Monitoring Chemotherapeutic Response by Hyperpolarized $^{13}$C-Fumarate MRS and Diffusion MRI

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Abstract

Targeted chemotherapeutic agents often do not result in tumor shrinkage, so new biomarkers that correlate with clinical efficacy are needed. In this study, we investigated noninvasive imaging protocols to monitor responses to sorafenib, a multikinase inhibitor approved for treatment of renal cell and hepatocellular carcinoma. Healthy cells are impermeable to fumarate, so conversion of this metabolite to malate as detected by $^{13}$C-magnetic resonance spectroscopy (MRS) has been suggested as one marker for cell death and treatment response in tumors. Diffusion MRI also has been suggested as a measure of therapy-induced cytotoxic edema because viable cells act as a diffusion barrier in tissue. For these reasons, we assessed sorafenib responses using hyperpolarized $^{13}$C-fumarate, diffusion-weighted MRI (DW-MRI) in a xenograft model of human breast cancer in which daily administration of sorafenib was sufficient to stabilize tumor growth. We detected signals from fumarate and malate following intravenous administration of hyperpolarized fumarate with a progressive increase in the malate-to-fumarate (MA/FA) ratio at days 2 to 5 after sorafenib infusion. The apparent diffusion coefficient (ADC) measured by DW-MRI increased in the treated group consistent with cytotoxic edema. However, the MA/FA ratio was a more sensitive marker of therapeutic response than ADC, with 2.8-fold versus 1.3-fold changes, respectively, by day 5 of drug treatment. Histologic analyses confirmed cell death in the sorafenib-treated cohort. Notably, $^{13}$C-pyruvate-to-lactate conversion was not affected by sorafenib in the breast cancer model examined. Our results illustrate how combining hyperpolarized substrates with DW-MRI can allow noninvasive monitoring of targeted therapeutic responses at relatively early times after drug administration. Cancer Res; 74(3); 686–94. ©2013 AACR.

Introduction

Sorafenib (Nexavar) was the first RAF kinase inhibitor to enter human clinical testing and is now approved for use in advanced or metastatic renal cell carcinoma and in unresectable hepatocellular carcinoma (1). This compound, initially developed as a selective inhibitor of RAF, has shown other biologically relevant targets, including VEGFR2/3, platelet-derived growth factor receptor (PDGFR), Fli-3, c-kit, and fibroblast growth factor receptor (FGFR-1; ref. 2). Sorafenib is therefore able to affect both tumor signaling and angiogenesis. Preclinically, Sorafenib shows broad-spectrum antitumor activity in renal, colon, hepatocarcinoma, breast, non–small cell lung, ovarian, thyroid, pancreatic, and melanoma xenograft models, involving either antiproliferative and/or antiangiogenic effects of the drug (3). Clinical studies using sorafenib as monotherapy have also been conducted in patients with malignant glioma (4), thyroid cancer (5–7), metastatic melanoma (8, 9), angiosarcoma (10), head and neck tumors (11), acute leukemias (12), and advanced soft tissue sarcomas (13).

The new targeted therapies and treatment options require timely and effective methods to evaluate an individual's response. Conventional anatomically based endpoints may be inadequate to monitor the tumor response to targeted agents that usually do not result in tumor shrinkage while used as monotherapy. Diffusion-weighted MRI (DW-MRI) seems to detect the loss of the cellularity, which is the end result of extensive necrosis (14), and has also been shown to be sensitive to other type of cell death, including mitotic catastrophe and apoptosis (15). However, tumor ADC (apparent diffusion coefficient) is not yet able to detect low levels of diffuse necrosis or early necrosis following administration of anticancer agents (16, 17). Therefore, the identification and use of complementary, earlier, and more sensitive noninvasive biomarkers are needed to optimize the schedule and dosage of novel therapeutics. Several novel imaging methods exploit altered metabolism and its normalization in treatment-responsive tumors as methods for the evaluation of the treatment response (18). Magnetic resonance spectroscopy (MRS) has been used to...
investigate biochemical changes associated with disease, and tumor response to targeted therapies (19). However, a limitation of MRS is low sensitivity, especially for nuclei other than protons. Dynamic nuclear polarization (DNP) can be used to increase the sensitivity >10,000-fold of in vivo 13C-MRS through hyperpolarization of nuclear spins of intravenously delivered 13C labeled substrates (20, 21). DNP transfers high electron spin polarization to nuclear spins via microwave irradiation in a strong magnetic field (3.35T) and at cryogenic temperature (1.4 K). Metabolic fluxes have been followed in experimental tumors using hyperpolarized [1-13C] pyruvate, [1,4-13C2] fumarate, or [13C] bicarbonate, among others (22, 23). The first clinical trial using hyperpolarized 13C-MR metabolic imaging with [1-13C] pyruvate has been successfully performed in patients with biopsy-proven prostate cancer and no dose-limiting toxicities were observed (24).

