BRAF Inhibition Decreases Cellular Glucose Uptake in Melanoma in Association with Reduction in Cell Volume

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Abstract

BRAF kinase inhibitors have dramatically affected treatment of BRAFV600E-driven metastatic melanoma. Early responses assessed using [18F]fluorodeoxyglucose uptake-poston emission tomography (FDG-PET) have shown dramatic reduction of radio-tracer signal within 2 weeks of treatment. Despite high response rates, relapse occurs in nearly all cases, frequently at sites of treated metastatic disease. It remains unclear whether initial loss of 18FDG uptake is due to tumor cell death or other reasons. Here, we provide evidence of melanoma cell volume reduction in a patient cohort treated with BRAF inhibitors. We present data demonstrating that BRAF inhibition reduces melanoma glucose uptake per cell, but that this change is no longer significant following normalization for cell volume changes. We also demonstrate that volume normalization greatly reduces differences in transmembrane glucose transport and hexokinase-mediated phosphorylation. Mechanistic studies suggest that this loss of cell volume is due in large part to decreases in new protein translation as a consequence of vemurafenib treatment. Ultimately, our findings suggest that cell volume regulation constitutes an important physiologic parameter that may significantly contribute to radiographic changes observed in clinic.

Introduction

Cancer cells frequently display high rates of fermentative glycolysis relative to benign cells under normoxic conditions (1–3), a characteristic often referred to as “aerobic glycolysis” that involves increased consumption of glucose. The benefits of this include increased precursors for macromolecule synthesis, generation of reducing agents, and production of adequate ATP for growth (4–7). Evidence of aerobic glycolysis can be observed clinically using the glucose analog radiotracer 18-fluorodeoxyglucose (18FDG) with positron emission tomography (PET) imaging, reflecting a relative increase in glucose uptake in tumors compared with most normal tissues. It is often used in tandem with tumor dimensional measurements, with classification of therapeutic responses defined by a set of guidelines known as Response Evaluation Criteria in Solid Tumors (RECISt) based on changes in the sum of the longest diameters of all computed tomography (CT) or MRI-measured lesions. Interestingly, during the initial phases of targeted therapy, the FDG-PET response may be greater than the degree of tumor shrinkage noted with CT imaging (8). Together, FDG-PET and RECIST serve as useful methods for tracking the efficacy of therapies in primary and metastatic tumors (9, 10). This occurs dramatically in melanoma tumors driven by oncogenic BRAF treated with BRAF inhibitors such as vemurafenib and dabrafenib (8, 11–13).

Approximately half of human melanomas harbor activating mutations at amino acid V600 in the protein kinase BRAF, leading to constitutive activation of the MAPK pathway (14, 15). The BRAF inhibitors vemurafenib and dabrafenib suppress downstream activity of the MAPK pathway in the majority of BRAF-mutant tumors (16, 17). Patients treated with vemurafenib or dabrafenib typically show decreased FDG-PET signal within 2 weeks, often before significant reduction in tumor mass (8, 13). In roughly 40% of patients, less than 30% maximal tumor shrinkage is ultimately observed on therapy, yet most tumors will have markedly decreased FDG-PET signal with a progression-free survival benefit (12). Subsequent increases in 18FDG uptake in treated tumors tightly correlate with emergence of resistance to vemurafenib, occurring between 2 and 18 months in most patients (11). Curiously, many tumors that become PET negative on therapy often regain 18FDG positivity later in the same location, likely signifying recurrence of residual disease at those sites. Both the precise mechanism for this early decrease and the mechanism behind subsequent increase of glucose uptake at relapse have yet to be conclusively determined.

In cancer cells, transmembrane GLUT carrier proteins that facilitate diffusion across the plasma membrane (18), and hexokinase enzymes I–IV (19), which phosphorylate glucose in the
uptake values (SUVmax) from 18FDG-PET imaging were as previously described (8, 16). Maximum standardized uptake was obtained in patients with correspondingly early during treatment (EDT) biopsies at 3 and 8 weeks. Here, we investigate how the metabolic and morphologic properties of melanoma cells on therapy interact to produce the observed changes in 18FDG uptake.

