ABSTRACT  Unlike many cancers that exhibit glycolytic metabolism, high-grade liposarcomas often exhibit low 2-[18F]fluoro-2-deoxy-d-glucose uptake by positron emission tomography (PET), despite rapid tumor growth. Here, we used liquid chromatography tandem mass spectrometry to identify carbon sources taken up by liposarcoma cell lines derived from xenograft tumors in patients. Interestingly, we found that liposarcoma cell lines consume nucleosides from culture media, suggesting nucleoside salvage pathway activity. The nucleoside salvage pathway is dependent on deoxycytidine kinase (dCK) and can be imaged in vivo by PET with 1-2′-deoxy-2′-[18F]fluoroarabinofuranosyl) cytosine (FAC). We found that liposarcoma cell lines and xenograft tumors exhibit dCK activity and dCK-dependent FAC uptake in vitro and in vivo. In addition, liposarcoma cell lines and xenograft tumors are sensitive to treatment with the nucleoside analogue prodrug gemcitabine, and gemcitabine sensitivity is dependent on dCK expression. Elevated dCK activity is evident in 7 of 68 clinical liposarcoma samples analyzed. These data suggest that a subpopulation of liposarcoma patients have tumors with nucleoside salvage pathway activity that can be identified noninvasively using [18F]-FAC–PET and targeted using gemcitabine.

SIGNIFICANCE: Patients with high-grade liposarcoma have poor prognoses and often fail to respond to chemotherapy. This report identifies elevated nucleoside salvage activity in a subset of liposarcomas that are identifiable using noninvasive PET imaging with FAC and that are sensitive to gemcitabine. Thus, we suggest a new treatment paradigm for liposarcoma patients that uses [18F]-FAC–PET in the clinic to delineate gemcitabine responders from nonresponders. Cancer Discov; 2(12):1109-17. ©2012 AACR.
INTRODUCTION

Sarcomas are tumors of mesenchymal origin with more than 50 distinct histologic subtypes (1). Liposarcomas are the most common soft tissue sarcomas, accounting for roughly 20% of sarcomas (2). On the basis of cell morphology, liposarcomas can be classified into well-differentiated/de-differentiated (WDLPS/DDLPS), myxoid/round cell, and pleomorphic subtypes (3). In more than 90% of cases, WDLPS/DDLPS show an amplification of chromosome 12q13–15, which harbors genes such as CDK4 (regulates cell cycle) and MDM2 (negatively regulates p53 stability; ref. 4).

Patients with high-grade liposarcoma have poor prognoses, and surgery is currently the best treatment option (5). However, tumors are frequently found in a location that makes surgery difficult or impossible. Patients with unresectable liposarcomas are commonly treated with anthracyclines (doxorubicin), DNA alkylating agents (ifosfamide), antimitic drugs (docetaxel), or antimetabolites such as gemcitabine, but response to treatment is rare (1, 6), and biomarkers that differentiate responders from nonresponders are currently lacking (7).

Liposarcoma cells are difficult to propagate under cell culture conditions, and few stable cell lines have been generated from tumor samples. We recently generated 3 dedifferentiated liposarcoma cell lines (LPS1–3) from patient-derived tumor xenografts in mice, all of which exhibit glycolytic metabolism by 2[18F]fluoro-2-deoxy-D-glucose positron emission tomography (FDG-PET; Supplementary Fig. S1). These cell lines recapitulate morphologic and gene expression characteristics of the primary tumors even after continuous passages as xenograft tumors in mice and under cell culture conditions in vitro (Smith et al., unpublished data).

In this study, we used an unbiased metabolomics approach to assess nutrient uptake of LPS cell lines and identified nucleoside salvage pathway activity. Using PET imaging with the cytidine derivative tracer 1-(2′-deoxy-2′-[18F]fluoroarabino-furanosyl) cytosine (FAC; ref. 8), we confirmed nucleoside salvage activity in liposarcoma xenografts. As the chemotherapeutic prodrug gemcitabine is metabolized the same way as FAC, we found that LPS cell lines are sensitive to nmol/L amounts of gemcitabine in vitro, and liposarcoma xenograft tumors in mice regress upon gemcitabine treatment. These results suggest that PET imaging with cytidine derivative tracers can be used to detect liposarcoma patients who will benefit from gemcitabine treatment.

RESULTS

As patients with high-grade liposarcoma frequently present to the clinic with low FDG uptake, despite rapid tumor growth (Supplementary Fig. S2), we took an unbiased approach to assess carbon sources other than glucose used by these fatal tumors to fuel proliferation. To this end, we analyzed changes in metabolite concentrations over time in liposarcoma cell culture media using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) and a NOVA BioProfile analyzer (Fig. 1A and Supplementary Figs. S3 and S4). We hypothesized that cellular uptake of nutrients would result in a continuous decrease in the concentration of media metabolite over time. Ten metabolites were consistently consumed by the 3 LPS cell lines analyzed (Fig. 1B). In accordance with previous reports for other cancer types, multiple amino acids and amino acid precursors were among the consumed metabolites identified (9–11). Interestingly, the nucleosides cytidine, thymidine, and uridine were also continuously consumed by the liposarcoma cells analyzed (Fig. 1C–E). These findings suggest that LPS cell lines have nucleoside salvage pathway activity.

