Targeting glycolysis through inhibition of lactate dehydrogenase impairs tumor growth in preclinical models of Ewing sarcoma

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Abstract

Altered cellular metabolism, including an increased dependence on aerobic glycolysis, is a hallmark of cancer. Despite the fact that this observation was first made nearly a century ago, effective therapeutic targeting of glycolysis in cancer has remained elusive. One potentially promising approach involves targeting the glycolytic enzyme lactate dehydrogenase (LDH), which is overexpressed and plays a critical role in several cancers. Here, we used a novel class of LDH inhibitors to demonstrate, for the first time, that Ewing sarcoma (EWS) cells are exquisitely sensitive to inhibition of LDH. EWS-FLI1, the oncogenic driver of EWS, regulated LDHA expression. Genetic depletion of LDHA inhibited proliferation of EWS cells and induced apoptosis, phenocopying pharmacological inhibition of LDH. LDH inhibitors impacted EWS cell viability both in vitro and in vivo by reducing glycolysis. Intravenous administration of LDH inhibitors resulted in the greatest intratumoral drug accumulation, inducing tumor cell death and reducing tumor growth. The major dose-limiting toxicity observed was hemolysis, indicating that a narrow therapeutic window exists for these compounds. Taken together, these data suggest that targeting glycolysis through inhibition of LDH should be further investigated as a potential therapeutic approach for cancers such as EWS that exhibit oncogene-dependent expression of LDH and increased glycolysis.

Statement of Significance

Lactate dehydrogenase A (LDHA) is a pharmacologically tractable EWS-FLI1 transcriptional target that regulates the glycolytic dependence of Ewing sarcoma.
Introduction

Glycolytic dependence of cancer cells was first described in 1927 by Otto Warburg, who hypothesized that tumor cells preferentially catabolized glucose to lactate even in the presence of oxygen (aerobic glycolysis), whereas normal cells preferentially catabolized glucose to carbon dioxide (oxidative phosphorylation) (1). Targeting this increased dependence on glycolysis in cancer cells presents an opportunity to inhibit their growth while potentially limiting the toxicity delivered to normal cells (2). Since this initial discovery, our understanding of these processes has evolved to reflect the role of genetic alterations in cancer cells, including the activation of genes, such as lactate dehydrogenase A (LDHA), the enzyme responsible for the conversion of pyruvate to lactate, the final enzymatic step in the glycolytic pathway (3). Several studies have shown that LDHA plays a key role in tumor initiation and maintenance, and that inhibition of LDHA reduces cellular growth and metastasis in preclinical cancer models (4-9). Intriguingly, many glycolytic tumors display elevated levels of LDHA, and it appears to be expressed primarily in cancer cells, as opposed to normal tissues (10, 11). LDHA is therefore used as a biomarker for many malignancies (11) and is a promising target for cancer therapeutics.

To date, much of the data on LDH inhibition has been generated through proof-of-principle genetic studies that reveal LDHA knockdown inhibits in vitro and in vivo growth of certain cancer cell lines (4-7, 9). While novel LDH inhibitors (LDHi) have been described, there are numerous issues with evaluating their translational potential, including suboptimal selectivity, potency, cellular permeability, and pharmacokinetic properties (4, 5, 12-14). Hence, the potential clinical applications of LDHi remain unrealized. Recently, renewed efforts have been made to more efficiently target LDH with agents developed through the National Cancer Institute Experimental Therapeutics (NExT) Program, a consortium that aims to develop drugs for difficult targets (15). NCI-737 and NCI-006 represent two novel LDHi that were developed and validated as part of the NExT Program.

In this study, we sought to evaluate the activity of NCI-737 and NCI-006, and to describe the impact of genetic and pharmacological inhibition of LDH on cellular metabolism, growth, and survival on in vitro and in vivo preclinical models of Ewing sarcoma (EWS). EWS is an aggressive malignancy of the bones and soft tissues that primarily affects adolescents and young adults and is driven by a reciprocal oncogenic translocation between EWSR1 and an ETS family member such as FLII or ERG that results in aberrant gene expression (16-18). EWS remains a disease for which new therapies are critically needed, given that outcomes for high-risk patients remain poor and have not improved in decades (19-21). In this study, we show that EWS cells are exquisitely sensitive to inhibition of LDH activity, both genetically and pharmacologically. Further, glycolytic inhibition with either NCI-006 or NCI-737 impairs growth and survival of EWS in vitro and in vivo.
Materials and Methods

Compounds

NCI-737 and NCI-006, equipotent against LDHA and LDHB, were obtained through the NExT Program (15). Details of compounds and their synthesis can be found in Supplemental Methods. Stock solutions for in vitro use were prepared in DMSO, aliquoted, and stored at -20°C. For in vivo use, powdered compound was dissolved in a volume of 0.1N NaOH, equivalent to 18% of the total solution volume and added to PBS. Dropwise addition of 1N HCl was performed to achieve a pH of 7.4-7.8. Solution was prepared weekly and kept at 4°C.

Cell Line Screen

The cell line screen was performed by Oncolead (Karlsfeld, Germany) using a panel of 94 cell lines and a 72-hour sulforhodamine assay.

Cell Lines

EWS cell lines TC32, TC71, EW8, and RDES have been previously described (2); SK-N-MC, and CHLA-258 were obtained from Dr. Lee Helman (Children’s Hospital of Los Angeles, Los Angeles, CA). 5838 was obtained from ATCC (Manassas, VA). Cell lines were authenticated by short tandem repeat (STR) DNA fingerprinting and compared to known sequences. TC32 (RRID: CVCL_7151), TC71 (RRID: CVCL_2213), EW8 (RRID: CVCL_V618), and CHLA-258 (RRID: CVCL_A058) were most recently authenticated in 2018 in the lab of Dr. Stephen Channock (National Cancer Institute, Rockville, MD). SK-N-MC (RRID: CVCL_0530) and RDES (RRID: CVCL_2169) were most recently authenticated in 2012 by Genetica Cell Line Testing (Burlington, NC). 5838 (RRID: CVCL_6255) has not been independently authenticated since purchase from ATCC.