Materials and Methods

Human melanoma tissue samples

Paired excision biopsies of 10 pretreatment (PRE) and corresponding early during treatment (EDT) biopsies at 3 and 8 weeks were obtained in patients with BRAF inhibitors in melanoma as previously described (8, 16). Maximum standardized uptake values (SUVmax) from 18FDG-PET imaging were obtained on day 15 of treatment (8) when available. Apoptotic cell counts represent average numbers of pyknotic tumor cells across 5 different 1 mm² areas per slide. Pyknotic cells were defined by hyperchromatic and shrunken nuclei with cytoplasmic compaction. Areas of ischemic, geographic necrosis were not included.

Cell culture and generation of acquired resistant lines

All specimens were collected with patients’ informed consent in accordance with the Health Insurance Portability and Accountability Act (HIPAA) under a Human Investigations Committee protocol. Authentication and mutational status were confirmed via expression profiling and Sanger sequencing as described previously (26). Cell lines were grown in OPTI-MEM (Invitrogen) supplemented with 1% penicillin-streptomycin and 10% FBS maintained in a 37°C incubator maintained at 5% CO₂. All lines utilized were derived from surgical melanoma resections and were provided by Dr. Ruth Halaban (Yale University; ref. 27) as part of the YSPORE tissue bank and passaged less than 6 months. Parental YUMAC and YUSIT1 lines were continuously grown in DMEM supplemented with varying concentrations of glucose along with a ratio of 1.8 μCi of 3-OMG (PerkinElmer) per mmol glucose for varying lengths of time. Uptake was halted with a quenching cocktail of 300 μmol/L phloretin (Sigma-Aldrich). Four washes were performed with the quenching cocktail before the residual pellet was lysed and added to Ultima Gold Scintillation Cocktail (PerkinElmer). Counts per minute were assessed with a Beckman Coulter LS 6500 Liquid Scintillation Counter (Beckman Coulter). Cell number was also corrected for using the CyQUANT NF Cell Proliferation Assay after washing. Results were also corrected for cell size using triplicate measurements of cell volume by Coulter Counter as described above.

Hexokinase activity assay

Total cellular hexokinase activity was assessed using an existing protocol (29), adapted from an earlier protocol (30). Cells were counted by hemocytometer. A volume of lysate containing 2.5 × 10⁵ cells or 30 μg of total protein was then added to a 96-well optical plate (Thermo Fisher Scientific) followed by an assay cocktail composed of 50 mmol/L triethanolamine buffer, 19 mmol/L Adenosine 5′-Triphosphate Solution, 100 mmol/L magnesium chloride, 14 mmol/L β-nicotinamide adenine dinucleotide phosphate, 125 units/mL glucose-6-phosphate dehydrogenase enzyme solution, and varying concentrations of glucose. Plates were read using a Spectramax M3 microplate reader (Molecular Devices) and maximum velocities computed using SoftMax Pro 6.2.2 (Molecular Devices). Correction for alterations in protein content per cell was performed using results of the Bradford assay in triplicate or CyQUANT NF Cell Proliferation Assay.

Flow cytometry

To measure glucose uptake, pellets were then resuspended in PBS supplemented with 300 μmol/L 2-deoxy-2-[1-14C]-d-glucose (2-NBDG; Cayman Chemical) for 10 minutes. After a single wash, pellets were stained using the BD Pharmingen Apoptosis Detection Kit II according to the manufacturer’s protocol (BD Biosciences). Samples were analyzed with the BD LSRII flow cytometer to at least 10,000 events per sample. Compensation for spectral overlap between 2-NBDG and propidium iodide was applied for each experiment. Each line was treated independently, and gates were fixed based on negative control signals. Plots were generated using FlowJo 9.6.2.

