBDG fluorescent glucose analogue (Invitrogen). The final 2-hour incubation was conducted in PBS- and glucose-free media. Cells were resuspended in 1 mL ice-cold PBS with 1 μg/mL propidium iodide and analyzed at 4°C on a FACSAria (BD Biosciences) using 488 nm excitation and 530 nm emission.

Hexokinase inhibition and c-Myc knockdown

To inhibit hexokinase, WM266.4 and CHL-1 cells were treated for 24 hours with 1 mmol/L of the glucose analogue 2-deoxyglucose (2-DG, Sigma-Aldrich). For c-Myc knockdown, WM266.4 cells were reverse transfected with 2 mmol/L of negative control siRNA (AllStars-target sequence proprietary) or c-Myc–directed siRNA (target sequence: CACAGGATGTTAATCCTTAAA) for 48 hours in 0.25% HiPerFect reagent using manufacturer’s protocol (Qiagen). Media samples were analyzed by 1H MRS for lactate as described above.

Melanoma xenografts

WM266.4 human melanoma cells (~5 × 10⁶) were injected subcutaneously into the flanks of 20 to 25 g, about 8-week-old NCr/Nu nude mice (Charles River), and when tumors reached about 500 mm³ in size (~3–4 weeks later), the mice were randomized into control- and selumetinib-treated groups. The control group (560 ± 77 mm³) received water only, whereas the treated group (560 ± 127 mm³) received 75 mg/kg selumetinib [dose within a previously reported range (2)] suspended in water twice daily by oral gavage for 3 days. Tumor volume was measured using calipers and the equation (length × depth × width) × π/6. Three hours after the last dose, tumors were excised, snap-frozen in liquid nitrogen, and stored at −80°C until further processing.

For MRS analysis, tumors were weighed and homogenized in lysis buffer (for protein analysis, see ref. 25) or ice-cold methanol (for metabolite extraction) at 4°C to minimize degradation and uncontrolled variations due to sample handling.

Animals were treated in accordance with local and national ethical requirements and with the UK National Cancer Research Institute (NCRI) Guidelines for the Welfare and Use of Animals in Cancer Research (28).

Statistical analysis

Two-tailed unpaired Student t test was used for statistical analysis, with P ≤ 0.05 considered to be significant. Pearson correlation analysis was conducted using GraphPad Prism (v5.01). Results represent the mean ± SD.

Results

CI-1040 induces a time-dependent reduction in extracellular lactate levels in BRAFV600D human melanoma cells concomitant with reduced p-ERK1/2 signaling and cell cycle arrest

Treatment of BRAFV600D WM266.4 human melanoma cells with CI-1040 decreased ERK1/2 phosphorylation at 30 minutes to 48 hours, indicating successful MEK1/2 inhibition. Cyclin D1 and pRB levels decreased from 6 hours up to 48 hours posttreatment (Fig. 1A), whereas p-MEK1/2 levels remained unchanged. Cell cycle changes were observed 16 to 24 hours following CI-1040 treatment with the majority of cells in the G1 phase and relatively few in the S and G2–M phases (Fig. 1B).

Figure 1C shows representative 1H MRS spectra illustrating the effect on extracellular lactate (lactateE) levels in CI-1040–treated versus control cells. LactateE was unchanged up to 6 hours but decreased significantly at 16 hours (79% ± 3%), 24 hours (76% ± 4%), and 48 hours (80% ± 6%) posttreatment relative to controls (Fig. 1D). Thus MEK1/2 inhibition by CI-1040 reduces lactateE in BRAFV600D WM266.4 cells.

Induction of G1 arrest in WM266.4 cells following 24-hour exposure to 1% to 2% dimethyl sulfoxide (DMSO), as previously documented in hybridoma B cells (29), had no effects on lactateE (Supplementary Fig. S1). Furthermore, lactateE was unchanged following induction of cell cycle arrest in WM266.4 cells with the cytotoxic DNA cross-linking agent and clinically relevant drug carboplatin (Supplementary Fig. S2). Thus, the
CI-1040–induced decrease in lactate\textsuperscript{E} is not a general anti-proliferative or cytotoxic effect.

