

## Evaluation of LDH-A and Glutaminase Inhibition *In Vivo* by Hyperpolarized $^{13}\text{C}$ -Pyruvate Magnetic Resonance Spectroscopy of Tumors

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### Abstract

Hyperpolarized  $^{13}\text{C}$  magnetic resonance spectroscopy provides a unique opportunity to detect real-time metabolic fluxes as a means to measure metabolic treatment responses *in vivo*. Here, we show that pharmacologic inhibition of lactate dehydrogenase-A suppressed the conversion of hyperpolarized  $^{13}\text{C}$ -pyruvate to lactate in murine xenografts of P493 human lymphoma. In contrast, a glutaminase inhibitor reduced conversion of  $^{13}\text{C}$ -pyruvate to alanine without affecting conversion of pyruvate to lactate. These results illustrate the ability to monitor biomarkers for responses to antimetabolic therapy in real-time, paving the way for clinical development of imaging biomarkers to monitor metabolic pharmacodynamics. *Cancer Res*; 73(14); 4190–5. ©2013 AACR.

### Introduction

Many cancer cells are characterized by high rates of glucose uptake and elevated lactate production. Oncogenes and tumor suppressors can be directly linked to oncogenic alterations of cancer metabolism and increased glucose metabolism (1–3). Glucose metabolism is initiated by glucose transporters and hexokinases, which result in glucose being trapped via intracellular phosphorylation. The 6-carbon glucose molecule is further catabolized to 3-carbon pyruvate, which is converted to alanine through transamination, and to acetyl-CoA by pyruvate dehydrogenase for oxidation in the tricarboxylic acid (TCA) cycle, or to lactate by lactate dehydrogenase-A (LDH-A). Increased glycolysis has a number of relevant sequelae, including acid production and the diversion of glucose-derived carbons to anabolic processes. Besides glycolysis, glutaminolysis, in which glutaminase converts glutamine to glutamate for oxidation in the TCA cycle, provides a major nitrogen and carbon source for the growing cells. The glutamine skeleton is also vital for the production of aspartate and other amino acids.

The tetrameric LDH-A enzyme kinetically favors the conversion of pyruvate to lactate, a hallmark of the Warburg effect, and hence is an attractive therapeutic target. Underscoring its appeal as a target are the high levels of LDH-A that have been documented in human cancers (4). We have recently observed that inhibition of LDH-A with a small drug-like molecule, FX11, curbed lymphoma and pancreatic tumor growth, with the caveat that there could be off-target effects *in vivo* (5). We further documented that an allosteric inhibitor of glutaminase, bis-2-(5-phenylacetamido-1,2,4-dithiazol-2-yl) ethyl sulfide (BPTES), could also delay the growth of lymphoma xenografts (6). Hence, cancer metabolism as a maturing field holds promise for new therapeutic agents. Currently, the ability to detect metabolism clinically is largely limited to  $^{18}\text{F}$ -fluorodeoxyglucose positron emission tomography (PET) imaging of glucose uptake into tumors (7). Notably,  $^{18}\text{F}$ -labeled tracers of glutamine metabolism are in development (8). Although it is highly sensitive, PET imaging is unable to dynamically measure metabolic conversion and is thus generally limited to static measures of tissue accumulation.

$^{13}\text{C}$  magnetic resonance spectroscopy (MRS) has long been used in the investigation of static metabolic processes *in vivo* (9). Recently, with the advent of dynamic nuclear polarization (DNP) techniques,  $^{13}\text{C}$ -MRS and imaging can measure dynamics of metabolic conversions *in vivo* (10). DNP ("hyperpolarization") can increase  $^{13}\text{C}$ -MRS sensitivity by 10,000-fold or more, allowing for detection of  $^{13}\text{C}$ -labeled compounds and their downstream metabolic products in real-time *in vivo* (10–12). In DNP, the large polarization of electron spins is transferred to the nuclear spins, enhancing the signal intensities for subsequent nuclear magnetic resonance (NMR) spectroscopy and imaging. Flux exchange of hyperpolarized  $^{13}\text{C}$  label between pyruvate and lactate is governed by the combination of: tumor perfusion, membrane transport of pyruvate, endogenous lactate concentration, and LDH activity (13–14). The principal drawback of DNP is the short spin-lattice relaxation