Immunoblotting

Immunoblots were conducted with the following primary antibodies all used at 1:1,000: Hexokinase II (cat. no. 2867; Cell Signaling Technology), β-actin (cat. no. 4970; Cell Signaling Technology), β-tubulin (cat. no. 2128; Cell Signaling Technology), GSK3B (cat. no. 9315), p-GSK3B S9 (cat. no. 9323; Cell

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Signaling Technology), p-p90RSK T573 (cat. no. 9346; Cell Signaling Technology), RSK1/RSK2/RSK3 (cat. no. 9355; Cell Signaling Technology), hsp60 (cat. no. sc-1052; Santa Cruz Biotechnology), GLUT1 (cat. no. 07-1401; Millipore), GLUT3 (cat. no. NBP1-89762; Novus Biologicals) and the secondary antibody Anti-rabbit IgG, HRP-linked Antibody (cat. no. 7074S; Cell Signaling Technology).

**Results**

**Cellular volume reduction occurs in clinical melanoma samples in response to BRAF inhibition**

$S_{LV_{max}}$ values from $^{18}$FDG-PET imaging in our paired biopsy PRE and EDT cohort were obtained on day 15 of therapy (8, 16). Evaluation of cellular size in the samples demonstrated a significant decrease in cellular and cytoplasmic volume following initiation of BRAF inhibitor therapy (Fig. 1A and B) without a significant decrease in average number of nuclei per tumor cross-sectional area (Fig. 1C). Contraction of cytosolic volume without loss of nuclei consequentially led to an increase in average intercellular distance in most patients (Fig. 1D). There was not an associated increase in the average number of apoptotic cells per mm$^2$ in the majority of cases (Fig. 1E).

**BRAF inhibition alters glucose uptake in melanoma**

After noting the morphologic changes, we evaluated changes in glucose metabolism in vitro to determine whether the phenotypes are linked. To quantify total glucose uptake changes in vitro, we incubated patient-derived melanoma cell lines harboring activating mutations in BRAF with $2\times [-(7$-nitrobenz-2-oxa-1,3-diazol-4-yl)$]amino)-2-deoxy-D-glucose (2-NBDG), a nonradioactive, fluorescent, nonmetabolizable form of glucose similar to $[^{18}$F]fluorodeoxyglucose, that is trapped within cells upon phosphorylation by hexokinase (31). Melanoma lines sensitive to vemurafenib, defined elsewhere as showing at least 50% growth inhibition at or below 3 $\mu$M drug (26) showed a significant decrease in 2-NBDG uptake per cell measured by flow cytometry after 12-hour exposure to vemurafenib, with maximum reduction in median fluorescent intensity observed by 72 hours (Fig. 2A). This was not associated with a significant increase in cell death as measured by Annexin-V and propidium iodide flow cytometry (Supplementary Fig. S1A).

Patients with acquired resistance to mutant BRAF inhibitors show elevated $S_{LV_{max}}$ relative to normal tissue even on BRAF inhibitors (11). To investigate this characteristic, we generated acquired vemurafenib-resistant clones by chronically growing two sensitive lines in vemurafenib and assessing 2-NBDG uptake. Drug resistance was verified by determination of $IC_{50}$ for proliferation at 72 hours (Supplementary Fig. S1B and S1C). As expected, 2-NBDG uptake in acquired resistant cell lines was maintained in the presence of vemurafenib (Fig. 2B).

Moreover, intrinsically vemurafenib-resistant mutant BRAF-driven melanoma lines and non-BRAF–mutant lines, showed equivalent or enhanced 2-NBDG uptake relative to vemurafenib-sensitive lines (Fig. 2C, Supplementary Table S1). These in vitro observations suggest that sensitivity to vemurafenib is closely associated with glucose uptake per cell, and phenocopies what is seen by $^{18}$FDG imaging in melanoma tumors from patients. Moreover, we observed that glucose uptake in sensitive lines was completely restored 72 hours after removal of vemurafenib, further suggesting that glucose uptake loss is a reversible effect of BRAF inhibition rather than a result of apoptosis (Fig. 2D). These results demonstrate that vemurafenib greatly reduces glucose uptake per cell within 72 hours of incubation with drug.