**CI-1040 and PD325901 decrease extracellular lactate levels in human cancer cells harboring highly active mutant BRAF**

MEK1/2 inhibition with CI-1040 and PD325901 reduced p-ERK1/2 signaling in BRAF\textsuperscript{VT} HCT116 (colorectal cancer), BRAF\textsuperscript{V600E} MDA-MB231 (breast cancer), and BRAF\textsuperscript{V600E} SKMEL-28 (melanoma) cells (Supplementary Fig. S3A) as also observed in BRAF\textsuperscript{V600E} WM266.4 melanoma cells (Fig. 1A). p-ERK levels were undetectable in BRAF\textsuperscript{VT} CHL-1 cells.

As with WM266.4 cells, p-MEK1/2 levels remained unaltered after MEK inhibition in SKMEL-28 cells harboring the highly active BRAF\textsuperscript{V600E}. In BRAF\textsuperscript{VT} cells and cells with an intermediate activity mutant BRAF (BRAF\textsuperscript{G464V}), p-MEK1/2 levels were induced post-treatment. The induction of p-MEK1/2 in non-BRAF\textsuperscript{V600E/V600D} cell lines following MEK1/2 inhibition is consistent with previous reports (30, 31). This effect is a result of MEK1/2 inhibitor–induced ablation of negative feedback regulation involving ERK1/2 and RAF proteins that is only present in BRAF-independent cells (30, 31). Furthermore, SRB assays indicated that CI-1040 and PD325901 inhibited cell proliferation with greater potency in BRAF-driven than in BRAF-independent cell lines (Supplementary Fig. S3B) consistent with previous reports (6).

Flow cytometry showed that CI-1040 and PD325901 induced cell cycle arrest in SKMEL-28, HCT116, and MDA-MB231 cells as observed in WM266.4 cells (Supplementary Fig. S3C).

\textsuperscript{1}H MRS showed that MEK1/2 inhibition with either CI-1040 or PD325901 was associated with decreased intracellular lactate levels (lactate\textsuperscript{I}) in BRAF\textsuperscript{V600E} WM266.4 and BRAF\textsuperscript{V600E} SKMEL-28 cells (to 31%–47% of controls, Table 2). Lactate\textsuperscript{I} also decreased in MDA-MB231 (BRAF\textsuperscript{G464V}) and HCT116 (BRAF\textsuperscript{WT}) cells in response to CI-1040 treatment (to ~68%–77% of controls) and in HCT116 cells following exposure to PD325901 (to ~71% of controls) as shown in Table 2. The extent of reduction was less than that seen in BRAF-driven cells.

Lactate\textsuperscript{E} remained unchanged following CI-1040 treatment in CHL-1, HCT116, and MDA-MB231 cells. Similarly, PD325901 did not alter lactate\textsuperscript{E} levels in HCT116 cells (Table 2). Importantly, however, cells harboring an activating BRAF mutation showed significantly decreased lactate\textsuperscript{E} in response to both CI-1040 and PD325901 treatments (Table 2). In BRAF\textsuperscript{V600D} WM266.4 and BRAF\textsuperscript{V600E} SKMEL-28 cells, lactate\textsuperscript{E} decreased to 69%–82% and 76%–77% respectively, of controls.

To determine whether the effect on lactate\textsuperscript{E} following MEK1/2 inhibition was more broadly related to BRAF mutational status, additional cell lines were assessed using a colorimetric assay. This method gave comparable results to \textsuperscript{1}H MRS as assessed in WM266.4 cells (Fig. 2A; \( P = 0.2 \)). As shown in Fig. 2A, only cancer cells harboring the BRAF\textsuperscript{V600E/V600D} mutation showed significantly reduced lactate\textsuperscript{E} following MEK1/2 inhibition (\( n = 3–5, P \leq 0.05 \)).

These data show that following MEK1/2 signaling inhibition, lactate\textsuperscript{E} decreases exclusively in BRAF-driven human cancer cell lines.