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time ( $T_1$ ) that leads to polarization decay. Notably, the  $T_1$  of [ $1\text{-}^{13}\text{C}$ ] pyruvate is about 30 to 40 seconds *in vivo*, which is sufficient to measure metabolic interconversion. The signal is observable for 5 times as long as the  $T_1$ , meaning that the pyruvate signal is visible approximately within 2 to 3 minutes post injection. Several orders of magnitude enhancement of the NMR signal, combined with the long  $T_1$  relaxation time, make this a promising technique for hyperpolarizing tracers with potential applications in medical imaging.

In the present study, we report that  $^{13}\text{C}$  MRS can assess the metabolic conversion of lactate from pyruvate and consequently evaluate the *in vivo* efficacy of a LDH-A inhibitor (FX11) in cancer treatment. We monitored the dynamic conversion of hyperpolarized [ $1\text{-}^{13}\text{C}$ ]-pyruvate to lactate and detected responses of tumors to FX11 treatment aimed at inhibiting LDH-A. Inhibition of glutaminase with BPTES, which could also curb tumor growth, did not affect the conversion of pyruvate to lactate and was comparable with dimethyl sulfoxide (DMSO) vehicle control-treated tumors. BPTES did, however, reduce the conversion of [ $1\text{-}^{13}\text{C}$ ]-pyruvate to alanine, which was also observable in these dynamic scans. FX11 had no significant effect on pyruvate-to-alanine conversion. Our studies establish for the first time the use of hyperpolarized [ $1\text{-}^{13}\text{C}$ ]-pyruvate to distinguish the responses of tumors to inhibition of LDH-A and glutaminase relative to vehicle controls.

## Materials and Methods

### Animal studies

All animal experiments were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals (Bethesda, MD) and were approved by the Institutional Animal Care and Use Committee of the University of South Florida (Tampa, FL), which regulates animal care and use at the Moffitt Cancer Center (Tampa, FL). To generate the xenograft model,  $2.0 \times 10^7$  P493 human lymphoma B cells were injected subcutaneously into male severe combined immunodeficient mice (National Cancer Institute, Bethesda, MD) as previously described (5). When the tumor volume reached about  $700 \text{ mm}^3$  (within 3–4 weeks), mice were injected daily with control 2% (v/v) DMSO or  $42 \mu\text{g}$  of FX11 (2.1 mg/kg bodyweight) and  $200 \mu\text{g}$  (10 mg/kg bodyweight) of BPTES via intraperitoneal injection to monitor therapy response. Tumor volumes were calculated using the following formula: [length (mm)  $\times$  width (mm)  $\times$  width (mm)  $\times$  0.52].

To prepare mice for  $^{13}\text{C}$ -MRS studies, a jugular catheter was surgically implanted to facilitate injections. The jugular vein, rather than tail vein, was used for MRS experiments as it accommodates larger injectate volumes (up to 0.5 mL compared with 0.2 mL), and multiple injections in tail veins of the same animal were associated with a high failure rate. Jugular vein catheterization allowed multiple injections over a week for therapy response study. The tumors were treated with FX11 or BPTES for 7 days, whereas the control group received DMSO.  $^{13}\text{C}$ -MRS acquisitions continued every 24 hours for a week.

For the magnetic resonance imaging (MRI)/MRS studies, mice were induced with isoflurane in a plastic anesthesia chamber with scavenging. Once unconscious, they were placed in a mouse-specific holder within the MRI coil, outfitted with a

mouse-specific nose cone inhalant anesthesia and scavenging system for imaging. This system also contained a pad for respiration monitoring, an endorectal fiber optic temperature monitoring probe, and a heated pad for maintaining core body temperature at  $37^\circ\text{C}$ .