Mycoplasma testing of these cell lines was most recently performed in January 2019 and confirmed negative results. Experiments were performed on cells that were passaged between 5 and 12 times.

Cells were maintained in RPMI growth medium (Life Technologies, Grand Island, NY) with 10% FBS (Sigma Aldrich, St. Louis, MO), 100 U/mL penicillin and 100 µg/mL streptomycin (Life Technologies), and 2 mM L-glutamine (Life Technologies) at 37°C in an atmosphere of 5% CO₂.

Cell Proliferation Assays

Cellular proliferation was monitored in real-time using the IncuCyte live cell analysis system (Essen BioScience, Ann Arbor, MI), and cellular viability was determined by MTT assay (Promega,
Madison, WI) according to the manufacturer’s instructions. For both methods, cells were plated at a density of 2,000 cells/well in 96-well plates overnight and treated the following day.

**Protein Analysis**

Cell lysates were prepared by plating one million cells/10-cm plate overnight and then treating cells for an additional 24 hours. Cells were harvested with 1X RIPA (Santa Cruz, Dallas, TX) plus phosphatase and protease inhibitor cocktail (ThermoFisher, Waltham, MA). Protein lysates (30 μg/lane) were quantified by BCA protein assay (ThermoFisher). Blots were prepared as previously described (22) and incubated with primary antibodies, as described in Supplemental Methods.

**Flow Cytometry**

Cells were plated at one million cells/10-cm plate and treated the following day. Cells were harvested using PBS-based, enzyme-free cell dissociation buffer (Life Technologies) at 72 hours and processed according to the annexin V-FITC apoptosis detection kit (Sigma), before being run through the LSRFortessa flow cytometer (BD Biosciences, San Jose, CA).

**siRNA Studies**

Lipofectamine RNAi Max (ThermoFisher) was used according to the manufacturer’s instructions. Additional details of siRNA sequences and experimental conditions can be found in Supplemental Methods.

**ChIP-seq Analysis**

FASTQ files from published FLI1 and H3K27ac ChIP-seq, as well as associated RNA-seq experiments, were downloaded from NCI GEO (GSE8826 and GSE89026). Reads were aligned to the hg19 reference genome using BWA (version 0.7.10) using an established pipeline (23). Resulting tdf files were visualized using IGV (version 2.3.40).

**Biochemical Analyses**

The YSI 2950D Biochemistry Analyzer (Xylem Inc., Yellow Springs, OH) was used to measure glucose and lactate in the media of cells treated with NCI-737 (187nM) or DMSO (control). Cells were plated in triplicate in 24-well plates as follows: 500,000 cells/well for TC71 and EW8, 600,000 cells/well for RDES, and 250,000 cells/well for TC32. Media was collected after 24 hours of treatment and the plate with cells was frozen at -80°C and subsequently lysed with 1X RIPA buffer (Santa Cruz) for protein assay.
Pyruvate Assay

Intracellular pyruvate concentrations were determined using the Pyruvate Assay Kit (Abcam). Cells were plated at two million/10-cm plate and treated with 187 nM NCI-737 for 24 hours. Cells were lysed using 200 μL pyruvate sample buffer. Proteins were precipitated from the lysate with perchloric acid and neutralized with KOH according to the manufacturer’s instructions. Pyruvate was measured using the fluorometric assay protocol.

NAD+/NADH Assay

5,000 cells/well were plated in ViewPlate-96 96-well microplates (PerkinElmer, Waltham, MA) and treated with up to 187 nM of NCI-737 for 13 hours. NAD+/NADH ratio was determined using the NAD+/NADH-Glo assay (Promega) according to the manufacturer’s instructions.

Extracellular Flux Analysis

Analyses of cellular bioenergetics were performed using the Seahorse XF96 Extracellular Flux Analyzer (Agilent, Santa Clara, CA). Glycolytic stress tests were performed according to the manufacturer's instructions, as previously described (24, 25). Additional experimental details can be found in Supplemental Methods.

LDH Activity Assay

Cells were plated overnight at 200,000 cells/well in 24-well plates. The following morning, cells were acclimated to XF complete media for 30 minutes prior to treatment with the indicated concentrations of LDHi or DMSO (control) for 1 hour (dose-response experiment) at 37°C in a non-CO2 incubator. For the time-point experiment, cells were incubated in XF complete media with the LDHi for the indicated time points. Cells were lysed using 100 μL LDH assay buffer and the oxidation of NADH measured spectrophotometrically at 340 nm.

13C Glucose Tracing

Details of sample preparation for LCMS and NMR analyses can be found in Supplemental Methods. LCMS analysis has been previously described (26), but further details of this and NMR analysis can be found in Supplemental Methods.

Statistical Analyses

Statistical significance was determined by two-tailed Student’s t-test. P<0.05 was considered significant.
In Vivo Studies

Animal studies were approved by and performed in accordance with the National Institutes of Health Animal Care and Use Committee (ACUC) guidelines. For all experiments described, female Fox Chase SCID beige mice (CB17.B6-Prkdcscid Lystbg/Crl) were purchased from Charles River Laboratories (Wilmington, MA). Two million cells in a solution of HBSS were injected orthotopically into the gastrocnemius muscle in the left hind leg of each mouse. Mice were randomized when palpable tumors developed, at which point treatment with agents began. Specific details for each set of experimental conditions may be found in Supplemental Methods.

Statistical Analyses

Tumor volumes were compared between groups using a two-tailed Student’s t-test at serial time points. \( P < 0.05 \) was considered significant. Measurements for mice that had already reached endpoint were carried forward until all mice in the group had reached endpoint.

Histopathology and image analysis

H&E stained slides were scanned using the Aperio XT ScanScope whole slide imaging system with a 20X objective lens (Aperio, Vista, CA). One whole slide image (SVS file) was prepared from each animal, capturing the entire tumor section on the slide. Details of image analysis can be found in Supplemental Methods.