**Vemurafenib-induced reduction in glucose uptake is not accounted for by alterations in transmembrane glucose transport**

The intracellular trapping of 2-NBDG after phosphorylation by hexokinase precludes its use as an accurate indicator of GLUT function. Therefore, we utilized the nonphosphorylatable tritiated glucose analog 3-O-methylglucose (3-OMG) to quantify transport rate changes independent of hexokinase activity. Normalizing to cell count, we observed a mild decrease in uptake in vemurafenib-treated vemurafenib-sensitive cell lines that was not observed in acquired vemurafenib-resistant cells (Fig. 3A). This was not associated with reduced protein levels of two of the major GLUT transporter enzymes known to be highly active in melanoma, GLUT1 and GLUT3 (32) (Fig. 3B). Accordingly, we did not observe a significant change in transporter mRNA levels in sensitive or resistant lines (Fig. 3C). These data indicated that changes in overall glucose transport are insufficient to explain the decrease in glucose uptake in these BRAF-inhibited cells.

**Reduction of hexokinase II protein and overall hexokinase activity per cell is observed in sensitive but not acquired resistant melanoma lines treated with vemurafenib**

We next investigated whether reduced hexokinase activity contributes to BRAF inhibitor induced reduction in cellular 2-NBDG levels. We first performed an in vitro total hexokinase activity assay in sensitive and resistant lines, normalizing to cell count. Vemurafenib-sensitive lines showed a significant decrease in hexokinase activity per cell after 72-hour vemurafenib treatment, in contrast to acquired vemurafenib-resistant derivatives, which showed no significant change (Fig. 4A). Consistent with previous literature identifying HKII as the isoenzyme most intimately tied to cellular growth and proliferation of tumor cells (21, 23), we observed reduction of HKII protein starting at approximately 6 to 8 hours. Marked reduction of HKII levels was seen by 72 hours in sensitive lines (Fig. 4B), which corresponded with the decrease in glucose uptake observed by 2-NBDG flow cytometry. Melanoma lines with...
BRAF inhibition induces a significant decrease in cell volume in treated human melanoma tumors. A significant reduction in mean cytoplasmic volume is observed in the majority of biopsies of patient tumors after commencement of BRAFi therapy (A and B), without a significant decrease in mean number of nuclei per HPF (C). D, the majority of cases showed a large increase in average intercellular distance after commencement of therapy. E, a significant increase in number of apoptotic nuclei was not observed in the majority of cases. *, *P < 0.05; **, *P < 0.01; ***, *P < 0.001; n.s., not significant.

Figure 1. BRAF Inhibition Alters Glucose Uptake and Morphology
Figure 2.
Vemurafenib causes a decrease in glucose uptake in drug-sensitive but not resistant lines. A, treatment with 3 μmol/L vemurafenib (Vem) causes a decrease in glucose uptake as assessed by flow cytometry using the fluorescent glucose analog 2-NBDG reaching maximum decrease by 72 hours. B, acquired resistant lines (e.g., YUMACr) that have been serially passaged in 5 μmol/L vemurafenib show a much lower decrease in glucose uptake than drug-naïve lines (e.g., YUMACs). C, changes in glucose uptake with BRAF inhibition mirror resistance status and are genotype specific. D, glucose uptake loss due to vemurafenib treatment is reversible. All cells were incubated in 3 μmol/L vemurafenib or DMSO vehicle for 72 hours. After 72 hours, the pulse-chase group had vemurafenib-supplemented media aspirated, was washed with PBS, and resupplied with normal media supplemented with vehicle.
acquired resistance to vemurafenib or those without BRAF mutations showed no significant differences in HKII protein levels upon BRAF inhibition (Fig. 4C).