13C-MRS–detectable conversion of hyperpolarized fumarate-to-lactate, catalyzed by fumarase, has been suggested as a marker of drug-induced cellular necrosis and treatment response in tumors (25). Healthy cells are impermeable to fumarate, thus fumarate-to-lactate conversion is thought to reflect the necrosis-mediated release of fumarase into the extracellular space of the surrounding tissue. Necrotic areas are poorly perfused and have high extracellular fumarase activity (25). Importantly, the background is low, as viable cells demonstrate slow uptake, and, consequently, there is little detectable malate production within the lifetime of the polarization (26). The levels of malate produced correlated with the levels of necrosis in vitro and in vivo (25, 27). This method could therefore be used as a very early marker of therapies that induce necrosis (28).

Pyruvate, an endogenous substrate, is generated by metabolism of glucose or oxidation of lactate (29). To date, this metabolite has been the most commonly used for DNP. The conversion of pyruvate-to-lactate may be used to help distinguish tumor from normal tissue (30) and can also serve as a diagnostic marker (31). The abnormality of pyruvate metabolism in diseased tissue can be detected by quantifying its downstream metabolites. Lactate conversion results from the reaction catalyzed by the enzyme lactate dehydrogenase (LDH). The conversion kinetics will depend on the delivery of hyperpolarized pyruvate to the tumor, the rate of pyruvate transport across the cell membrane and the activity of LDH (26). LDH activity, in turn, depends on the concentration of the enzyme and substrate (NAD+, NADH, pyruvate, and lactate) concentrations at steady state as well as the intracellular pH (26). There is increasing evidence for an early reduction in pyruvate–lactate exchange in a range of cancer models following treatment with cytotoxic chemotherapy (27, 32), targeted drug (28, 33, 34), and radiotherapy (35). In addition, 13C-MRS–detectable hyperpolarized pyruvate-to-lactate conversion has recently been suggested as a response marker for LDH A (36) and MAPK inhibition (18) as well.

The aim of this study was to assess the response to sorafenib using 13C-MRS of hyperpolarized fumarate and pyruvate in mammary xenografts, in comparison with DW-MRI and histologic markers.

Materials and Methods

Tumor model

All animals were maintained in accordance with Institutional Animal Care and Use Committee (University of South Florida, Tampa, FL) standards of care in pathogen free rooms, in the Moffitt Cancer Center (Tampa, FL) Vivarium. All imaging and measurements were performed within the facility. As a model for tumor therapeutic response, human tumor xenografts were grown in NMRI nude mice. MDA-MB-231 human breast cancer cells were grown in Dulbecco’s Modified Eagle Medium-F12 media supplemented with 10% FBS and 1% penicillin–streptomycin. Cells were removed from flasks by either treating with trypsin. About 10 million cells in 100 μL of media were immediately injected into the leg of 3- to 6-week-old female severe combined immunodeficient mice. For imaging and histology, xenografts were allowed to grow 3 to 6 weeks into tumors of suitable volume. The 13C-MRS study was initiated when tumor volume reaches about 200 mm3. Mice were weighed and tumor volumes were measured twice-weekly using calipers and calculated as (length × width × width)/2, with the length and width defined as the long and short diameters, respectively. Moreover, tumor volume was measured more precisely during MRI experimentation using T2-weighted imaging (fast spin-echo). Only healthy mice were used for imaging and spectroscopy. Magnetic resonance (MR) experiments were carried out before injection of any drug, after 2 and 5 days of daily sorafenib treatment (40 mg/kg dissolved in dimethyl sulfoxide, DMSO). Drug was administered by intraperitoneal injection (35 μL).