Hyperpolarized MRS

Hyperpolarized \(^{13}\)C-MRI study was performed as described previously (27). Additional experimental details can be found in Supplemental Methods.

Intratumoral Drug Measurement

Measurement of tumor concentrations of LHDi was by mass spectrometric analysis with internal standards (pure NCI-737 and NCI-006) and was performed by Quintara Discovery (Hayward, CA).

Intratumoral LDH Activity Measurement

Frozen tumor sections were pulverized in a liquid nitrogen cooled pestle-mortar apparatus. Pulverized tumors were lysed in 10 volumes of LDH assay buffer and LDH activity measured as described above.
Results

**EWS cells are sensitive to genetic and pharmacological inhibition of LDHA, which decreases cellular proliferation and activates apoptotic pathways**

To evaluate the functional activity of NCI-737 and NCI-006, we screened a panel of 94 cancer cell lines using the Oncolead cell panel assay, which represents a diverse array of cancer cell lines. Across this panel, EWS cell lines emerged among the top ten and top twelve most sensitive cell lines to NCI-006 and NCI-737, respectively, with IC\(_{50}\) values of 100-200 nM (median IC\(_{50}\) values for all cell lines in the panel were 1260 nM for NCI-006 and 845 nM for NCI-737) (Figure 1A). Of note, other pediatric-type sarcoma cell lines (rhabdomyosarcoma and osteosarcoma) in the panel had median IC\(_{50}\) values in line with the general panel (1037 nM and 712 nM, for NCI-006 and NCI-737, respectively) (Supplemental Table 1). To validate these findings, additional in vitro studies of cellular viability were performed on a broader panel of EWS cell lines. By MTT assay, all EWS cell lines displayed nearly identical dose-dependent sensitivity to NCI-737 and NCI-006, with IC\(_{50}\) values ranging from 100 nM (TC71 and TC32), to 1 \(\mu\)M (RDES and EW8) for each compound at 72 hours of treatment (Supplemental Figure 1A). Given the potential for metabolic inhibitors to affect reagents in this assay, we verified these findings using IncuCyte live cell analysis, which confirmed that both NCI-737 and NCI-006 inhibited cellular proliferation at doses in the 100-500 nM range across multiple cell lines (Figure 1B and Supplemental Figures 1B and 1C). Notably, the IC\(_{50}\) values for each of the compounds were very similar for each cell line tested, indicating equal potency of NCI-737 and NCI-006 in the EWS cell line models.

To evaluate the mechanisms behind this loss of viability, we performed western blot analysis of cells treated with NCI-737, which revealed activation of pro-apoptotic proteins, cleaved PARP and cleaved caspase 7 at 24 hours (Figure 1C). Flow cytometry analysis of EWS cells treated with NCI-737 for 72 hours demonstrated a significant increase in the percentage of cells in both early and late apoptosis (Figure 1D). Taken together, these findings suggest that the observed decrease in EWS cellular proliferation caused by LDHi is due to apoptotic cell death.

Next, we examined the result of genetic loss of LDHA and LDHB in EWS cell lines by knocking down the enzymes using siRNA to determine whether the results of pharmacological LDH inhibition would be recapitulated. LDHA knockdown resulted in the expected loss of LDHA protein in EWS cell lines. As with the LDHIs, genetic knockdown of LDHA resulted in decreased cellular proliferation and induction of apoptotic markers (Figures 1E, 1F and Supplemental Figures 2A and 2B), consistent with the conclusion that LDHA is important for EWS cell survival. In contrast, knockdown of LDHB with four siRNA sequences in multiple cell lines neither induced apoptosis nor negatively affected proliferation of EWS cells (Supplemental Figures 2C, 2D and 2E).
**LDHA is regulated by EWS-FLI1**

EWS is characterized by a reciprocal translocation between chromosomes 11 and 22, resulting in the oncogenic transcription factor EWS-FLI1, which acts as the primary driver of the disease (17, 18). While attempts to target EWS-FLI1 directly have yet to yield an effective therapeutic, examining the downstream effects of EWS-FLI1 has the potential to reveal insights into EWS biology that may be translated in clinically meaningful ways (28, 29). Given the marked sensitivity to both genetic depletion and pharmacological inhibition of LDHA in EWS cells, as well as the recent findings that EWS-FLI1 regulates a shift away from oxidative metabolism towards glycolysis, which is NAD+ dependent (30, 31), we sought to determine whether EWS-FLI1 might play a role in the regulation of LDHA and/or LDHB. Depletion of EWS-FLI1 with multiple siRNA sequences resulted in a decrease in LDHA protein, as well as a decrease in NROB1 protein, a known direct target of EWS-FLI1, in each of the four EWS cell lines tested (Figure 2A and Supplemental Figure 3A), suggesting that LDHA expression is regulated, at least in part, by EWS-FLI1. In contrast, depletion of EWS-FLI1 had no effect on LDHB expression in each of the four EWS cell lines tested (Supplemental Figure 3B).

To evaluate whether LDHA might be a direct EWS-FLI1 target, we examined publicly available chromatin immunoprecipitation sequencing (ChIP-seq) data generated using the shFLI1-transfected EWS cell line SK-N-MC (32). In the FLI1 knockdown, EWS-FLI1 and H3K27-acetylation deposition at the LDHA locus were decreased, and a corresponding decrease in LDHA mRNA expression was observed (Figure 2B). An expected decrease in LDHA protein with EWS-FLI1 depletion in SK-N-MC was similarly observed (Figure 2C). Furthermore, an analysis of publicly available ChIP-seq data on primary EWS tumors (33) also demonstrated H3K27ac deposition at the LDHA enhancer (Supplemental Figure 3C). Analysis of ChIP-seq data from the same shFLI1-transfected EWS cell line SK-N-MC revealed that there was neither EWS-FLI1 nor H3K27-acetylation present at the LDHB locus (Supplemental Figure 3D). Similarly, there was no change in LDHB protein with EWS-FLI1 depletion in this cell line (Supplementary Figure 3E). Collectively these data suggest that LDHA, but not LDHB, is a direct target of EWS-FLI1, and supports further investigation of the translational potential of pharmacological LDH inhibition in EWS.