Quantitative measurement of HKII mRNA showed virtually no change after 72 hours of vemurafenib treatment in sensitive or resistant lines (Supplementary Fig. S2A), suggesting a post-transcriptional mechanism of HKII loss. We observed a significant loss of HKII protein in innately vemurafenib-resistant lines YUKSI and YUKOLI comparable with sensitive lines (Fig. 4D), despite the increased uptake of 2-NBDG observed by flow cytometry (Fig. 1C). We hypothesized that a compensatory increase in other HK isoforms present in intrinsic resistant lines might be responsible for the increase of glucose uptake. Interestingly, we found that both vemurafenib-sensitive and intrinsically resistant lines showed a significant rise in HKI levels at 72 hours (Fig. 4E). In contrast, all acquired vemurafenib-resistant lines as well as non-BRAF–mutant lines showed no significant change in HKI levels (Fig. 4F), underscoring the complex relationship between changes in HK levels and 2-NBDG uptake with BRAF inhibition (Fig. 4G). Furthermore, immunohistochemical staining of our biopsy cohort for HKII revealed moderate to high levels in all cases but revealed no
significant difference between levels in PRE and EDT samples (Supplementary Fig. S2B). These conflicting data suggest that changes in HK activity and regulation with vemurafenib treatment do not show a consistent relationship with glucose uptake, hinting that an additional alteration might be responsible for clinical therapeutic responses.

Figure 4.
Vemurafenib (Vem) induces significant loss of HKII in both sensitive and intrinsically resistant lines. A, 3 μmol/L vemurafenib induces a decrease in overall hexokinase activity per cell after 72 hours in sensitive cell lines. Vemurafenib (+) induces near complete loss of HKII in sensitive (B) but not acquired resistant or non-BRAF cell lines at 72 hours relative to DMSO (-; C). D, intrinsically resistant cell lines show a decrease in HKII comparable with sensitive cell lines. Sensitive and intrinsically resistant lines show a significant increase in HKI with vemurafenib treatment by 72 hours (E) that is not observed in acquired resistant or non-BRAF–mutant lines (F).
Cell volume decreases predict extent of reduction of glucose uptake in vitro

SUVmax alterations measured by 18 FDG-PET account for the total radioactivity signal emitted from a tumor with a particular gross tumor volume. These measurements do not distinguish between the relative proportions of cell volume and extracellular matrix and edema that comprise the tumor microenvironment. As cellular volume is another major determinant of total solute movement into a cell, including glucose, we microscopically examined morphologic changes in a wide range of melanoma lines using the cell volume measurement functions of a Z2 Coulter Counter. Over multiple cases, we quantified an average volume decrease of 43% across three highly vemurafenib-sensitive lines: YUMACS, YUSIT1s, and YUGEN8, but there was no substantial change in cell volume in lines resistant to BRAF inhibition (Fig. 5A and B). Moreover, we observed that the intrinsically vemurafenib-resistant lines YUKSI and YUKOLL, which showed mild increase in 2-NBDG uptake with vemurafenib, also showed increases in median cell volume. Mean adherent area calculations also supported these trends (Supplementary Fig. S3). Ultimately, when calculated across 13 melanoma lines, changes in cell volume following vemurafenib treatment were found to predict 83% of the variance in NBDG uptake in our model (Fig. 5C, P < 0.001, Student t test). This relationship also showed a strong correlation with vemurafenib concentrations required to inhibit growth of cells by 50% (GI50), with GI50 in our model predicting 86% of the variance in volume change (Fig. 5D, P < 0.001, Student t test). We found the same relationship between cell volume and uptake changes when cells were treated with the MEK inhibitor AZD-6244 (Supplementary Fig. S4A and S4B), an effect not explained by cell-cycle arrest alone (Supplementary Fig. S4C and S4D).

Applying correction for changes in cell volume to calculated transmembrane glucose transport (radiolabeled 3-OMG uptake) greatly reduced the perceived decrease in glucose transport kinetics (Fig. 5E). Similarly, normalization to protein content, as opposed to cell number, greatly reduced differences in pan-HK activity seen in treated versus untreated cells (Fig. 5F). Taken together, these data strongly suggest that measured alterations in glucose transport and phosphorylation are markedly influenced by cell volume changes.