MR experiments

Mice bearing MDA-MB-231 xenografts were imaged at day 0 (tumor size of 150-200 mm3), day 2, and day 5 of daily treatment with 40 mg/kg of sorafenib. Mice were anesthetized with a mixture of 1.0 L/min O2 and 3% isoflurane (AErrane; Baxter) for induction, and 1% isoflurane for maintenance. Body temperature was maintained constant (37°C ± 0.5°C) in the gradient coils using an air heater and monitored using a rectal temperature probe.

[1-13C] pyruvic acid or [1,4-13C2] fumaric acid, were mixed with 15 mmol/L trityl radical (OX63) and hyperpolarized by an Oxford DNP Polarizer (HyperSense). The polarized substrate was quickly dissolved in Tris/EDTA, NaCl, and NaOH solution at 37°C, yielding 80 mmol/L pyruvate or 20 mmol/L fumarate at neutral pH, before injecting into the mouse via jugular vein catheter. Mice were imaged using a double tuned 1H–13C–volume coil in an Agilent ASR 310 7T small animal imaging system. After administration of 0.45 mL of hyperpolarized fumarate, 13C spectra were acquired with a T1 of 2,000 ms and flip angle 15° for 5 minutes from a 3- to 4-mm-thick slice across the tumor. After 1 hour of fumarate injection, 0.35 mL of hyperpolarized pyruvate was administered and 13C spectra were acquired with a T1 of 1,000 ms and flip angle 9° for 5 minutes from the same tumor. Low flip angle pulses are required to preserve the polarization over the whole imaging period that allow more efficient use of the available polarization. DW-MRI was performed before the injection of
hyperlaminated substrate using a spin-echo sequence with three b values (50, 500, and 1,000). T1 = 1,500 ms, T2 = 86 ms. All the MRI data were compared before and after 2 or 5 days of treatment.

**Histologic analysis**

Tumors were excised at day 5 for histologic analysis and compared with untreated tumors. The center section of the subcutaneous tumor of each animal was fixed in 10% formalin and paraffin embedded before staining one 5-μm-thick cross-sectional sample per animal with hematoxylin and eosin (H&E) for histology. Another 5-μm slice was taken for cleaved caspase-3 (CC3) staining. Histology slides of both H&E and CC3 were scanned using the Aperio ScanScope XT with a 200×/0.8 NA objective lens at a rate of 8 minutes per slide via Basler trilinear array. Histologic pattern recognition was conducted using the Genie (Aperio) software platform to segment and classify necrosis, viable tumor, and other nontarget tissues (i.e., adipose, muscle, and skin; ref. 37). Furthermore, Spectrum algorithms were applied the entire slide’s digital image and to determine the percentage of necrosis by detecting the number of pixels that satisfy a color and intensity predefined (necrotic), divided by the number of pixels in nonnecrotic tissue. Similar analyses were performed to evaluate the number of positive nucleus in CC3 slides. All the training algorithms developed above were quality controlled by a practicing pathologist.

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**Statistical analysis**

All results are expressed as mean ± SEM. ANOVA and Bonferroni post-test, t test were performed to assess the statistical significance between the different groups and timings. Statistical significance was considered at the P < 0.05 level.

**Results**

Daily intraperitoneal sorafenib injections (40 mg/kg) for 9 days were able to significantly reduce MDA-MB-231 tumor growth. Tumor volumes were measured twice-weekly using calipers (n = 4). We observed a significant difference at days 2, 6, and 9 (P value < 0.001) between the sorafenib-treated group and the control group, which received only DMSO vehicle (Fig. 1A).

By day 5, we observed a significant increase in tumor ADCw during Sorafenib treatment (P < 0.01; one-way ANOVA, Bonferroni post-test). These results suggest a significant decrease in cellularity in the tumor after 5 days of treatment (Fig. 1B). ADCw values within the segmented tumor regions of interest were also used to generate histograms. As shown in Fig. 1C, a right shift in tumor water diffusion was observed at 5 days of treatment. ADC maps from a representative animal before and after treatment with sorafenib (day 0 vs. day 5) are shown on Fig 1D. This is better displayed using cumulative (Fig. 1E) and cumulative difference histograms (Fig. 1F).