### Pyruvate polarization and injection procedures

Experiments were carried out using  $30 \mu\text{L}$  [ $1\text{-}^{13}\text{C}$ ]-labeled pyruvic acid (Cambridge Isotope Labs; <http://www.isotope.com>) containing 15 mmol/L trityl radical OX63 (GE Healthcare),  $\text{Gd}^{2+}$ -dotarem (Guerbet; <http://www.guerbet-us.com>) at 1.4 K and a 3.35 T field strength and was hyperpolarized for an hour at 94.082 GHz microwaves using an Oxford Instruments DNP polarizer (HyperSense). Before the injection into the mouse via jugular vein catheter, the polarized substrate was quickly dissolved in Tris/ETDA and NaOH at  $37^\circ\text{C}$ , yielding 80 mmol/L pyruvate at physiologic pH. At the start of each dynamic  $^{13}\text{C}$ -MRS scan,  $350 \mu\text{L}$  of the hyperpolarized pyruvate was injected over a period of 12 to 15 seconds. The injection was immediately followed by a  $100 \mu\text{L}$  saline flush to clear the pyruvate solution from the tubing.

### *In vivo* $^{13}\text{C}$ -MRS

All  $^{13}\text{C}$  spectra were acquired using a 7 T, 31 cm horizontal bore magnet (Agilent) using a 35 mm dual tuned  $^1\text{H}$ - $^{13}\text{C}$  volume coil (M2M; <http://www.m2mimaging.com>). Anatomic reference images to determine location and size of the xenograft tumors were acquired with  $T_2$ -weighted FSE (fast spin echo) multislice ( $T_R = 4$  seconds,  $TE = 60$  ms, echo train length = 8, matrix  $128 \times 128$ , slice thickness = 1 mm, 15 slices). *In vivo* data acquisition started immediately before the pyruvate injection with a repetition time ( $T_R$ ) of 1 second and flip angle  $9^\circ$  and single transient spectra were acquired over a period of 150 seconds from a 6 mm thick tumor slice oriented at an oblique angle to acquire signal from tumor.

### Statistical analysis

Values reported are means  $\pm$  SD. Statistical significance between the control and treatment groups was assessed by using a 2-sample *t* test assuming unequal variances. Significant differences were assessed by using a paired 2-sample *t* test for means. Statistical significance was considered at the  $P < 0.05$  level.

## Results

The conversion of hyperpolarized [ $1\text{-}^{13}\text{C}$ ]-pyruvate to lactate was monitored with MRS to assess the response of human P493 B cell lymphoma xenografts to FX11, an inhibitor of LDH-A, or to BPTES, an inhibitor of glutaminase. Tumor xenografts were established and animals were treated with DMSO vehicle or drug via intraperitoneal injection. Fig. 1A and B show relevant metabolic pathways and the  $^{13}\text{C}$ -MR spectrum obtained from a 6 mm thick slice across the tumor after hyperpolarized pyruvate injection, respectively. It displays the prominent peak of pyruvate (171 ppm) and its conversion by LDH to lactate (183 ppm) along with its conversion by glutamate-pyruvate transaminase (GPT) to alanine (176 ppm). Pyruvate hydrate (179 ppm) is formed nonenzymatically in solution and is in