**LDHi act through impairment of glycolysis in EWS cells**

To evaluate on-target activity of these LDHi, we characterized the enzymatic inhibition and downstream metabolic effects of these agents. First, we assessed expression levels of LDHA and LDHB, and baseline LDH activity level in EWS cell lines, and determined there was uniform expression and enzymatic activity across the panel (Supplemental Figures 4A and 4B). Next, we examined the change in
basal LDH activity with increasing doses of NCI-737 or NCI-006 and found a dose-dependent inhibition of LDH activity in EWS cell lines for each of the compounds (Figure 3A). For each of the cell lines tested (TC71, TC32 and EW8), the IC_{50} values for LDH inhibition were similar for both agents, at approximately 100 nM, consistent with the observation that LDH appears to be uniformly expressed and active in these cell lines. Notably, the LDHi resulted in rapid inhibition of LDH upon exposure to EWS cells, with LDH activity decreasing within 30 seconds of drug exposure and reaching maximal inhibition by approximately 10 minutes in vitro (Supplemental Figure 4C).

Next, biochemical analyses of glucose and lactate content in the culture media of treated and untreated cells were performed using YSI analysis and demonstrated a significant decrease in glucose consumption and lactate production in four EWS cell lines treated with NCI-737 (Figure 3B). Furthermore, LDHi treatment induced an increase in intracellular pyruvate concentration in all four cell lines (Figure 3C). Confirmatory biochemical studies, performed using mass spectrometry-based analysis of intracellular pools of pyruvate and lactate in EWS cells treated with LDHi, demonstrated a similar increase in steady-state levels of pyruvate and a decrease in steady-state levels of lactate in EWS cells treated with NCI-737 (Supplemental Figures 5A and 5B).

We next examined the impact of LDHi treatment on trace labeling of intracellular pyruvate pools and lactate release using 13C-labeled glucose. In both EWS cell lines tested, we observed a significant increase in accumulation of 13C-labeled intracellular pyruvate (M+3) after six hours of treatment with NCI-737 (Supplemental Figures 5C and 5D). We also noted a significant decrease in the amount of 13C-labeled lactate released into the media of treated cells, consistent with the data generated by YSI analysis (Supplemental Figure 5E). Taken together, these data are consistent with NCI-737-mediated inhibition of LDH, resulting in accumulation of pyruvate that cannot be converted to lactate, resulting in a reduction of overall glucose consumption.

Since LDH is a major cytosolic regulator of redox homeostasis, we next examined the effect of NCI-737 on the cellular NAD^{+}/NADH ratio. Treatment with NCI-737 resulted in a dose-dependent decrease in the NAD^{+}/NADH ratio in all four EWS cell lines tested, compared to control (Figure 3D), indicating that loss of NAD^{+}/NADH homeostasis is a key outcome of treatment with these LDHi.

To further understand the bioenergetic effects of LDH inhibition on EWS cells, we utilized extracellular flux analysis to perform a glycolytic stress test (24, 25) on cells treated with NCI-737 to examine changes in extracellular acidification rate (ECAR). In both TC71 and TC32 cell lines, NCI-737 treatment for 6 hours resulted in a dose-dependent decrease in both glycolysis (change in ECAR upon glucose addition) and glycolytic capacity (change in ECAR upon oligomycin treatment), with the effect on glycolytic capacity being more pronounced (Figure 3E and Supplemental Figure 6A). Notably, upon stimulation of glycolysis by addition of oligomycin, untreated cells were able to increase ECAR, whereas
this effect was blunted or lost completely in cells treated with NCI-737. A comparison of the effects of NCI-006 with those of NCI-737 on glycolytic flux demonstrated that the two agents inhibit ECAR in a nearly identical manner across a range of doses (Supplemental Figure 6B).

Additionally, we examined the effect of short-term NCI-737 treatment on oxidative phosphorylation by measuring the oxygen consumption rate (OCR) of EWS cells. When treated with NCI-737 at doses that impacted ECAR, no changes in OCR were observed in EWS cells (Supplemental Figures 7A and 7B). Further, when cells were exposed to higher doses of LDHi, concentrations between 10 and 30 μM were required to produce a decrease in OCR, whereas decreases in ECAR were noted at concentrations starting at less than 1 μM (Supplemental Figures 7C and 7D). Taken together with the proliferation data, these findings suggest that at doses under 10 μM these LDHi induce metabolic changes within the glycolytic pathway that directly impact the ability of the cells to proliferate and survive.

**Pharmacologic effects on bioenergetics predict effect on proliferation in vitro**

While all EWS cell lines tested exhibited sensitivity to LDHi, there were slight differences noted in the degree of the antiproliferative effects. To understand the basis for this differential sensitivity, we interrogated the EWS cell lines for differences in various characteristics. As previously described, no correlations were noted between cell line sensitivity to LDHi and basal LDH activity, LDHA or LDHB expression, basal glucose consumption, basal pyruvate level, or basal NAD\(^+\)/NADH ratio (Figures 3B-3D, and Supplemental Figures 4A and 4B). We did observe that the more sensitive cell lines had higher basal lactate production and a greater change in NAD\(^+\)/NADH ratios upon drug treatment, suggesting that LDH may play a greater role in maintaining the redox balance in sensitive cells (Figures 3B and 3D). In addition, further evaluation of extracellular flux data revealed a dose-dependent decrease in both glycolysis (Supplemental Figure 8A) and glycolytic capacity (Supplemental Figure 8B) in all cell lines tested, with the extent of change for each parameter varying across the cell lines (Figures 4A and 4B). For example, when treated with NCI-737 at 250 nM, TC71 cells underwent an 87% decrease in glycolysis and a 100% decrease in glycolytic capacity, whereas EW8 cells underwent a 19% decrease in glycolysis and a 38% decrease in glycolytic capacity. Notably, cell lines exhibiting larger decreases in glycolysis and glycolytic capacity with drug treatment were more sensitive to the effects of the drug on proliferation, whereas cell lines exhibiting smaller decreases required higher doses of NCI-737 for growth suppression. (Figure 4C). Thus, the magnitude of the biochemical effect of the LDHi on ECAR was predictive of functional response.