**Dependency on activated BRAF signaling dictates modulation of extracellular lactate levels following MEK1/2 inhibition**

To confirm that the MEK1/2 inhibitor–induced lactate\textsuperscript{E} effects were due to the presence of activated BRAF, we used murine Ba/F3 cells isogenic for BRAF mutation. BRAF\textsuperscript{VT}-expressing Ba/F3 cells depend on IL-3 for growth. This dependency is abolished upon enforced expression of mutant BRAF\textsuperscript{V600E}, which renders the cells dependent on BRAF for ERK1/2-driven survival. The addition of IL-3 to BRAF\textsuperscript{V600E}-expressing Ba/F3 cells, however, abrogates their BRAF dependency (24).
MEK1/2 Inhibition Lowers Lactate in BRAF-Driven Cancer Cells

Western blotting showed that baseline p-MEK1/2 levels were detectable only in BRAFV600E-expressing Ba/F3 cells and were induced by CI-1040 in all except BRAFV600E-expressing Ba/F3 cells grown without IL-3 (i.e., BRAFV600E-dependent, Fig. 2B). Relative to BRAFVT Ba/F3 and BRAFV600E Ba/F3 cells grown with IL-3, baseline p-ERK1/2 levels were elevated in BRAFV600E Ba/F3 grown without IL-3 (i.e., BRAFV600E-dependent) and an increase in the G1 phase population in both BRAFV600E Ba/F3 and NRASV600E Ba/F3 cells (Fig. 2C). Importantly, baseline p-ERK1/2 levels in NRASV600E Ba/F3 were induced by CI-1040 in all except BRAFVT Ba/F3 and NRASV600E Ba/F3 cells grown with IL-3 (Fig. 2D; 94% reduction from baseline following CI-1040 treatment (98% decrease from baseline in Na+/K+-ATPase activity). These data show that it is the dependency of cells on mutant BRAF that controls the decrease in lactate5 following MEK1/2 inhibition.

CI-1040 reduces glucose metabolism in BRAFV600E/V600D but not BRAFVT human melanoma cells

In cancer cells, the major route for lactate production is glycolysis (12). Concentrating on melanoma cells, we assessed the effect of CI-1040 on glucose metabolism by monitoring its uptake, using 1H MRS and the fluorescent glucose analogue 2-NBDG, together with the expression of hexokinase-I and hexokinase-II using Western blotting.

1H MRS showed that CI-1040 reduced glucose uptake in BRAF-driven WM266.4 but not in BRAF-independent CHL-1 cells (Supplementary Table S1). As shown in Fig. 3A, CI-1040 reduced 2-NBDG uptake to 88% ± 2% of controls (P = 0.001) in BRAFV600E WM266.4 cells. These results confirmed that MEK1/2 inhibition with CI-1040 reduced glucose uptake in BRAF-dependent WM266.4 cells in agreement with the 1H MRS findings.

While hexokinase-I levels were mostly unchanged, hexokinase-II expression was markedly reduced by CI-1040 in BRAFV600E WM266.4 cells. These results confirm that CI-1040 reduced glucose uptake in BRAF-dependent WM266.4 cells in agreement with the 1H MRS findings.

Table 2. The effect of CI-1040 and PD325901 on 1H MRS-detectable lactate

<table>
<thead>
<tr>
<th>Lactate</th>
<th>Control</th>
<th>CI-1040</th>
<th>PD325901</th>
</tr>
</thead>
<tbody>
<tr>
<td>WM266.4 (BRAFV600E)</td>
<td>Lactate</td>
<td>18,000 ± 2,300</td>
<td>13,000 ± 1,400</td>
</tr>
<tr>
<td>Lactate</td>
<td>1.5 ± 3</td>
<td>6 ± 1</td>
<td>5 ± 0.8</td>
</tr>
<tr>
<td>SKMEL-28 (BRAFV600E)</td>
<td>Lactate</td>
<td>26,000 ± 5,300</td>
<td>20,000 ± 4,300</td>
</tr>
<tr>
<td>Lactate</td>
<td>39 ± 17</td>
<td>18 ± 9</td>
<td>15 ± 7</td>
</tr>
<tr>
<td>CHL-1 (BRAFVT)</td>
<td>Lactate</td>
<td>21,000 ± 1,200</td>
<td>22,000 ± 820</td>
</tr>
<tr>
<td>Lactate</td>
<td>20,000 ± 3,000</td>
<td>20,000 ± 2,600</td>
<td>NT</td>
</tr>
<tr>
<td>Lactate</td>
<td>27 ± 1</td>
<td>21 ± 4</td>
<td>NT</td>
</tr>
<tr>
<td>HCT116 (BRAFVT)</td>
<td>Lactate</td>
<td>11,000 ± 640</td>
<td>11,000 ± 1,100</td>
</tr>
<tr>
<td>Lactate</td>
<td>5 ± 1</td>
<td>3 ± 1</td>
<td>3 ± 0.8</td>
</tr>
</tbody>
</table>

NOTE: Levels are femtomoles per cell.