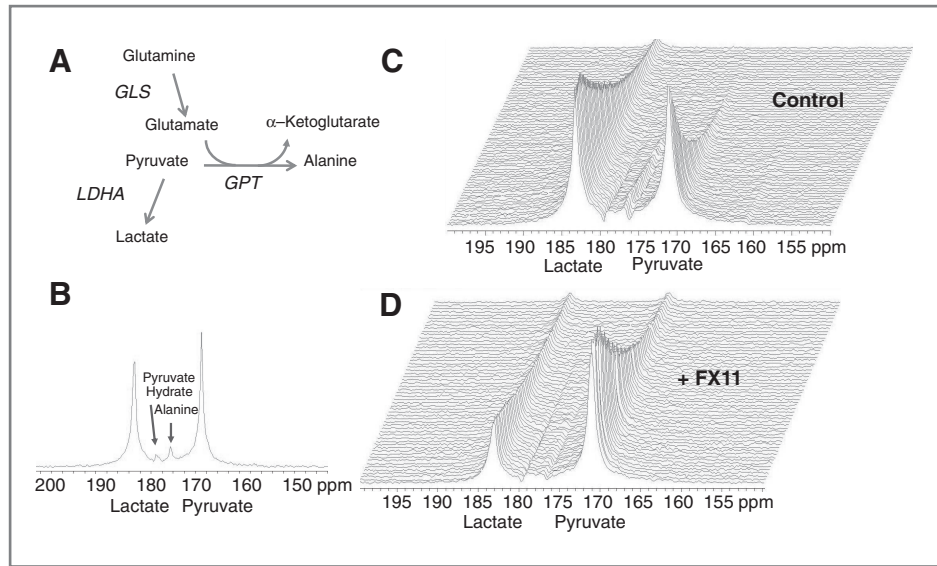


Figure 1. A, schema depicting metabolic pathways relevant to  $^{13}\text{C}$ -pyruvate MRS spectra (glutaminase, GLS). B, hyperpolarized  $^{13}\text{C}$ -MRS acquired from a 6 mm thick slice across the tumor after 20 seconds of pyruvate injection (i.v.) into a mouse. Representative dynamic  $^{13}\text{C}$ -MR spectra after delivering hyperpolarized pyruvate in DMSO-treated (control; C) and FX11-treated (D) mice for 6 days. Spectra are acquired every second.

equilibrium with dehydrated pyruvate. Figure 1C displays sequential dynamic spectra acquired from a 6 mm tumor slice over a total acquisition time of 100 seconds in DMSO-treated mice (control), illustrating a robust conversion of pyruvate to lactate in tumor. After 6 days of FX11 treatment, the pyruvate-to-lactate conversion flux in tumors diminished considerably (Fig. 1D). There is an overall signal decay due to  $T_1$  relaxation of the hyperpolarized substrate. Fig. 2A and B document the lactate and pyruvate (Lac/Pyr) peak intensities as a function of time in DMSO-treated and FX11-treated mice respectively for 4

days. A reduction in lactate flux was evident in the FX11-treated tumor. The flux ratio of tumor lactate and pyruvate was considered to be a drug therapy response marker in this study. The Lac/Pyr flux ratio was calculated from area under the curve (regarded as a "Model-Free" approach) of the metabolic profile from the dynamic scan. The Lac/Pyr flux ratios of FX11-treated tumors were compared with DMSO-treated tumors at different treatment days to assess the response to therapy. The Lac/Pyr ratio increased with time in DMSO-treated animals and progressively lowered in the FX11-treated group, ( $P < 0.01$ ),