Extrinsic regulation of cell size regulates cellular glucose uptake independent of BRAF inhibition

To establish a mechanistic link between cell volume changes and glucose uptake independent of metabolic changes from vemurafenib, we increased cell volume by decreasing solution tonicity. We found that decreasing the overall solution tonicity by 50% and 75% during 2-NBDG incubation significantly increased 2-NBDG uptake (Fig. 6B) under otherwise identical conditions without significant cell death or membrane disruption (Supplementary Fig. S5). These findings suggest that changes in cell size alone are sufficient to alter glucose uptake in melanoma. Extending these findings to BRAF-inhibited cells, we found that incubating cells exposed to vemurafenib for 72 hours in hypotonic 2-NBDG solutions was sufficient to rescue glucose uptake relative to untreated controls incubated at normal tonicity (Fig. 6C). As a second method for verification of this concept, we treated cells for 24 hours with 50 μg/ml of the ribosomal translation inhibitor cycloheximide. Cycloheximide treatment of vemurafenib-sensitive cells produced a decrease in both cell volume (Fig. 6D) and glucose uptake (Fig. 6E) that was comparable with the decrease induced by vemurafenib. Cycloheximide treatment in acquired or primary vemurafenib-resistant lines also resulted in marked reduction of cell volume and 2-NBDG uptake that was significantly greater than vemurafenib treatment in these lines. Cycloheximide treatment of additional lines revealed a positive linear correlation between cell volume and 2-NBDG uptake (Fig. 6F). Recent studies have demonstrated strong links between cell volume and changes in total cellular protein content (19, 33), as well as significant inhibition of 5’ cap-dependent translation with BRAF inhibition (34, 35). These studies, in addition to our cycloheximide data, suggested that changes in cap-dependent protein translation may be at least partially responsible for the noted decrease in cell size and glucose uptake loss. Consistent with this conclusion, we also noted a decrease in HKII levels with cycloheximide treatment that was comparable with that induced by vemurafenib (Fig. 6G), as well as a significant decrease in overall protein synthesis after 72 hours of drug treatment (Fig. 6H). This underlying mechanism was also supported by studies showing significant inhibition of assembly of the cap-dependent translation initiation complex, as well as decreased HKII de novo translation (Supplementary Fig. S6A and S6B). In parallel, dephosphorylation of ribosomal protein p70S6K and S6 downstream of mTOR, both of which are involved in initiation complex assembly and activity (36), occurs within 24 hours of treatment, mirroring observed alterations in cell volume and protein content (Supplementary Fig. S7). Additional loss of S6 and p70S6K continues out to 72 hours, with later loss of upstream Akt phosphorylation, further paralleling increasing loss of glucose uptake and volume over this timeframe (Supplementary Fig. S8). In line with previous studies (37), loss of S6 phosphorylation was greatly abrogated in resistant lines, showing a consistent relationship between drug sensitivity, volume, new protein synthesis, and S6 activity. The importance of decreases in protein translation over increased protein degradation was also supported through the inability of the proteasome inhibitors bortezomib or MG132 to significantly rescue HKII loss (Supplementary Fig. S9). This suggests that targeted destruction of HKII at the proteasome is not primarily responsible for the observed decreases in HKII levels.

Discussion

RECIST criteria are currently widely utilized to evaluate therapy efficacy. Though response rates are defined by the sum of complete and partial responses, it is also apparent that patients with stable disease may derive progression free and overall survival benefit from treatment (12). Poor correlation between tumor shrinkage over an initial time window and overall survival is frequently seen in the melanoma immunotherapy, where increases in tumor size can occur before eventual complete regression with survival benefit (6, 38). This suggests that therapy-induced changes in tumor volume taken in isolation may not be the optimal predictors of patient survival. Incorporation of additional data may be needed, necessitating improved understanding of the mechanism of tumor shrinkage.