*; n = 3; \( \frac{a}{b} \); n = 4; \( \frac{c}{d} \); n = 5; \( \frac{e}{f} \); n = 8; \( ^{P} \leq 0.05 \).

Abbreviation: NT, not tested.

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whose expression is known to be modulated by ERK1/2 signaling (32).

These results indicate that the reduction in lactate observed after MEK1/2 inhibition is associated with down-regulation of glucose metabolism, and particularly hexokinase-II expression, which could result from c-Myc depletion. This effect was only observed in BRAF-driven melanoma cells.

**MEK1/2 inhibition with selumetinib reduces lactate levels in BRAF-driven melanoma xenografts**

WM266.4 melanoma xenografts were used to monitor the in vivo effects of MEK1/2 inhibition by selumetinib on tumor lactate. This drug was chosen as it was in clinical testing when the research was being conducted.

After 3 days of dosing, selumetinib-treated WM266.4 tumors were not significantly different in volume compared with pre-treatment values (100% ± 22%, \( P = 0.98 \)) indicating a growth arrest. In contrast, vehicle-treated tumors progressed to 143% ± 18% \( (P = 0.002) \).

Immunoblotting confirmed MEK1/2 inhibition in tumor xenografts as shown by reduced p-ERK1/2 and pRB expression (Fig. 5A). \(^1\)H MRS of excised tumor tissue revealed a 55% ± 23% reduction in lactate concentration in the drug-treated tumors relative to controls \( (P = 0.0001, \text{Fig. 5B}) \). Furthermore, the ratio of lactate to total metabolites present in the 0.8 to 3.1 ppm spectral range, containing many glycolytic intermediates and amino acids, was also reduced to a similar level (Fig. 5B). Thus, the decline in lactate content is not simply a reflection of reduced overall metabolite levels or tissue mass following selumetinib therapy. These results show that MEK1/2 inhibition reduces lactate levels in BRAF-driven tumors treated in vivo, in agreement with the in vitro effects.

**Discussion**

The BRAF inhibitor vemurafenib has recently gained U.S. Food and Drug Administration approval for the treatment of BRAF-driven melanoma (33), and many other BRAF and MEK1/2 inhibitors are undergoing clinical development (34).

Earlier studies have shown that oncogenic RAS increases the glycolytic activity of murine fibroblasts and human cancer cells (35, 36). The ERK1/2 target c-Myc increases lactate dehydrogenase (LDH)-A expression and lactate generation in rat fibroblasts (37), and activation of NF-κB, another ERK1/2 target, in mouse embryonic fibroblasts increases glucose...
transporter expression and lactate production (21). Furthermore, human cancer cells with high-activity BRAF mutants exhibit enhanced glucose uptake and lactate production (23). We therefore hypothesized that inhibition of MEK1/2 signaling decreases lactate production in human cancer cells allowing monitoring of drug effects by MRS.

MEK1/2 inhibition with CI-1040 and PD325901 caused a reduction in cell proliferation with increased potency in the human cancer cells containing the highly active BRAFV600E/V600D variants compared with cells with WT or intermediate activity mutant BRAF, in agreement with previous reports (6, 38). Treatment of BRAFV600D WM266.4 melanoma cells with CI-1040 caused a time-dependent inhibition of ERK1/2 signaling output and cell cycle progression, as previously reported (6). These molecular and cellular effects were concomitant with a time-dependent 1H MRS–detectable reduction in lactate E levels.