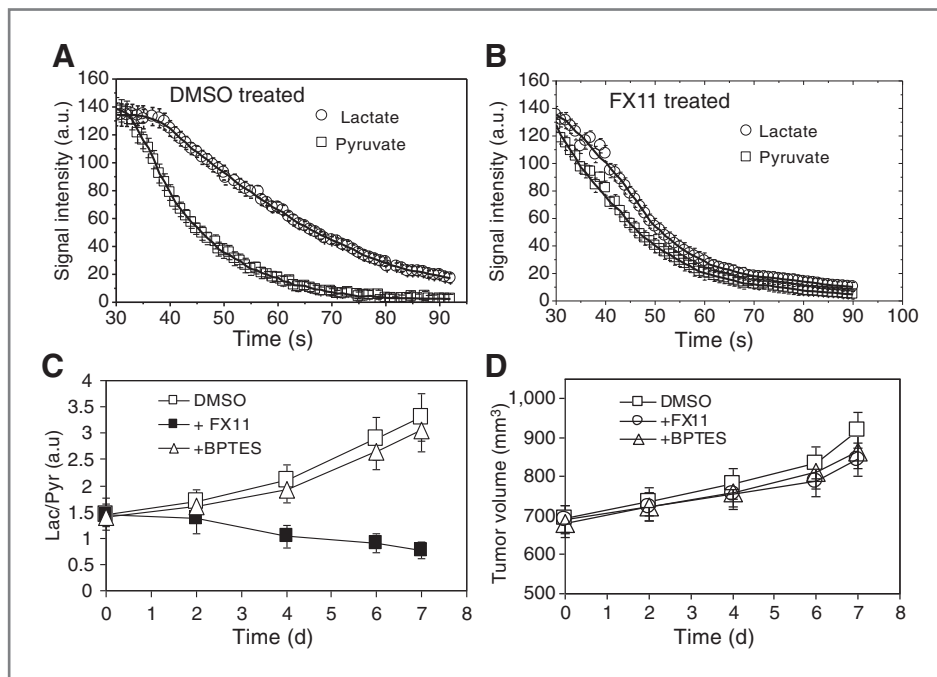
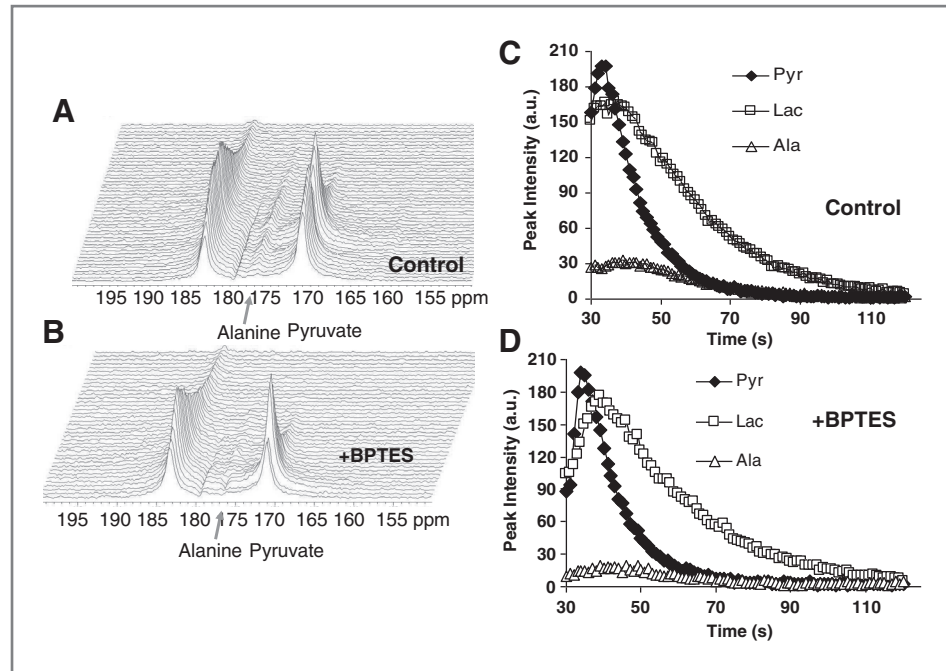


Figure 2. Tumor  $[1-^{13}\text{C}]$  lactate and  $[1-^{13}\text{C}]$  pyruvate peak intensities with time after intravenous injection of hyperpolarized  $[1-^{13}\text{C}]$  pyruvate in DMSO-treated (control; A) and FX11-treated (B) tumors for 4 days. The initial 30 seconds of data were not shown because that time was taken for pyruvate delivery and uptake by the tumor. The data were also fitted to two-site exchange model to estimate the rate constants  $k_P$  and  $k_L$ . C, Lac/Pyr flux ratio increased with treatment days in DMSO- and BPTES-treated mice and decreased in FX11-treated mice. D, a slight increment of tumor volume (measured from  $T_2$ -weighted MRI) was observed with no significant differences between groups. Error bars are the SD from the mean values;  $n = 8$  for each group of mice.

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**Figure 3.** Representative dynamic  $^{13}\text{C}$ -MR spectra after delivering hyperpolarized pyruvate in DMSO-treated (control; A) and BPTES-treated (B) mice for 6 days. Peak intensity profile for pyruvate, lactate, and alanine with time in DMSO-treated (C) and BPTES-treated mice (D).



confirming the FX11 drug response (Fig. 2C). We have also evaluated the conversion rate constants ( $k_p$  = pyruvate-to-lactate and  $k_L$  = lactate-to-pyruvate) using two-site exchange model (14). The ratio of rate constants ( $k_p/k_L$ ) decreases with FX11 treatment and increases in DMSO-treated tumors (Table 1). The tumor volume was monitored during the treatment time window using  $T_2$ -weighted MRI. Although there is a slight growth of tumor in all groups (Fig. 2D), no significant differences in tumor volumes between groups were observed.