Additionally, we examined cell lines for potential differences in their use of oxidative phosphorylation, since metabolic plasticity has been shown to affect sensitivity to LDH inhibition (34). To elucidate the relative contributions of the basal rates of glycolysis and oxidative phosphorylation, an
energy map of basal and on-treatment ECAR and OCR was generated for several cell lines following 24-hour treatment with NCI-737 at 100 nM (Figure 4D). Interestingly, while all cell lines experienced an expected decrease in ECAR with treatment, the OCR response varied. The more sensitive cell lines (TC71 and TC32) experienced a relative decrease in OCR whereas the less sensitive cell lines (RDES and EW8) experienced an increase in OCR, suggesting that increased oxidative phosphorylation may compensate, in part, for the loss of glycolysis in a subset of EWS cell lines, reflecting differences in sensitivity. In addition, data from the in vitro study using 13C-labeled glucose revealed a more robust increase of 13C-labeling of TCA cycle intermediates in EW8 compared to TC71 cells, consistent with a greater ability of EW8 cells to redirect pyruvate towards the TCA cycle upon inhibition of LDH (Figure 4E.)

**LDH inhibition impairs glycolysis and affects cell survival in aggressive xenograft models of EWS**

To evaluate the translational potential of NCI-737 and NCI-006, we treated several orthotopic xenograft models of EWS with the compounds to examine on-target activity, intratumoral drug concentration, toxicity and efficacy. For all xenograft studies described, treatment was initiated after tumors became palpable. Initial in vivo studies were aimed at establishing the optimal route and dose of LDHi in orthotopic xenografts. Oral administration of the agents given at the maximum tolerated doses (MTD) of 75 mg/kg total daily dose for NCI-737 and 50 mg/kg total daily dose for NCI-006 given for three weeks either once or twice daily to mice bearing TC71, TC32 or EW8 tumors resulted in minimal efficacy, although mice in the TC71 and TC32 groups treated with NCI-737 on the twice daily schedule experienced a slightly decreased tumor growth rate (Supplemental Figure 9A). Analysis of the effect of the compounds on LDH activity in the tumors revealed an inconsistent pattern of suppression of enzyme activity (Supplemental Figure 9B). Furthermore, intratumoral drug levels were noted to be variable, establishing that oral dosing was insufficient to achieve adequate intratumoral drug levels and clinically relevant inhibition of LDH in these xenograft models (Supplemental Figure 9C).

Given the limitations of oral dosing, follow-up studies were performed to evaluate IV dosing in TC71 tumor-bearing mice. NCI-737 was selected for the IV studies due to the slightly more efficacious antitumor effect noted in the oral dosing studies. Initial pilot studies were performed using doses of 25 and 40 mg/kg of NCI-737, given IV on a M/W/F schedule. At 25 mg/kg, no impact on tumor growth rate was observed, and at 40 mg/kg, a minimal change in tumor growth rate was noted (Supplemental Figure 10A). Samples of plasma and tumor drug levels taken four hours post-dosing revealed no differences in plasma concentrations (2.9 μM for 25 mg/kg and 2.8 μM for 40 mg/kg) and a slightly greater tumor concentration for the 40 mg/kg group (9 μM vs. 10.3 μM; Supplemental Figures 10B and 10C).
mg/kg dosing, no inhibition of LDH activity was noted in the tumors four hours post-dosing; with 40 mg/kg dosing, 60% inhibition of LDH activity was seen at this time-point (Supplemental Figure 10D).

Based on the lack of LDH inhibition seen at 25 mg/kg and the minimal efficacy observed with the 40 mg/kg dose given on the M/W/F schedule, we next examined doses of 40, 50 and 75 mg/kg given on a more frequent schedule of five consecutive days per week by IV to animals bearing TC71 tumors. At these doses, a dose-dependent reduction in tumor growth rates was noted, although the differences did not reach statistical significance (Supplemental Figure 11A). However, at 75 mg/kg on this schedule, the mice displayed evidence of toxicity, so this dose could not be pursued further.

Pharmacokinetic and pharmacodynamic studies were performed on the animals in the 40 mg/kg and 50 mg/kg dosing groups after tumor-bearing animals were treated with three consecutive days of IV NCI-737. Samples of tumor and plasma drug levels taken one-hour post-dosing revealed average intratumoral concentrations of 12.3 μM (40 mg/kg group) and 18.5 μM (50 mg/kg group) and average plasma concentrations of 8.9 μM (40 mg/kg group) and 11.6 μM (50 mg/kg group). (Figure 5A and Supplemental Figure 11B). Pharmacodynamic studies indicated 82% and 93% inhibition of intratumoral LDH activity in the 40 mg/kg and 50 mg/kg groups, respectively (Figure 5B). Notably, intratumoral drug concentration and LDH inhibition rapidly diminished over a short interval. When animals treated at the same doses underwent tumor sampling four hours post-dose (versus one hour), a rapid loss (28%) of intratumoral compound and a corresponding decrease in the degree of LDH inhibition (82% to 58%) were observed between the one- and four-hour sampling times (Figures 5C and 5D). These data were consistent with the known pharmacological properties and full PK profile of NCI-737, which indicate a half-life of approximately 5 hours in plasma.