Though induction of apoptosis has been assumed to be a principal mechanism of action of BRAF inhibitors, there is...
A

YUMACs
DMSO

YUMACs
72 h Vem

B

Cell volume (μm^3)

DMSO

VEM

C

\[ r^2 = 0.85*** \]

% NBDG change

% Volume change

D

\[ r^2 = 0.86*** \]

gI50

E

\[ \Delta \text{CPM min}^{-1} \text{per } 1 \times 10^6 \text{cells} \]

YUMACs

\[ \mu \text{mol glucose phosphorylated/min per } 1 \times 10^6 \text{cells} \]

YUSIT1

F

\[ \mu \text{mol glucose phosphorylated/min per } 1 \times 10^6 \text{cells} \]

YUMACs
relatively little evidence that this is the primary mode of effective response in patients. At odds with extensive cell death is the lack of observation of tumor lysis syndrome in several large clinical trials of BRAF inhibitors (1, 2, 9, 10), which might be expected from toxic chemotherapy treatments producing comparable RECIST responses. Evaluation of a series of melanoma patients EDT demonstrated that geographic tumor necrosis occurred at a higher rate than observed in PRE biopsies (8); however, geographic necrosis is typically secondary to vascular insufficiency (ischemia or infarct). A clear increase in individual apoptotic cells was not observed. In contrast, significant decrease in proliferation (Ki67) and Cyclin D1 levels noted in previous staining of a subset of this cohort suggest G0–G1 arrest (8). In vitro, high doses of BRAF inhibitors typically induce low levels of apoptosis (0%–10%), with some cell lines (e.g., A375) exhibiting higher rates. In essentially all BRAF-mutant lines, resistant clones emerge following chronic treatment in vitro, mimicking the high rate of relapse in melanoma patients treated with BRAF inhibitors. On the basis of these data, tumor shrinkage is likely a complex phenomenon that integrates occasional geographic (ischemic) necrosis, cell-cycle arrest, and a minor component of apoptotic cell death. The sum of these factors does not appear to fully account for and correlate with clinical tumor shrinkage in BRAF inhibitor–treated melanoma patients.

Here we provide evidence for BRAF inhibitor–induced reduction in cell volume as a contributing factor in clinical tumor shrinkage and FDG-PET responses. We observed a significant decrease in cell size in melanoma patient samples EDT, and a trend toward correlation between magnitude of shrinkage and SLVmax decrease. The observed similar level of HKII staining, reduction in cytoplasmic volume, and increase in intercellular distance early during treatment suggests that decrease in cell size precedes the slow, gradual reduction in tumor size that is typically observed. These findings suggest that early/rapid decrease in FDG-PET signal without corresponding tumor shrinkage as determined by CT imaging may be due to decreased tumor cellular mass per imaging voxel, intermixed with increased edematous changes. Resolution of the extracellular edematous changes would be predicted to result in gradual tumor shrinkage associated with residual to slightly elevated FDG-PET signal, which is typically observed. On a broader scale, these findings raise the possibility that imaging-based responses to targeted signal transduction inhibitors may be partially mediated by modulation of cell volume in addition to cell death.

Recent studies have suggested that MAPK inhibition may influence glucose metabolism in BRAFV600E mutant cells by inducing decreases in GLUT transporter or HKII protein levels (5, 11, 24, 25). Decreased metabolic activity has also been observed in the form of decreased lactate production (25), oxygen consumption (39), and metabolic enzyme expression (24). All generally normalized to protein mass or cell count. In this study, we provide evidence in melanoma that cell size alone can significantly regulate glucose uptake in BRAF-mutant melanomas treated with vemurafenib. We did note decreases in glucose uptake and HK activity per cell; however, following additional normalization for cell volume, the changes in HK activity were markedly diminished. This suggests that while previously demonstrated metabolic enzyme changes do contribute, they are likely to be only a part of the mechanism for 18FDG signal loss. This finding may also help explain the metabolic changes induced by BRAF inhibition in previous reports (11, 24, 25, 40) which have largely focused on changes in the first 24 hours after induction of therapy without exploration of later steady state. Taken together, these findings emphasize a broader need for consideration of cell size changes in metabolic and biochemical studies, especially in melanoma biology, where cell populations may consist of multiple subclones with heterogeneous morphologic properties (16, 20).