To test the specificity of the hyperpolarized  $[1-^{13}\text{C}]$ -pyruvate in the assessment of LDH-A inhibition *in vivo*, we used BPTES, a glutaminase inhibitor, which has previously been observed to reduce growth of P493 tumor xenografts (6). BPTES did not affect *in vivo* pyruvate-to-lactate conversion, and the Lac/Pyr ratio was compared with DMSO-treated control as shown in Fig. 2C. BPTES inhibits the conversion of glutamine to glutamate in tumor cells (15). Because the resulting glutamate is a substrate for the transamination of pyruvate to alanine by GPT (Fig. 1A), we sought to determine whether hyperpolarized

$[1-^{13}\text{C}]$ -pyruvate conversion to alanine might be diminished by BPTES. In this regard, the BPTES-treated animals had a reduced pyruvate-to-alanine conversion compared with controls (Fig. 3A–D). Figure 4A illustrates the significant ( $P < 0.001$ ) reduction of alanine-to-pyruvate (Ala/Pyr) flux ratio by BPTES in replicated experiments. The Ala/Pyr flux ratio, however, was not significantly ( $P = 0.112$ ) diminished by FX11 (Fig. 4B). These data represent the first use of the pyruvate-to-alanine conversion flux ratio and document an effect of BPTES *in vivo*.

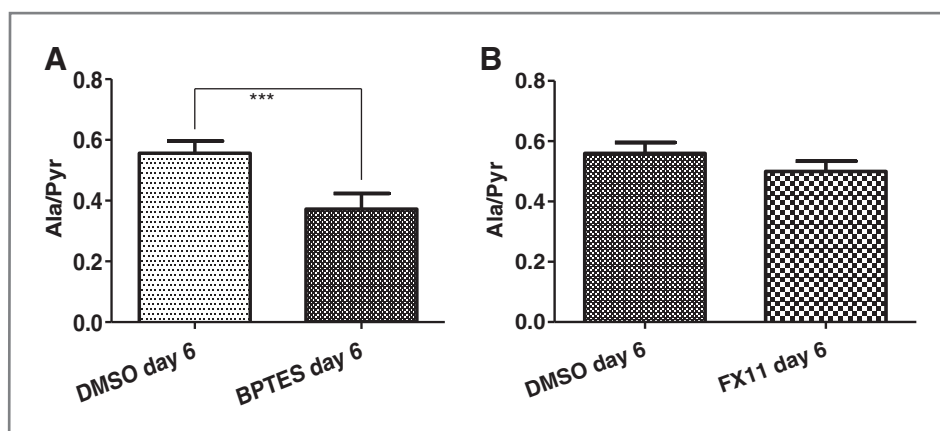
## Discussion

The resurgence of interest in cancer metabolism has increased expectations for targeting specific metabolic pathways in cancers. Advances in molecular metabolic imaging have emerged with new tools to measure tumor metabolism *in situ*. Hence, we sought to provide a proof-of-concept that hyperpolarized  $[1-^{13}\text{C}]$ -pyruvate MRS could be deployed as an imaging biomarker of therapeutic response to inhibition of LDH and/or glutaminase in lymphoma tumor xenografts.

**Table 1.** FX11 and DM SO treatment response

Days of treatment	FX11 treatment		DMSO treatment	
	Lac/Pyr (area under the curve)	$k_p/k_L$ (two-site exchange)	Lac/Pyr (area under the curve)	$k_p/k_L$ (two-site exchange)
0	1.45 ± 0.04	3.7 ± 0.4	1.46 ± 0.04	3.8 ± 0.4
4	1.32 ± 0.05	2.9 ± 0.5	2.11 ± 0.05	4.3 ± 0.6
6	1.07 ± 0.03	2.1 ± 0.6	2.89 ± 0.03	4.7 ± 0.5
7	0.84 ± 0.04	1.8 ± 0.5	3.31 ± 0.04	5.5 ± 0.6

NOTE: Summarization of the FX11 and DMSO treatment response in terms of Lactate-to-Pyruvate flux ratio (Lac/Pyr) and conversion rate constant ratio ( $k_p/k_L$ ).



**Figure 4.** Differences in alanine-to-pyruvate flux ratio (Ala/Pyr) between DMSO and BPTES groups (A;  $n = 8$  for each group; error bars, SD from the mean;  $P < 0.001$ ) and DMSO and FX11 groups (B;  $n = 8$  for each group; error bars, SD from the mean;  $P = 0.112$ ).