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**Figure 1.** A, MDA-MB-231 tumors growth curve with or without sorafenib treatment (*P < 0.01). B, average tumor ADCw following administration of sorafenib (*P < 0.01 after 5 days of treatment). C, typical ADCw histograms of control and treated tumors (day 5). A right shift in tumor water diffusion was observed at 5 days of treatment. D, typical diffusion maps before treatment and after 5 days of treatment with sorafenib. ADCw is observed after 5 days of treatment with sorafenib. E, cumulative histograms before and after treatment, percentage pixels above ADC value. F, cumulative change in percentage pixels above ADC value after 2 days and 5 days of treatment.
Intravenous administration of hyperpolarized fumarate resulted in detectable signals from fumarate (176 ppm) and malate (179 ppm) following 2 days of treatment (Fig. 2B). No detectable malate was observed before treatment (Fig. 2A). Because the two $^{13}$C carbons of $[1,4-^{13}$C$_2]$ fumarate are equivalent, they give a single peak. However, $[1,4-^{13}$C$_2]$ malate should yield two peaks. These are merged into a single anisotropic peak in this study, likely because of low spectral resolution resulting from the 4 mm tumor slice from which the spectra were obtained. In our study, 3 ppm chemical shifts between fumarate and malate were observed in vivo, which is consistent with the results of Witney and colleagues in similar tumors (27). However, larger chemical shifts have been described in other studies between malate and fumarate, especially in vitro (25). There was a time-dependant change in fumarate-to-malate conversion that became significant after 5 days of treatment with sorafenib ($n = 3$). The flux ratio of tumor malate and fumarate was assessed as a drug therapy response marker in this study. The evolution of this ratio reflects the evolution of cell death in response to sorafenib treatment. The malate-to-fumarate (MA/FA) ratios calculated as the 30-second integrals following peak (Fig. 2C) progressively increased from day 2 ($n = 5$) until day 5 ($n = 3$), time at which the relative increase of the ratio is significant ($P < 0.01$, one-way ANOVA; Bonferroni post-test; Fig. 2D). The MA/FA ratio reached its highest value at 2.8 after 5 days of sorafenib treatment.

Notably, the ADC$_w$ changes in sorafenib-treated tumors at day 2 ($n = 9$) and day 5 ($n = 5$) were in accordance with changes observed with MA/FA ratio. Indeed, a positive correlation was observed between the relative change in MA/FA and the relative change in ADC$_w$ over time (Pearson correlation test, $P < 0.05$; Fig. 3). In comparing the sensitivities of the two methods, a 2.8-fold increase was observed for $^{13}$C fumarate/malate versus 1.3-fold for ADC$_w$. However, the reproducibility of ADC$_w$ measurements is expected to be higher than those of MA/FA, as they do not require injection of exogenous substrate.

One hour after fumarate injection, hyperpolarized $^{13}$C pyruvate was administered and resulted in two detectable peaks: lactate (183 ppm) and pyruvate (171 ppm; Fig. 4A and B). Pyruvate-to-lactate conversion and their quantitative analysis for pretreated and treated animals were performed. The flux ratio (Lac/Pyr) were calculated from area under the curve of total pyruvate pool and lactate pool with time and this ratio did not change in pre- and post-treated tumors as shown in Fig 4C. In addition, we calculated the apparent rate

![Figure 2](image1.png)

**Figure 2.** A, typical $^{13}$C-MRS spectra from an MDA-MB-231 untreated tumor after hyperpolarized fumarate injection. B, $^{13}$C-MRS spectra from a treated tumor: fumarate (176 ppm) and malate (179 ppm) peaks. C, corresponding areas under the curves for fumarate and malate signals. D, $^{13}$C flux modifications in the malate/fumarate ratio after sorafenib treatment ($P < 0.01$).
constants \( k_P \) (pyruvate-to-lactate) and \( k_L \) (lactate-to-pyruvate) using modified Bloch equations (32, 34) as shown in Fig. 4D and E. The fitting results indicated that the rate constants did not change significantly between pre- and post-treated tumors as shown in Table 1.