Given the rapid loss of LDH inhibition in the tumor, we hypothesized that the tumor growth response might be blunted due to inability to maintain continuous inhibition of tumor LDH on the five days/week schedule. To test this hypothesis, we evaluated a continuous seven days/week dosing schedule. A daily dose of 60 mg/kg was chosen to attempt to minimize toxicity and maximize efficacy. NCI-737 treatment for seven days/week resulted in statistically significant tumor growth suppression in mice bearing TC71 xenografts (Figure 6A). Additionally, histopathologic analysis of tumors revealed that treated tumors had a nearly two-fold increase in necrosis over vehicle treated tumors (59% v. 33%). Similarly, tumors from EW8-bearing xenografts treated with NCI-737 at 60 mg/kg displayed a statistically significant increase in necrosis compared to control (43% v. 28%) (Figures 6B, 6C and Supplemental Figure 12A). However, treatment at this dose was not sufficient to impact overall tumor growth in the EW8 model, suggesting that the relative sensitivity differences noted in vitro may be relevant in vivo (Supplemental Figure 12B).
Finally, to confirm the in vivo on-target activity of the LDHi, we performed hyperpolarized MR spectroscopy, using hyperpolarized $^{13}$C-pyruvate, of orthotopic xenograft EWS tumors in mice to quantify the ratio of $^{13}$C-lactate generated from $^{13}$C-pyruvate 30 minutes after a single intravenous (IV) dose of NCI-737 at the efficacious dose of 60 mg/kg. NCI-737 treatment resulted in a 57% and 49% reduction in the labeled lactate/pyruvate ratio in TC71 and EW8 tumors, respectively (Figures 6D and Supplemental Figure 13). Taken together, these findings verified the on-target activity of the agent in vivo and were consistent with the in vitro results. Furthermore, the in vivo data indicate that to achieve tumor growth inhibition, sustained inhibition of LDH through frequent dosing at the highest tolerable dose is required.

**Hemolysis is the primary dose-limiting toxicity of LDH inhibition**

Assessments of toxicity via observations of general appearance, blood parameters, and necropsy were performed on animals treated via a five days/week IV dosing schedule with NCI-737 at 40, 50, or 75 mg/kg. Toxicity assessments were performed at day three and at tumor or humane endpoint. While mice tolerated treatments without weight loss (Supplemental Figure 14A), a dose-dependent intolerance of NCI-737 was noted. All mice treated at 40 mg/kg displayed no toxicity, while one of three mice treated at 50 mg/kg and two of three mice treated at 75 mg/kg displayed decreased physical activity by day two of treatment. Laboratory and pathological assessments of all mice revealed that hemolysis was the primary dose-limiting toxicity observed, with a dose-dependent decrease in hemoglobin noted within three days of starting treatment (Supplemental Figure 14B). Within three days, hemoglobin values began to fall below the lower limit of the normal range of 11 g/dL in mice receiving the lowest (40 mg/kg) dose. At higher doses, hemoglobin values were below 8 g/dL. By endpoint, average hemoglobin levels had decreased further to 9.1, 4.6, and 5.3 g/dL in the 40, 50, and 75 mg/kg treated groups, respectively, indicating that toxicity was cumulative with continued dosing.

A corresponding rise in total bilirubin was also noted both at day three of treatment and at endpoint (Supplemental Figure 14C). While measurements of direct bilirubin were not obtained, normal liver studies were noted in all mice, suggesting that the rise in total bilirubin was the result of an increase in the indirect bilirubin fraction, which is consistent with red blood cell breakdown. In addition, mice treated at all dose levels were noted to have an increase in splenic weight at endpoint, further supporting hemolysis as the major toxicity (Supplemental Figure 14D). Red blood cells lack nuclei and mitochondria and therefore rely entirely on glycolysis for bioenergetics (35). A previous study describing the phenotypes associated with genetic loss of LDHA in murine models reported non-lethal hemolytic anemia as a main finding, supporting the idea that this toxicity is on-target (4). Of note, no other lab abnormalities were seen on complete blood counts or chemistry panels performed as part of the toxicity assessment.
Discussion

In this study we have demonstrated that EWS cells display a marked dependency on LDHA, due, in part, to the regulation of LDHA by the oncogenic transcription factor EWS-FLI1. We have shown that genetic or pharmacological inhibition of LDHA reduces proliferation and induces apoptosis in EWS cells, and that this is associated with suppression of glycolytic flux and perturbation of the NADH/NAD⁺ ratio. Additionally, we have explored the translational potential of this target through in vivo characterization of two novel LDHi, performing analyses of drug delivery routes, pharmacokinetics, pharmacodynamics, toxicity, and efficacy.

We show that LDHA is an important enzyme for the survival of EWS cells, which are remarkably sensitive to both genetic and pharmacological inhibition of LDH. Pharmacological targeting of LDH using NCI-737 and NCI-006 inhibited proliferation of EWS cells both in vitro and in vivo through inhibition of glycolysis and induction of apoptosis. This finding is consistent with published literature describing targeting of LDH in other sensitive cancer types (9, 36). Notably, the effect of these agents on proliferation of EWS cells exceeded the effects seen on other types of cancer cells. Our work suggests that the biology of EWS, in particular the presence of the driving EWS-FLI1 fusion oncogene (17, 18), contributes to the sensitivity of these cells to glycolytic inhibition. Specifically, we have shown that the EWS-FLI1 fusion oncoprotein directly regulates LDHA, but not LDHB, expression. This finding is supported by the recent report that EWS-FLI1 regulates metabolic pathways in EWS, where it shifts glucose consumption away from oxidative metabolism and towards glycolysis (30), and by evidence that EWS cells are highly glycolytic and highly susceptible to glucose deprivation and disruption of glucose metabolism (31, 37-40). In addition, there is clinical data to suggest that outcomes for EWS patients are worse for those who exhibit higher plasma levels of LDH (41-43).