Notably,  $^{13}\text{C}$ -MRS imaging involves no ionizing radiation and, because it is a multispectral modality, it has the ability to detect simultaneously the metabolic fluxes of a variety of substrates (16). The conversion of hyperpolarized  $^{13}\text{C}$ -labeled pyruvate to lactate or alanine provides a noninvasive method for assessing directly LDH and indirectly glutaminase activities *in vivo* within the same acquisition.

In this study, we document that P493 lymphoma xenografts displayed high levels of native  $^{13}\text{C}$ -pyruvate-to-lactate conversion, consistent with the findings in previous studies of mouse lymphoma xenografts, and other tumor models (14, 17–19). The LDH-A inhibitor FX11, but not the glutaminase inhibitor BPTES, diminished the conversion of  $^{13}\text{C}$ -pyruvate to lactate, suggesting that  $^{13}\text{C}$ -pyruvate-to-lactate conversion could serve as a biomarker of LDH-A inhibition. This may be a generalized response biomarker, however, as decreased pyruvate-to-lactate conversion has also been observed in response to etoposide in murine lymphoma, apparently through a different mechanism (14). Etoposide was reported to induce apoptosis and necrosis, leading to PARP-mediated depletion of the coenzyme NADH pool and consequently to a decrease in the apparent pyruvate-to-lactate flux through LDH (14). Steady-state lactate levels in MR spectra have also been observed to decrease within 1 to 3 days of chemotherapy or radiotherapy of murine models of sarcoma (RIF-1) and breast cancer (EMT6; ref. 20). In the current study, the specificity of the FX11 response was substantiated by a lack of effect of BPTES on pyruvate-to-lactate conversion. However, while our studies showed a selective alteration of Lac/Pyr flux ratio by FX11 versus BPTES, these other studies offer a cautionary note about other mechanisms leading to altered lactate production after treatment. Although BPTES was observed to not affect conversion of pyruvate to lactate, it was observed to reduce the conversion of pyruvate to alanine as shown in Fig. 3A–D, presumably through deprivation of the glutamate pool. This documents for the first time, the potential use of Ala/Pyr flux ratio, to monitor glutaminase inhibition *in vivo*.

The current use of hyperpolarized  $^{13}\text{C}$ -pyruvate MR to study tumor xenografts *in vivo* has shown the ability of this technique to examine living animals serially throughout the course of a disease and its response to different therapies. This work has shown the potential for hyperpolarized  $^{13}\text{C}$ -MRS to follow

metabolic pathway fluxes *in vivo*, noninvasively, particularly to monitor and understand metabolically targeted cancer therapies, which are likely to emerge clinically in the next several years.

## Conclusion

This study documents that the metabolic consequences of treatment with an LDH-A inhibitor in lymphoma can be detected by monitoring the diminished pyruvate-to-lactate conversion *in vivo* using hyperpolarized  $^{13}\text{C}$ -MRS. It also documents for the first time the use of pyruvate-to-alanine conversion as a pharmacodynamic marker of glutaminase inhibition. Our results indicate that monitoring aerobic glycolysis using  $^{13}\text{C}$ -MRS with hyperpolarized pyruvate is a promising technique that could potentially detect the molecular effect of various emerging therapies that target cell signaling and metabolism, and thus provide a radiation-free method to assess tumor response longitudinally.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Authors' Contributions

**Conception and design:** P. Dutta, A. Le, C.V. Dang, R.J. Gillies  
**Development of methodology:** P. Dutta, G.V. Martinez, C.V. Dang, R.J. Gillies  
**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** P. Dutta, A. Le, G.V. Martinez  
**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** P. Dutta, A. Le, G.V. Martinez, C.V. Dang, R.J. Gillies  
**Writing, review, and/or revision of the manuscript:** P. Dutta, A. Le, T. Tsukamoto, G.V. Martinez, C.V. Dang, R.J. Gillies  
**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** A. Le, T. Tsukamoto, R.J. Gillies  
**Study supervision:** R.J. Gillies  
**Provided the research chemical FX11 needed for this study:** D.L. Vander Jagt

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