Importantly, analysis of additional cell lines (SKMEL-28, MDA-MB231, CHL-1, and HCT116), in which ERK1/2 signaling

Figure 3. Effect of CI-1040 on glucose uptake, hexokinase, and c-Myc protein expression in human melanoma cells. A, flow cytometric data showing the reduction in 2-NBDG uptake in BRAFV600D WM266.4 cells following CI-1040 treatment (24 hours, 1 μmol/L; n = 3). B, Western blot analyses showing reduced hexokinase-II expression in BRAF-driven WM266.4, SKMEL-28, and A2058 cells but not BRAFWT CHL-1 or DO4 cells following CI-1040 treatment (24 hours, 1 μmol/L). C, CI-1040-induced changes in hexokinase-II signal relative to total ERK1/2 as determined by densitometric analysis of the Western blot analyses shown in B (, P < 0.05, n ≥ 3). D, the reduction in lactate E versus hexokinase-II expression, ERK1/2 signaling, and cell counts in WM266.4 cells exposed to varying concentrations of CI-1040 for 24 hours. E, depletion of c-Myc following CI-1040 treatment (24 hours, 1 μmol/L) in WM266.4 and SKMEL-28 but not CHL-1 or DO4 cells. *, P < 0.05, n ≥ 3.
was also inhibited with CI-1040 (except CHL-1 cells where p-ERK1/2 was undetectable) indicated that lactateE was only significantly decreased in SKMEL-28 melanoma cells containing the highly activating BRAFV600E mutation but not in MDA-MB-231 breast cancer cells harboring intermediate activity BRAFV600K or CHL-1 melanoma and HCT116 colorectal cancer cells expressing BRAFWT. Similar effects were observed with BRAFV600G464V or CHL-1 melanoma and HCT116 colorectal cancer cells harboring intermediate activity BRAFV600K. The differences in the lactateE effects could not be attributed to differences in cell cycle changes, which were similar in all 5 cell lines. Furthermore, treatment of WM266.4 with DMSO or the cytotoxic DNA cross-linking agent carboplatin for 24 hours at concentrations that caused inhibition of cell cycling had no effect on p-AKT levels (Supplementary Fig. S5). Hence, the treatment-induced decrease in lactateE observed here could not be due to PI3K/AKT inhibition.

LactateE reflects the total lactate levels accumulated over time. In contrast, lactateI represents only 0.05% to 0.15% of lactateE and is a snapshot of what is present at the time of metabolite extraction. The reduction in lactateI seen following MEK1/2 inhibition was always more pronounced in cells containing the oncogenic BRAFV600E/V600D mutants relative to non-BRAF-driven cells. This suggests that the BRAF-independent cells are experiencing a less profound metabolic response to treatment as lactateE remained unchanged.

To further corroborate this conclusion, lactateE was analyzed in a larger cancer cell line panel previously sequenced in-house for BRAF (24). Our data show that following MEK1/2 inhibition, human cancer cell lines harboring an activating BRAF mutation consistently display a significant decrease in lactateE that is not observed in BRAFWT cells.

To ascertain whether the differences in metabolic response were related directly to BRAF dependence, we used isogenic Ba/F3 cells expressing either BRAFWT or BRAFV600E. The Ba/F3 cells harboring BRAFV600E are driven by BRAF only in the absence of IL-3. Western blotting showed signaling output profiles following CI-1040 treatment in Ba/F3 cells consistent with MEK1/2 inhibition under conditions of BRAF dependency or independency, namely, reduced P-ERK1/2 levels in all cases and induction of P-MEK1/2 in BRAF-independent cells only (30).

31H MRS showed that CI-1040 decreased lactateE significantly in BRAFV600E-driven Ba/F3 cells. Importantly, when the BRAF dependency was removed by the addition of IL-3, the effect on lactateE was abolished. LactateE was unchanged in BRAFWT-expressing Ba/F3 cells. These experiments showed that the reduction in lactateE levels observed following MEK1/2 inhibition is controlled by the dependence of cancer cells on mutant BRAF.
The molecular drivers mediating the BRAF dependency of the lactate response following MEK1/2 inhibition remain unclear. MEK1/2/ERK1/2 signaling is regulated by cross-talk from other signaling proteins such as CRAF (40) and AKT (41), and glycolytic alterations occur with modulation of various oncogenic signaling effectors (14–16). Whether the differential lactate response observed here is linked to compensatory effects from such proteins or from alternative signaling or metabolic pathways warrants further investigation.