Figure 2A depicts the nucleoside salvage pathway, which enables nucleoside uptake and conversion to nucleotide triphosphates that can be incorporated into DNA (12). Deoxycytidine kinase (dCK) is required to catalyze the initial and rate-limiting phosphorylation of the nucleoside that traps the nucleotide inside the cell (13). To assess nucleoside salvage pathway activity in LPS cell lines, we measured dCK activity and FAC uptake in vitro. LPS cell lines exhibited dCK expression and activity (Fig. 2B) as well as tritiated FAC ([3H]-FAC) uptake in vitro (Fig. 2C). Stable knockdown of dCK using short hairpin RNA (shRNA) reduced dCK activity and [3H]-FAC uptake to background levels (Fig. 2B and C). These results confirm dCK-dependent nucleoside salvage activity in LPS cell lines in vitro.

To determine whether liposarcoma cells exhibit nucleoside salvage activity in vivo, we established liposarcoma xenografts in immunocompromised as NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) mice by subcutaneously injecting 5 × 10⁶ liposarcoma cells into the neck area of the mouse. Imaging the mice with microPET/computed tomography (CT) using D-[18F]-FAC (Fig. 2D) revealed a biodistribution similar to that seen in C57/BL6 mice (14), and quantification of 3-dimensional regions of interest (ROI) showed medium-high to high [18F]-FAC uptake in liposarcoma xenografts (percentage injected dose per gram of tissue [%ID/g > 9]), confirming nucleoside salvage activity in vivo.

To determine whether nucleoside salvage pathway activity contributes to proliferation in LPS cell lines, we compared proliferation rates in the presence and absence of media nucleosides and dCK expression. Incubation of cultured liposarcoma cells in medium lacking nucleosides had no effect on proliferation rates (data not shown). In addition, dCK knockdown affected neither proliferation rates nor glucose consumption, glutamine consumption, or lactate production rates (Supplementary Figs. SSA and SSB). These data suggest that although liposarcoma cells exhibit nucleoside salvage activity, they are not dependent on this metabolic pathway for proliferation and survival. This phenotype is consistent with previously published results on the dCK knockout mouse, which has a phenotype predominantly restricted to T and B lymphogenesis (15).

To determine whether the nucleoside salvage pathway activity observed in LPS cell lines and xenograft tumors is reflective of the primary tumor from which they were derived, we measured dCK activity in protein lysates from the primary tumor, xenograft, and cell line for LPS1–3. As shown in Fig. 2E, the amount of dCK activity was unchanged between samples from LPS2 and LPS3, whereas the amount of dCK activity in the LPS1-derived cell line was 2-fold lower compared with the corresponding primary and xenograft tumor tissue samples. These data suggest that all 3 primary liposarcoma...
tumors likely also exhibited nucleoside salvage pathway activity and that nucleoside salvage activity is unlikely to be an artifact of cell culture conditions.

A variety of chemotherapeutic prodrugs depend on nucleoside salvage activity in tumors for uptake and drug activation (16). For example, gemcitabine, a derivative of cytidine and FAM, is critically dependent on dCK for its activation. Recent studies have suggested use of dCK as a potential prognostic marker for gemcitabine sensitivity in patients with pancreatic cancer (17–20). Phosphorylated gemcitabine can be incorporated into DNA, causing DNA synthesis to stall (21). In addition, diphosphorylated gemcitabine can bind and block the function of ribonucleotide reductase, an enzyme required for de novo nucleotide synthesis (17). This block in de novo synthesis causes a positive feedback loop that increases nucleoside salvage activity and potentiates the cytotoxic effect of gemcitabine (18). The nucleoside salvage pathway activity in LPS cell lines and xenograft tumors suggests potential sensitivity to gemcitabine and other nucleoside derivative prodrugs.