This result was also confirmed \textit{ex vivo} by lactate measurements of tumor extracts. There were no significant differences in lactate concentrations between control (7.6 ± 1.1 μmol/g) and treated (6.1 ± 1.1 μmol/g) tumors \((P = 0.41)\). Moreover, regardless of the tracer used, the times to maximum peak of the signals after tracer injection were not significantly different between control and sorafenib tumors. For pyruvate, the times to peak were 13 s ± 1.0 s versus 11 s ± 1.0 s for control and treated tumors, respectively, \((P = 0.22)\). For fumarate, the times to peak were 14.0 s ± 2.0 s versus 16.0 s ± 2.0 s for control and treated tumors, respectively, \((P = 0.58)\). These observations indicate that the delivery of hyperpolarized substrates, and hence the vasculature and perfusion, were not modified by sorafenib treatment.

Histologic studies of tumor sections were obtained post-mortem on control mice and mice treated with daily sorafenib for 5 days. These included H&E and caspase-3 analysis. H&E histologic analysis showed a significant \((P < 0.008)\) increase in tumor necrosis (40.7 % increase) between untreated tumors and tumors treated with sorafenib for 5 days (Fig. 5A and B). Differences in CC3 staining probing apoptotic cell death were also observed between treated and control tumors. Quantitative analyses showed that average CC3 stained–positive pixel values normalized by constant area of tissue section were much larger \((P < 0.0001)\) following 5 days of treatment with sorafenib compared with control (untreated) tumors (Fig. 5C and D). Notably, tumor size did not change between day 0 and day 5 under sorafenib treatment, whereas it did in the controls (Fig. 1A). This suggests that the lack of tumor volume change was associated with an increase in apoptotic and necrotic cell death.

Discussion

The introduction of new drugs and targeted treatments to individual patient has led to the need for specific biomarkers of treatment response that are good predictors of final outcome (38). Indeed, targeted therapies usually result in tumor stabilization rather than in tumor shrinkage, at least in monotherapy, reducing the sensitivity of standard metrics of response, such as Response Evaluation Criteria in Solid Tumors (39). There is therefore a need for quantitative biomarkers to assess treatment response. Noninvasive imaging can provide sensitive and specific measures of tumor response in the absence of

Table 1. Fitted values of apparent rate constants \( k_P \) (pyruvate-to-lactate) and \( k_L \) (lactate-to-pyruvate) using modified Bloch equations for the pre- and post-sorafenib-treated tumors \((n = 4; P < 0.05, \text{Student } t \text{ test})\)

<table>
<thead>
<tr>
<th>Tumors</th>
<th>( k_P ) (per second)</th>
<th>( k_L ) (per second)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-sorafenib (day 0)</td>
<td>0.054 ± 0.004</td>
<td>0.023 ± 0.003</td>
</tr>
<tr>
<td>Post-sorafenib (day 2)</td>
<td>0.055 ± 0.003</td>
<td>0.022 ± 0.004</td>
</tr>
<tr>
<td>Post-sorafenib (day 5)</td>
<td>0.053 ± 0.004</td>
<td>0.021 ± 0.003</td>
</tr>
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change in tumor size. Such methods include DNP and DW-MRI, which alone or in combination can noninvasively assess metabolic or oedematous responses in vivo.

Tumor response to sorafenib has been assessed using overall survival, time to symptomatic progression and time to progression in phase II and III clinical studies in hepatocellular carcinoma (40). Objective evidence of tumor shrinkage has been seen in renal cell carcinoma (41), melanoma (42), pancreatic, and papillary thyroid carcinoma (43). More sensitive and quantitative biomarkers that correlate with clinical efficacy are required in the transition toward individualized therapy; especially given the financial and patient costs of targeted treatments, such as sorafenib. Notably, response biomarkers can also serve as nonresponse biomarkers, to spare patients cycles of futile therapy, and possibly allow them to move to other, possibly experimental therapies.

Imaging techniques to quantify response have included 2[18F]fluoro-2-deoxy-D-glucose–positron emission tomography to assess metabolism (44), dynamic contrast enhanced MRI (DCE-MRI) to assess tumor blood flow and perfusion (45), and DW-MRI to assess cellularity (14). Newly emerging techniques include hyperpolarized 13C-MRI, which cannot only measure catabolism and trapping of tracer, but can also measure metabolic conversion.