Next we set out to explore the metabolic processes underlying the lactate\(^e\) effect following MEK1/2 inhibition in human melanoma cells. The predominant source for lactate production in cancer cells is glucose metabolism. Glucose is taken up into cells via glucose transporters then phosphorylated via hexokinase, particularly hexokinase-II, to produce glucose-6-phosphate. This is subsequently metabolized via a multistep process to pyruvate, which is then converted to lactate via LDH (12). Pyruvate can also be metabolized to other intermediates, mainly alanine, acetyl-CoA, and oxaloacetate (42). No significant changes in \(^1H\) MRS-detectable alanine were observed in CI-1040-treated BRAF\(^{V600D}\) WM266.4 melanoma cells that could indicate pyruvate-alanine transamination and that could lead to decreased lactate\(^e\) (data not shown). Detailed metabolic flux analysis is required to assess whether the effects observed here lead to altered flux to oxaloacetate and/or acetyl-CoA.

We assessed glucose metabolism by measuring its uptake into cells (using \(^1H\) MRS and 2-NBDG) and the expression of hexokinase-I/II using immunoblotting. MEK1/2 inhibition by CI-1040 in BRAF\(^{V600D}\) WM266.4 cells caused approximately 12% decrease in glucose uptake as measured with \(^1H\) MRS and 2-NBDG. More importantly, CI-1040 caused a substantial reduction in hexokinase-II expression in oncogenic BRAF-driven WM266.4, SKMEL28, and A2058 melanoma cells but not in BRAF-independent CHL-1 or DO4 melanoma cells, correlating positively with lactate\(^e\) levels.

Hexokinase-II is a key glycolytic enzyme that is overexpressed in many human cancers (43). Decreased hexokinase-II expression following MEK inhibition suggests reduced glycolytic flux to pyruvate leading to a decrease in lactate formation as previously shown (44). CI-1040 had no significant effect on LDH activity in WM266.4 cells (data not shown), supporting our conclusion that the effect on lactate\(^e\) occurs upstream of pyruvate.

Exposing WM266.4 cells to varying CI-1040 concentrations showed that the decrease in lactate\(^e\) mirrored the reduction in hexokinase-II levels but not cell counts, indicating that lactate\(^e\) changes reflect hexokinase-II inhibition rather than antiproliferative effects following MEK1/2 blockade. Indeed the hexokinase inhibitor 2-DG was able to reproduce the decrease in lactate\(^e\) induced by CI-1040 indicating that inhibition of glucose utilization is a key contributor to the reduction in lactate production following MEK inhibition in BRAF-driven melanoma cells.

The changes in hexokinase-II expression reported here are likely to result in reduced 2-deoxy-2\(^{18}\)F fluoro-D-glucose (FDG) uptake (which is influenced by both tracer uptake and phosphorylation via hexokinase-II) as reported in a hexokinase-II knockdown model (45). Consistent with our findings, recent work has shown reduced FDG uptake in vitro and in vivo post-treatment with the BRAF inhibitor vemurafenib in BRAF-driven human melanoma cells, associated with reduced hexokinase-II expression (46, 47).

CI-1040 also induced depletion of c-Myc in BRAF-mutant WM266.4 and SKMEL28 but not in BRAF\(^{WT}\) CHL-1 or DO4 melanoma cells, and siRNA-mediated knockdown of c-Myc in WM266.4 cells also led to depletion of hexokinase-II with a concomitant decrease in lactate\(^e\). Thus the decrease in hexokinase-II expression could be caused by depletion of its transcriptional regulator c-Myc (20) following ERK1/2 signaling inhibition.

In BRAF-WT/NRAS-mutant DO4 cells, and despite reduced P-ERK1/2 levels following CI-1040 treatment, c-Myc and hexokinase-II expression was not altered, concomitant with no changes in lactate\(^e\). Thus the lactate effects observed are not simply readouts of MEK blockade but likely reflect downstream inhibition of hexokinase-II and possibly also c-Myc.