The metabolic consequences of NCI-737 and NCI-006 on EWS were suppressed glycolytic flux through inhibition of the conversion of pyruvate to lactate, which was observed in both in vitro and in vivo models, concomitant with disruption of the NAD⁺/NADH ratio. By extracellular flux analysis, NCI-737 treatment resulted in decreased ECAR, the magnitude of which was predictive of the effect on cellular viability across multiple EWS cell lines. The ability of the cells to engage oxidative phosphorylation through the TCA cycle, which has been reported as a resistance mechanism in other cancer types (7, 34) also emerged as a potential factor contributing to differential sensitivity to LDHi.

Given the critical need for novel therapies for patients with EWS (19-21), the remainder of our work was focused on determining the translational potential of LDHi for this disease. While suppression of glycolysis was profound in the single treatment MR spectroscopy experiments, the long-term in vivo studies revealed that effect on xenograft tumor growth was dependent on achievement of consistently
high and sustained levels of the inhibitors intratumorally, which required frequent IV dosing. This requirement may also explain why a maximal intratumoral drug concentration in the micromolar range could result in less antitumor activity than would be expected based on the \textit{in vitro} dosing range. While an increase in the delivered dose or a more frequent dosing interval may have been able to overcome this, the on-target dose-dependent hemolytic anemia observed in the mice limited further dose-escalation. Thus, there appears to be a narrow therapeutic window for these LDHi to move forward into clinical development as single agents. Strategies to overcome these challenges include development of novel delivery methods for LDHi, or combinatorial approaches that might allow for lower doses of LDHi to be effective. Based on published data, kinase inhibitors, HSP90 inhibitors, oxidative phosphorylation inhibitors, and inducers of reactive oxygen species represent several potential combination partners worthy of further study (5, 44-47). In conclusion, our findings suggest that further translational preclinical work is necessary to optimize the potential of LDHi as anti-cancer agents, but that due to their exquisite sensitivity to glycolytic impairment, EWS may be one of the best indications for their use.

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References


**Figure Legends**

**Figure 1. Ewing sarcoma (EWS) cells are sensitive to inhibition or loss of LDH activity.**

A. Relative IC₅₀ values for top 12 most sensitive cell lines to LDH inhibitors NCI-737 and NC1-006, compared to median IC₅₀ for 94 cancer cell lines (striped bar) tested in the Oncolead cell panel assay. EWS cell lines MHHES1 and A673 are shown in black. B. IncuCyte live cell analysis of EWS cell lines TC71 and TC32 treated with NCI-737 at doses between 0.05 and 1 μM, 24 hours after plating. C. Western blot analysis of EWS cell lines TC32 and TC71 with and without treatment with 1 μM NCI-737 for 24 hours. D. Percent of TC71 and TC32 cells in early apoptosis (annexin V positive, PI negative) and late apoptosis (annexin V positive, PI positive) with and without treatment with 1 μM NCI-737 for 72 hours, measured by flow cytometry. Asterisks denote p-values, as compared to control, as follows: **p<0.01, ***p<0.001. E. Western blot analysis of LDHA and cleaved PARP in EWS cell lines (TC71, TC32, and EW8) 72 hours after LDHA siRNA knockdown. F. Relative viability of EWS cells (TC71, TC32, and EW8) following knockdown with siLDHA sequence #5 for 72 hours, expressed as percent of control confluence ± SEM. Asterisks (****) denote p-value < 0.0001, as compared to control.

**Figure 2. EWS-FLI1 regulates LDHA expression.**

A. Western blot analysis of EWS-FLI1 and LDHA 72 hours following EWS-FLI1 siRNA knockdown with sequence #8 in EWS cell lines (TC71, TC32, RDES, and EW8). B. Representative ChIP-seq tracks for EWS-FLI1 (dark blue), ELF1 (light blue), and H3K24ac (yellow) from SK-N-MC EWS cells expressing either control shRNA (top) or EWS-FLI1 shRNA (bottom). RNA-seq tracks at the LDHA locus are shown for each cell line (green). C. Western blot analysis of LDHA protein level in SK-N-MC cells 72 hours following EWS-FLI1 siRNA knockdown.
with sequence #8.

Figure 3. LDH inhibitors act through impairment of glycolysis in EWS. A. Dose-response curves for change in LDH activity with increasing doses of NCI-737 or NCI-006 in EWS cell lines (TC71, TC32 and EW8). B. YSI analysis of glucose consumption and lactate production in EWS cells 24 hours after treatment with 187 nM NCI-737. Error bars indicate SD. Asterisks denote p-values between groups as follows: *p<0.05, **p<0.01, ***p<0.001. C. Intracellular pyruvate concentration in EWS cells 24 hours after treatment with 187 nM NCI-737. Error bars indicate SD. Asterisks denote p-values between groups as follows: **p<0.001, ****p<0.0001. D. NAD+/NADH ratio in EWS cells treated with various doses of NCI-737 at 13 hours. Error bars indicate SD. Asterisks denote p-values, as compared to controls, as follows: *p<0.05, ***p<0.001, ****p < 0.0001. E. Extracellular flux analysis was used to perform glycolytic stress tests of TC71 and TC32 cells treated with increasing doses of NCI-737. Glycolysis time-point indicates flux after glucose addition; glycolytic capacity time-point indicates flux after oligomycin addition. X-axis reflects NCI-737 dose in nM. Error bars indicate SD. Asterisks denote p-values, as compared to controls, as follows: *p<0.05, ***p<0.001, ****p<0.0001.