13C fumarate provides positive contrast and is described to be a marker of cellular necrosis (25). In necrotic cells, the plasma membrane permeability barrier is compromised; fumarate is therefore rapidly transformed into malate. In viable cells, however, there is a very slow plasma membrane transport and thus, no detectable conversion of fumarate-to-malate within the short lifetime of the 13C polarization is observed (27). The rate of labeled malate production has shown good correlation with the level of tumor cell necrosis (25). The lack of a malate signal in the absence of cell death makes this a highly sensitive measurement. In this study, we observed a 280% increase of the malate/fumarate ratio following sorafenib treatment. These results were consistent with the decrease in cellularity assessed using DW-MRI. Because there is water diffusion in normal tissues, there is a higher background and hence, a smaller magnitude change of only 30%. Both the increase in malate and ADC were consistent with the increase of necrosis and apoptosis measured by H&E and caspase-3 analyses, respectively. The level of tumor cell necrosis after treatment has been described both in preclinical and in clinical studies as a good prognostic indicator for treatment outcome in the absence of any change in tumor size (46). Our results show that the net change in 13C fumarate conversion into malate marker was more sensitive than DW-MRI. However, it must be remembered that the value of a biomarker is directly related to the magnitude of change, normalized to its test–retest reproducibility, and this has yet to be assessed in a larger cohort and others tumor models before generalization, although our results are in agreement with other recent preclinical studies comparing fumarate with pyruvate and ADC (28, 47). Also, ADC has been reported to be highly reproducible within a single institution. No such data are yet available for fumarate, yet the real-time availability of the input function can mitigate the greatest source of error for an injectable kinetic model (48). Bohndiek and colleagues showed that the changes in fumarate conversion were earlier and more sensitive than the changes in ADC in lymphoma tumors treated with combrestatatin-A4-phosphate (28). In this model, they did not observe any change in ADC at 6 hours after treatment, although there was a 32% increase in necrotic fraction at 24 hours. This was consistent with the fact that in xenograft tumors with small or diffuse regions of necrosis, there may be no change in ADC with necrotic fractions of up to 40% (17). We also do see a more sensitive response using fumarate than ADC, but in our case, timing of response is similar and even positively correlated at day 2 and day 5. So, in this study, the major advantage of fumarate is the higher dynamic range, which might be balanced by the disadvantage of requiring the injection of an exogenous substrate with respect to DW-MRI.

Cohyperpolarized pyruvate and fumarate preparations could not be used in vivo as malate production is masked by
overlapping signals from lactate and the pyruvate hydrate formed from labeled pyruvate (27). However, injections of hyperpolarized fumarate and pyruvate were performed one hour apart and were consecutive to the DW-MRI scans, so that all measurements were performed on the same tumors during the same MRI session.

Importantly, the monitoring pyruvate-to-lactate flux did not show any sensitivity to treatment with sorafenib in this study on MDA-MB-231 xenografts. This is in contrast with other studies that have shown significant effects of targeted and nontargeted chemotherapy on pyruvate-to-lactate fluxes. For example, the LDH-catalyzed interconversion of hyperpolarized label between pyruvate and lactate was observed to decrease early after etoposide chemotherapy (32), which was explained by a loss of the coenzyme NADH, decreases in tumor cellularity, and reduced LDH concentrations (23). Also, with respect to targeted therapies, inhibition through the phosphoinositide 3-kinase (PI3K/Akt/mTOR) pathway was shown to correlate with a drop in hyperpolarized [1-13C] lactate levels in breast cancer and glioblastoma cells and xenografts (34). This was attributed to a drop in LDH expression as a result of reduced levels of the transcription factor, hypoxia-inducible factor-1α, which regulates expression of the LDH gene (23). Finally, the pyruvate-to-lactate flux observed in transgenic prostate tumors has been shown to be consistent with tumor cellularity and necrosis (23).