Taken together, these data show that decreased hexokinase-II expression is a key contributor to the reduction in lactate\(^e\) in BRAF-driven melanoma cells following MEK1/2 inhibition, which could be caused by depletion of the ERK1/2 target c-Myc (32). These changes were observed only in BRAF-dependent melanoma cells displaying reduced lactate\(^e\). Further work is required to assess these effects in cell lines from other tissue types with mutant BRAF- or RAS-driven ERK1/2 signaling and investigate the impact of MEK inhibition on additional glycolytic enzymes.

Finally, and to assess the translatability of the lactate changes to in vivo tumor models, we assessed the effects of the MEK1/2 inhibitor selumetinib on lactate levels in tissue extracts from BRAF\(^{V600D}\) WM266.4 melanoma xenografts. Ex vivo \(^1H\) MRS analysis of tumor tissue showed that concomitant with decreased ERK1/2 signaling and induction of tumor stasis, lactate concentration (including both lactate\(^1\) and lactate\(^e\)) decreased in the selumetinib-treated tumors compared with controls. The ratio of lactate/total metabolites contained in the 0.8–3.1 ppm spectral region (including various amino acids and glycolytic intermediates) was also reduced in the selumetinib-treated tumors relative to controls to a comparable extent to the absolute lactate concentration. Thus, the decrease in lactate is not simply a reflection of reduced overall tumor and metabolite mass following treatment and constitutes a genuine metabolic effect of MEK1/2 inhibition.

The tumor lactate changes seen in responding tumor xenografts are consistent with our in vitro findings in BRAF\(^{V600E/T}\) cancer cells. Although it is unclear whether this lactate effect is functionally important to the antitumor activity of MEK inhibitors, our data highlight the potential of lactate as an in vivo biomarker of MEK1/2 signaling blockade. Further studies are required to assess whether changes in lactate following BRAF-MEK1/2 targeted therapy can be detected noninvasively using in vivo MRS in animal tumor models and, more importantly, in patients.

\(^1H\) MRS has been used to monitor tumor lactate in vivo both preclinically (48) and in patients (49). \(^13\)C MRS combined with dynamic nuclear polarization has been used to monitor real-
time pyruvate–lactate exchange in vivo (50) in response to therapy in preclinical models (9, 51) and, more recently, in patients with cancer. The possibility of translating our findings to clinical MR systems supports the investigation of lactate as a clinically applicable noninvasive biomarker of MEK1/2 signaling inhibition. This would complement current work using FDG-positron emission tomography (PET) to investigate metabolic response to BRAF/MEK1/2-targeted therapy in the clinic (52). MRS assessment of lactate, especially when combined with multiparametric MR imaging of the tumor microenvironment (e.g., cellularity and vascularity), will offer valuable complementary information to FDG-PET. The current study provides proof of principle for investigating lactate metabolism using MRS in BRAF/MEK inhibitor–treated patient tumors.

The observation that downregulation of hexokinase-II and c-Myc via alternative approaches also results in an MRS-detectable reduction in lactate could widen the applicability of this lactate response is dependent on BRAF oncogene addiction. This effect, which was also observed in tissues from MEK1/2 inhibitor–treated BRAF-driven human melanoma xenografts, was time-dependent and correlated with reduced hexokinase-II expression, which could be caused by c-Myc depletion. These data provide important new insights into the cellular consequences of oncogenic BRAF signaling modulation and support the development of lactate as a clinically applicable noninvasive metabolic imaging biomarker of MEK1/2-targeted therapeutics in BRAF-driven cancers.

Disclosure of Potential Conflicts of Interest
P. Workman is a consultant/advisory board member of Wilex. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: M.F. Miniotis, P. Workman, M.O. Leach, M. Beloueche-Babari
Development of methodology: M.F. Miniotis, T.R. Eykyn, M. Beloueche-Babari
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M.F. Miniotis, T.R. Eykyn, M. Beloueche-Babari
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M.F. Miniotis, T.R. Eykyn, P. Workman, M.O. Leach, M. Beloueche-Babari, R. Marais
Writing, review, and/or revision of the manuscript: M.F. Miniotis, P. Workman, M.O. Leach, M. Beloueche-Babari, R. Marais
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): V. Arunan, P. Workman, M. Beloueche-Babari
Study supervision: P. Workman, M.O. Leach, M. Beloueche-Babari, R. Marais

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