Figure 4. LDH inhibitor sensitivity correlates with cellular bioenergetics. A-B. Relationship between degree of LDH inhibitor effect on glycolysis or glycolytic capacity and EWS cell line sensitivity. Error bars represent SEM. Relative cell line sensitivity determined based on experiments in Figures 1B and Supplemental Figure 1B. C. Pearson correlation between change in ECAR (glycolytic capacity measurement after treatment with 250 nM NCI-737) and cellular viability (as measured by percent inhibition compared to control after treatment with 500 nM NCI-737 using MTT assay). Correlation coefficient =-0.69 with p=0.038. D. Energy map of EWS cell lines (TC71, TC32, RDES, and EW8) with and without treatment with 100 nM NCI-737 for 24 hours. Error bars indicate SD. p<0.05 for comparisons of treated and untreated OCR for EW8, RDES, and TC71 (NS for TC32). p<0.01 for comparisons of treated and untreated ECAR for EW8, RDES, and TC71, p <0.05 for TC32. E. Percent $^{13}$C incorporation from glucose into major TCA cycle intermediates. Asterisks denote p-values as follows: *p>0.05, ***p<0.001, using t-test with Welch correction.

Figure 5. LDH inhibitors have a dose- and time-dependent effect on tumor cell LDH activity in vivo. A. Average intratumoral drug concentrations one-hour post-dosing of NCI-737 on day 3 in TC71-bearing xenografts (n=3 mice/group). B. Intratumoral LDH activity one-hour post-dosing of NCI-737 on day 3 in TC71-bearing xenografts (n=3 mice/group). Asterisk denotes p<0.05, compared to vehicle. C. Average intratumoral drug concentrations after one- and four-hours following treatment with NCI-737 at 40 mg/kg.
in TC71-bearing xenografts (n=3 mice/group). **D.** Percent change in intratumoral LDH activity after one- and four-hours following treatment with NCI-737 at 40 mg/kg in TC71-bearing xenografts (n=3 mice/group). Asterisk denotes p<0.05 between groups.

**Figure 6.** **LDH inhibition impairs glycolysis and affects cell survival in vivo.** **A.** Tumor growth curves for TC71-bearing xenografts treated seven days per week with IV NCI-737 at 60 mg/kg (n=5 mice/group). Error bars indicate SEM. Asterisks denote p-values, as compared to vehicle, as follows: *p<0.05, **p<0.01. **B.** Percent necrosis in tumor tissue of TC71- and EW8-bearing xenografts treated with IV NCI-737 on a seven day per week schedule (n= 5 mice/group). Error bars indicate SEM. Asterisk denotes p<0.05, for comparison between vehicle and treated tumors. **C.** Representative H&E stained whole slide digital images of entire tumor sections resected from TC71-bearing xenografts treated with vehicle (left) or NCI-737 (right). Viable tissue is shown in dark purple. Visual image analysis can be found in Supplemental Figure 12A. **D.** $^{13}$C lactate to pyruvate ratio change 30 minutes post injection of NCI-737 at 60 mg/kg in xenografted TC71 and EW8 tumors (n=3 mice/group). Lines indicate changes in each animal. Asterisk denotes p<0.05 comparing pre- and post-treatment animals.
Figure 1

A) Top 12 Most Sensitive Cell Lines to 737

B) TC71

C) Cleaved caspase 7

D) Cleaved PARP

E) LDHA

F) Cleaved PARP

GAPDH

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Figure 2

A

B

C

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Figure 3

A

B

Glucose Consumption

Lactate Production

C

Pyruvate Concentration

D

NAD+/NADH

E

TC71

TC71 Glycolysis

TC71 Glycolytic Capacity

TC32

TC32 Glycolysis

TC32 Glycolytic Capacity

Glucose Consumption (µmoles/mg protein)

Lactate Production (umoles/mg protein)

Average Pyruvate Concentration (nM per cell)

NAD+/NADH Ratio

ECAR (mP/min)

ECAR (mP/min)

ECAR (mP/min)

ECAR (mP/min)

Veh    125    187     250

Veh    125    187    250

Veh    125   187    250

Veh    125   187    250

Veh    125   187    250

Veh    125   187    250

Veh    125   187    250

Veh    125   187    250

0

0

200

400

600

800

1000

-9

-8

-7

-6

-5

-4

-3

-2

-1

0

Glucose Consumption (µmoles/mg protein)

Lactate Production (umoles/mg protein)

NAD+/NADH Ratio

Average Pyruvate Concentration (nM per cell)

Veh 125 187 250

Veh 125 187 250

Veh 125 187 250

Veh 125 187 250

Veh 125 187 250

Veh 125 187 250

Veh 125 187 250

0

0

200

400

600

800

1000

-9

-8

-7

-6

-5

-4

-3

-2

-1

0

ECAR (mP/min)

ECAR (mP/min)

ECAR (mP/min)

ECAR (mP/min)

Veh 125 187 250

Veh 125 187 250

Veh 125 187 250

Veh 125 187 250

Veh 125 187 250

Veh 125 187 250

Veh 125 187 250

0

0

200

400

600

800

1000

-9

-8

-7

-6

-5

-4

-3

-2

-1

0

ECAR (mP/min)

ECAR (mP/min)

ECAR (mP/min)

ECAR (mP/min)
Figure 4

A Effect on Glycolysis at 250 nM

B Effect on Glycolytic Capacity at 250 nM

C Percent change in ECAR relative to control

D OCR v. ECAR

E TC71

TCA Intermediates

EW8

TCA Intermediates

Labeling from 13C-Glucose (%)

Control

Treated

ns

* ***

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Figure 5

A. Average Intratumoral Drug Concentration

B. Intratumoral LDH Activity

C. Average Intratumoral Drug Concentration

D. Percent Decrease in LDH Activity

* Denotes statistical significance.
Figure 6

A

Average Tumor Volume (mm$^3$) vs Days Post Injection

- Vehicle
- 737 60 mg/kg

B

Percent necrosis

<table>
<thead>
<tr>
<th></th>
<th>TC71</th>
<th>EW8</th>
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</thead>
<tbody>
<tr>
<td>NCI-737</td>
<td>70%</td>
<td>50%</td>
</tr>
<tr>
<td>Vehicle</td>
<td>20%</td>
<td>30%</td>
</tr>
</tbody>
</table>

C

Histological images:
- Vehicle
- NCI-737

D

13C-Lac/Pyr Ratio Change

- TC71
- EW8

- Pre-treatment
- Post-NCI-737

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