Two studies reporting a lack of change in pyruvate-to-lactate conversion (18, 49), attributed the lack of response to a putative role played by the monocarboxylate transporters (MCT) 1 and MCT4, in pyruvate-based molecular imaging and the concomitant low MCT1 expression in their tumor cell lines. MCT1 is not detected in MDA-MB231 cells (50). However, the hypothesis that the expression of the transporter is rate limiting is not consistent because other groups observed a decrease in lactate-to-pyruvate ratio in MDA-MB-231 tumors in response to doxorubicin (27) or to PI3K inhibition (34).

A factor that could be involved in the lack of change in pyruvate-to-lactate ratio is a potential normalization effect of sorafenib. Indeed, this could be the result of compensating effects between an increase in delivery of pyruvate (due to potential normalization effects of sorafenib) and a potential decrease in pyruvate-to-lactate conversion. Pyruvate results have been discussed in the same context in other studies: Bohndiek and colleagues (47) had similar results using bevacizumab in colorectal cancer xenograft (HT29). In this study they observed a significant increase in apoptotic and necrotic areas (5%-10% at 72 hours) accompanied by a paradoxical increase in 13C flux from hyperpolarized pyruvate. In contrast, the same treatment in another colorectal cancer model that was more sensitive to the anti-VEGF therapy showed a decrease in 13C pyruvate flux. The likely explanation of these discrepancies was the difference in the angiogenic response and in pyruvate delivery depending on the tumor type. In our study, normalization effects of sorafenib were not directly assessed, although the time to maximum peak intensity (reflecting the delivery of the substrates) in both fumarate and pyruvate studies did not differ significantly from day 0 to day 5. Time-to-maximum is, however, not as robust as is DCE-MRI or ex vivo studies of microvesSEL density to assess spatially explicit change in delivery/perfusion that can be induced by sorafenib. With this regard, the literature reports that the MDA-MB-231 breast cancer model was shown to be sensitive to sorafenib treatment in terms of normalization. Daily oral administration of a 30 or 60 mg/kg dose of sorafenib for 5 days strongly decreased the number and area of microvesSELS in the sorafenib-treated tumors, showing significant inhibition of angiogenesis in this tumor model, as shown by ex vivo staining of CD31 (2). A hypothesis is therefore that a potential vessel normalization could contribute to an increase in delivery of pyruvate at day 2 of 5, which could in turn compensate for a potential decrease in the pyruvate-to-lactate conversion. Notably, a decrease in pyruvate-to-lactate conversion has been described by other groups in response to doxorubicin or PI3K inhibition in this same tumor model (27, 34). If this were the case, the final lactate-to-pyruvate ratio would not be modified. Although we did observe a trend for ex vivo lactate levels to decrease at day 5, these were not significant. This would also require further "normalization" studies, such as DCE-MRI, to attest this hypothesis. Finally, the malate/fumarate ratio is a more direct measure of cell death than is the lactate/pyruvate ratio. For example, dying cells are permeable and, unless this results in a loss of cofactor NADH, the LDH reaction may continue to proceed in a MCT-independent fashion. This same increase in permeability allows access of fumarate to fumarase and subsequent conversion to malate. The fumarate results are coherent with histologic results, whereas pyruvate results seem to be also influenced by the vascularization state and normalization state of the tumor, as suggested by Bohndiek and colleagues.

Conclusion

We show that hyperpolarized MRS using 13C-fumarate is an early in vivo marker of response to sorafenib in MDA-MB-231 tumors and is positively correlated with DW-MRI, with a higher sensitivity for MA/FA ratio with respect to DW-MRI (dynamic ranges of 2.8 vs. 1.3, respectively, at day 5). Results are in accordance with ex vivo H&E and CC3 analysis. The lactate-to-pyruvate ratio does not seem to be an in vivo marker of tumor response to sorafenib in MDA-MB-231 tumors, likely due to vessel normalization.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: L. Mignion, P. Dutta, R.J. Gillies, B.F. Jordan
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L. Mignion, P. Dutta, G.V. Martinez, P. Foroutan, B.F. Jordan
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L. Mignion, P. Dutta, G.V. Martinez, R.J. Gillies, B.F. Jordan
Writing, review, and/or revision of the manuscript: L. Mignion, P. Dutta, P. Foroutan, R.J. Gillies, B.F. Jordan
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L. Mignion, P. Dutta
Study supervision: G.V. Martinez, B.F. Jordan
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