To determine whether LPS cell lines are sensitive to gemcitabine treatment, we incubated 3 LPS cell lines for 5 days in the presence of various concentrations of gemcitabine and assessed cell viability. As shown in Fig. 3A, all 3 LPS cell lines tested showed decreased viability upon incubation with gemcitabine, with 50% lethal concentration ($L_{50}$) values in the low nmol/L range. We confirmed cytotoxicity in the LPS cell lines by observing increased propidium iodide staining upon treatment with 100 nmol/L gemcitabine (Supplementary Fig. S6). To determine whether gemcitabine also has a cytotoxic effect on liposarcoma cells in vivo, we used gemcitabine to treat mice with liposarcoma xenograft tumors and measured the effect on tumor growth (schematic in Fig. 3B). As shown in Fig. 3C–E, gemcitabine treatment led to immediate and complete regression of tumors derived from each of the LPS cell lines, whereas tumors in vehicle-treated mice exhibited continuous growth (see also Supplementary Figs. S7A–S7D). Similar results were obtained on passaged xenograft tumors derived from a patient’s primary liposarcoma sample (Supplementary Fig. S8). These data suggest that gemcitabine has a cytotoxic effect on liposarcoma tumors that exhibit nucleoside salvage pathway activity.

To estimate the proportion of liposarcoma patients with tumor nucleoside salvage pathway activity, we analyzed our previously published mRNA expression data generated from the tumors of patients with various liposarcoma subtypes (22). As shown in Supplementary Fig. S9A, we assessed the transcriptional expression levels of the genes encoding nucleoside transporters (SLC28A1, SLC29A1) and enzymes in the nucleoside salvage pathway (dCK, cytidine deaminase (CDAs), deoxy-ribonucleotide deaminase (dCTD), and S'-nucleotidase, ecto (NTS5E)).
Ten of 74 patient samples (13.5%) showed increased dCK mRNA expression levels. We also assessed dCK, CDA, and dCTD protein expression levels in 9 primary liposarcoma tumors, including those used to generate the LPS cell lines, LPS1–3. As shown in Supplementary Figs. S9B and S9C, dCK, CDA, and dCTD protein expression varied across liposarcoma samples. However, recent publications have shown that dCK can be posttranslationally modified (23); therefore, the relationship between dCK expression and activity is not precise. To overcome this limitation, we measured dCK activity in liposarcoma tumor samples from patients. Tumor tissue samples from 68 liposarcoma patients with various histologic subtypes were analyzed for dCK activity. As shown in Fig. 4A, 4 additional liposarcoma patient samples (LPS12, LPS17, LPS51, and LPS55) had dCK kinase activity levels comparable to the liposarcoma samples from which we generated cell lines (LPS1–3). Together, 7 of 68 (10%) tumor tissues showed elevated dCK kinase activity. These results raise the interesting possibility that a subset of liposarcoma patients have tumors with nucleoside salvage activity that...
Identification of Nucleoside Salvage Activity in Liposarcoma

May be detectable using PET with FAC (or a FAC derivative) and targeted using gemcitabine.

To determine whether nucleoside salvage activity is required for gemcitabine sensitivity in liposarcomas, we reduced nucleoside salvage activity in the LPS2 liposarcoma cell line by stable knockdown of dCK and examined the resultant effects on gemcitabine response in vitro and in vivo.

Similar to the results shown in Fig. 2, dCK knockdown in LPS2 cells led to reduced dCK activity, reduced dCK protein expression, and reduced [3H]-FAC uptake when compared with the scrambled shRNA-expressing control cells (Supplementary Figs. S10A–C). dCK knockdown in LPS2 cells showed a 1,000-fold decrease in gemcitabine sensitivity—the LC_{50} values for gemcitabine in scrambled shRNA-expressing cells were in the nanomolar range, whereas the LC_{50} values for gemcitabine in dCK shRNA-expressing cells were in the micromolar range (Fig. 4B).

To determine whether PET imaging using [18F]-FAC can distinguish between xenografted tumors from dCK knockdown versus scrambled shRNA-expressing control cells in the same mouse, we injected 5 \times 10^5 cells of each liposarcoma cell line into the flanks of immunocompromised mice (Scr cells into the left and ΔdCK cells into the right flanks). We imaged the mice with [18F]-FAC-PET and quantified tumor FAC uptake using AMIDE software (Fig. 4C and Supplementary Figs. S10D and S10E). Tumors from LPS2 cells expressing scrambled shRNA had a 2.8-fold higher FAC uptake than did tumors of the same size expressing dCK knockdown shRNA (Fig. 4C). Mice bearing both dCK knockdown and scrambled shRNA control tumors of equal size were treated with gemcitabine. As shown in Fig. 4C and D, tumors expressing scrambled shRNA decreased in size when treated with gemcitabine; however, dCK knockdown tumors exhibited continuous growth. In contrast, both tumors in PBS-treated mice grew at roughly the same speed and exhibited similar [18F]-FDG uptake when imaged with microPET/CT (Supplementary Figs. S10E and S10F). These results suggest that dCK and nucleoside salvage activity are necessary for gemcitabine response in liposarcomas, and PET imaging with...
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[18F]-FAC (or an FAC derivative) could be conducted to identify liposarcoma patients with this metabolic phenotype.