In this study, we demonstrate an increase in HKI levels in all melanoma cell lines that show HKII decrease. This could suggest that HKI may help compensate for changes in HKII in melanoma to maintain glycolytic activity, as indicated by our evaluation of overall HK activity, and consistent with previous studies in breast and lung cancer (23). Interestingly, we found that intrinsically vemurafenib-resistant melanomas show a mild increase in glucose metabolism and decrease in proliferation with treatment, yet display the same HKII/HKI switch seen in sensitive BRAF-mutant melanomas. Further studies will be required to determine the functional effects of upregulation of HKI, as well as the functional contributions of individual HK isoforms to glucose uptake.

Evidence provided by this study suggests that total protein translation per cell is significantly decreased with BRAF inhibition, in agreement with other studies (34, 35). This occurs in parallel with vemurafenib-induced decreases in p70S6K and S6 activity observed previously (37), and is largely absent in lines that have acquired resistance to the drug. The synchronicity of loss of cell volume, decrease in protein translation, and downregulation of p70S6K and S6 activity, as well as the correlation of these phenomena with drug sensitivity, further support the proposed explanation for decreases in glucose uptake. Our findings also expand on previous melanoma metabolomics literature, providing a mechanistic explanation for the previously noted downregulation of multiple key metabolic enzymes (24, 25). Furthermore, our study provides evidence for the importance of global protein synthesis to maintenance of cell size, and the consequences for the clinically relevant characteristic of glucose uptake capacity when this process becomes dysregulated, emphasizing the previously established relationship between cell size, cell-cycle progression, and translation (20, 34). Indeed, the G0–G1 cell-cycle arrest and markedly reduced bromodeoxyuridine incorporation we observed in vemurafenib-treated cells also suggests that new nucleic acid production is also greatly decreased in the

**Figure 5.**

Vemurafenib (Vem, 3 μmol/L) induces a cellular volume decrease in sensitive lines, which largely accounts for alterations in glucose uptake per cell. A, adherent sensitive but not resistant cell lines show visual decrease in cell size by light microscopy. B, vemurafenib-sensitive but neither acquired nor intrinsically resistant cell lines show a decrease in total cell volume with treatment as assessed by Coulter Counter. ∗, *P < 0.05; †, ††, †††, **P < 0.001; n.s., not significant. C, changes in cell volume show a strongly significant correlation with changes in measured glucose uptake (*r* = 0.8483, *P* < 0.01). D, changes in cell size also show a strong correlation with vemurafenib GI50 for mutant BRAF lines (*r* = 0.8568, *P* < 0.01). E, changes in transmembrane glucose transport are largely abrogated when corrected for changes in cell volume. F, changes in hexokinase activity are also largely abrogated when normalized to whole lysate protein concentration.
setting of BRAF inhibition; a finding which may reflect broader changes in glucose carbon utilization that merits further exploration. Nevertheless, the inability of cell-cycle arrest to independently fully account for decreased size and glucose uptake suggests that additional MAPK-specific regulation of translation and volume maintenance are also being inhibited. These findings taken together suggest a quiescent metabolic phenotype, implying that inhibition of mutant BRAF removes the driving
force behind neoplastic growth and metabolism without desta-
bilizing cells enough to cause apoptosis, levels of which have
previously been noted to be variable and frequently minimal
(39, 41).

In summary, our study suggests that cell size is an important
determinant of glucose uptake in melanoma and can affect
many of the observed changes in metabolic activity. It also
provides a possible explanation for the re-emergence of resis-
tant tumors at sites identical to previous metastases. Furt-
hermore, these findings underscore the importance of correcting
for physical parameters when undertaking studies in cellular
metabolism, as well as the dynamic interplay between growth,
metabolism, and signaling.

Disclosure of Potential Conflicts of Interest

G.V. Long is a consultant/advisory board member for GSK, Novartis, and
Roche. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: N. Theodosakis, M.A. Held, A. Marzuka-Alcala,
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