**DISCUSSION**

Liposarcomas are deadly tumors, and surgical intervention remains the best treatment option. However, surgery is not an option for patients with tumors in visceral locations. Unfortunately, the success rate for chemotherapeutic agents in liposarcoma is low, and biomarkers indicating patients likely to respond are lacking. In this study, we set out to identify carbon sources other than glucose used by LPS cell lines derived from patient-derived xenograft tumors. To this end, we conducted mass spectrometry-based metabolic footprint analysis, and identified nucleosides among other metabolites consumed by LPS cell lines. We confirmed nucleoside salvage activity in LPS cell lines and xenograft tumors using the cytidine-derivative PET tracer FAC.

Nucleoside derivative prodrugs such as gemcitabine rely on nucleoside salvage activity, which is dependent on the activity of various proteins, including nucleoside transporters and kinases. The rate-limiting step in the nucleoside salvage pathway is the initial phosphorylation catalyzed by dCK (20). Although expression of nucleoside transporters and dCK is critical for nucleoside salvage activity, metabolic flux through this pathway is complex and determined by various mechanisms of regulation. For instance, dCK is posttranscriptionally regulated by HuR (24), posttranslationally regulated by phosphorylation at Ser-74 by DNA
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In summary, we used a mass spectrometry-based metabolomics approach to analyze liposarcoma metabolism and identified nucleoside salvage pathway activity in a subset of patients’ tumors. In addition, we showed that liposarcoma xenograft tumors with nucleoside salvage activity can be imaged in vivo using the cytidine-derivative PET tracer [18F]-FAC and respond to treatment with the nucleoside-based prodrug gemcitabine. By analyzing dCK activity in primary liposarcoma samples, we estimate the population of liposarcoma patients with tumor nucleoside salvage activity to be around 10%. Together, these findings could directly affect management and treatment of liposarcoma patients, as they suggest that PET imaging with cytidine-derivative PET tracers may identify potential responders to gemcitabine treatment. However, further work is needed to determine whether liposarcoma nucleoside salvage activity can successfully stratify gemcitabine responders and nonresponders in the clinic. Nonetheless, identification of nucleoside salvage activity in a subset of liposarcomas suggests a promising new strategy to identify patients with this otherwise fatal disease who will benefit from nucleoside prodrug treatment.

METHODS

Generation and Culture Conditions of LPS

The generation of the LPS cell lines has been described previously (10) and was confirmed by histologic study and microarray analysis. All cell lines were originally grown in Dulbecco’s Modified Eagle’s Medium (DMEM)/F12 medium; LPS2 was subsequently grown in DMEM medium (10% FBS, 1% Pen/Strep). Cells were counted using a Beckman Coulter Z1 Particle Counter.

Xenograft Models, Drug Efficacy Assay, and MicroPET/CT Imaging

Liposarcoma tumor xenografts were generated by subcutaneously injecting 2.5 × 10^5 (LPS2) or 5 × 10^5 (LPS1 and 3) cells into female NSG mice. Drug treatment was started once tumors reached a size of 5 × 5 to 8 × 8 mm^2 and was conducted by intraperitoneal injection of 360 mg/kg gemcitabine (Eli Lilly) dissolved in PBS and, subsequently, 180 mg/kg every fourth day. Control animals were injected with an equal volume of PBS. Tumor size was monitored every second day with a caliper, and animals were euthanized once the tumors reached a size of 15 × 15 mm^2. For microPET imaging; animals were anesthetized with 1.5% isoflurane, USP (Phoenix Pharmaceutical Inc.) and injected intravenously with 200 μCi 18F-FAC. PET imaging was conducted on a Focus 220 microPET scanner (Siemens) and, subsequently, CT recorded using a MicroCAT II CT instrument (Siemens). Data were analyzed by drawing 3-dimensional ROIs using AMIDE software (29).

Mass Spectrometry-Based Metabolomic Analysis of Cell Culture Medium

A total of 5 × 10^6 liposarcoma cells per well were seeded onto a 6-well plate, medium was replaced after 24 hours, and 20 μl cell-free medium samples were taken every 24 hours thereafter. Metabolites were extracted by adding 300 μl 80% methanol (−80°C) to the medium samples followed by centrifugation for 5 minutes at 13 krpm at 4°C. The supernatant was transferred into a fresh tube, and the solvent was evaporated using a SpeedVac. Dried metabolites were resolved in high-performance liquid chromatography–grade water and analyzed as described previously (30).
Disclosure of Potential Conflicts of Interest

O.N. Witte is an inventor with national and Patent Cooperation Treaty (PCT) patent applications for FAC technology referred to in the article. O.N. Witte is also involved in Soofi Biosciences, a startup company that has licensed this intellectual property. O.N. Witte has an ownership interest in Soofi Biosciences. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

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Metabolomics Strategy Reveals Subpopulation of Liposarcomas Sensitive to Gemcitabine Treatment

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