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Label-free high-throughput assays to screen and characterize novel lactate dehydrogenase inhibitors

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ABSTRACT

Catalytic turnover of pyruvate to lactate by lactate dehydrogenase (LDH) is critical in maintaining an intracellular nicotinamide adenine dinucleotide (NAD^+) pool for continuous fueling of the glycolytic pathway. In this article, we describe two label-free high-throughput assays (a kinetic assay detecting the intrinsic reduced nicotinamide adenine dinucleotide (NADH) fluorescence and a mass spectrometric assay monitoring the conversion of pyruvate to lactate) that were designed to effectively identify LDH inhibitors, characterize their different mechanisms of action, and minimize potential false positives from a small molecule compound library screen. Using a fluorescence kinetic image-based reader capable of detecting NADH fluorescence in the ultra-high-throughput screening (uHTS) work flow, the enzyme activity was measured as the rate of NADH conversion to NAD^+. Interference with NADH fluorescence by library compounds was readily identified during the primary screen. The mass spectrometric assay quantitated the lactate and pyruvate levels simultaneously. The multiple reaction monitoring mass spectrometric method accurately detected each of the two small organic acid molecules in the reaction mixture. With robust Z' scores of more than 0.7, these two high-throughput assays for LDH are both label free and complementary to each other in the HTS workflow by monitoring the activities of the compounds on each half of the LDH redox reaction.

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Cancer cells have been shown to have an altered metabolism to meet the demands of faster cell division and higher energy consumption [1–3]. The altered metabolism in cancer cells is often accompanied either by a change in the expression level of an enzyme or its isoform or by an introduction of mutations or different posttranslational modification patterns that can affect functional activity of a given protein. For example, the biosynthetic pathways involved in generating fatty acids or nucleotides can be up-regulated to supply these building blocks in the fast proliferating cancer cells [4,5]. In many tumor cells, an increase in the aerobic glycolytic flux and a reduction in the oxidative phosphorylation of glucose metabolism in mitochondria was observed; these phenomena are known as the Warburg effect [6,7]. Several oncogenes such as Akt, Myc, and Ras have been linked to the Warburg effect and can activate the aerobic glycolysis pathways [8]. In addition, the loss of tumor suppressor genes such as p53 can cause a switch from cellular respiration to aerobic glycolysis, directly contributing to the Warburg effect [9,10]. The increase in the glucose uptake and glycolytic flux allows the tumor cells to quickly produce ATP as an energy source through the conversion of glucose to pyruvate, especially under hypoxic conditions that fast-growing solid tumor cells often experience. For every glucose molecule that is consumed, glycolysis reduces two molecules of nicotinamide adenine dinucleotide (NAD^+)[1] to reduced nicotinamide adenine dinucleotide (NADH) in order to generate two molecules of ATP. To continue fueling this glycolytic pathway in tumor cells, NADH needs to be efficiently recycled back to NAD^+. The decrease in aerobic glucose metabolism in the mitochondria of tumor cells also reduces NADH oxidation by the TCA cycle; thus, an alternative reaction is required. Under anabolic conditions, the conversion of the NADH back to NAD^+ is catalyzed by lactate dehydrogenase (LDH), and this oxidation is coupled to the reduction of the glycolytic product pyruvate to lactate, which will then be secreted from the cells. In many glycolytic-addicted tumor cells, the expression of LDH (especially the isoform LDHA) is found to be elevated [11,12].

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[1] *Abbreviations used:* NAD^+, nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; LDH, lactate dehydrogenase; LDHA, lactate dehydrogenase A; uHTS, ultra-high-throughput screening; BGG, bovine gamma globulin; TCEP, tris(2-carboxyethyl)phosphine; FDSS, Functional Drug Screening System; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTT, dithiothreitol; RFU, relative fluorescence unit; DMSO, dimethyl sulfoxide; MRM, multiple reaction monitoring; SPE, solid-phase extraction; AUC, area under the curve.

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The enzymatic reaction catalyzed by LDH has been characterized since the 1960s, with numerous publications primarily using the detection of NADH absorbance or intrinsic fluorescence. The enzyme family is capable of carrying out both the forward (pyruvate to lactate) and reverse (lactate to pyruvate) reactions, with the preference varying among different LDH isoforms [13,14]. Furthermore, the forward enzymatic reaction has been shown to proceed as an ordered reaction, with the enzyme binding NADH first followed by the substrate, pyruvate [13,14]. Because of the ability to use NADH or NAD^+ as substrate, LDH was used extensively as coupling enzymes for NADH or NAD^+ detection. Even though the elevated expression of LDH in cancer had been noted a few decades ago [15], it was not until 2006 when Fantin and coworkers demonstrated the link between LDHA and tumor metabolism [16] that the LDH enzyme was transformed from a supporting role of being used in coupling enzyme assays to center stage and being used as a potential therapeutic target for cancer. This also explains in part why there has been a lack of validated inhibitors for LDH despite the fact that this enzyme has been well characterized for decades. Until recent months, the only validated inhibitor known to LDH is a small organic acid, oxamate, which is a pyruvate mimetic with a reported biochemical IC_{50} ranging from 17 to 150 μM, depending on the assay conditions [17,18]. This weak and nonselective inhibitor that has poor cell permeability is less than an ideal tool compound for target validation in cells or in vivo.

With an increasing interest in exploring metabolic pathways for potential cancer therapeutic strategies, we sought to develop assay platforms that are amenable to ultra-high-throughput screening (uHTS) to identify chemical tool compounds with various mechanisms to probe the effect of LDH inhibition on cancer cell growth. LDHA has been implicated in disease progression for tumor cells that rely on glycolytic flux to provide the requisite fast energy source. LDHA is expressed to efficiently recycle NADH back to NAD^+ to continue fueling glycolysis; thus, the enzyme has been found to drive the reaction in the forward direction (i.e., pyruvate to lactate and NADH to NAD^+) [16,19]. Using LDHA that carries out predominantly the forward reaction as a model target, here we describe a robust 1536-well NADH fluorescence kinetic assay for screening and characterizing LDHA inhibitors. To avoid the potential for fluorescence interference from the use of NADH fluorescence as a readout, we optimized the protocol to readily identify compound fluorescence interference observed at the excitation and emission wavelengths of NADH. We also describe a label-free mass spectrometric assay set up to monitor the conversion of pyruvate to lactate by LDHA. This label-free assay was complementary to the higher throughput NADH fluorescence assay because it detected pyruvate to lactate conversion and was effective in further eliminating fluorescence artifacts as well as in identifying additional false positive mechanisms of inhibition. Finally, taking advantage of the well-characterized ordered enzyme reaction, the primary screening assay was designed to capture, and has successfully identified, uncompetitive or noncompetitive inhibitors with the NADH–LDHA complex in addition to conventional inhibitors that are competitive with the substrates.

Materials and methods

Chemicals

NADH was purchased from Roche Diagnostics (Indianapolis, IN, USA). Oxamate, pyruvate, bovine gamma globulin (BGG), tris(2-carboxyethyl)phosphine (TCEP), NaCl, and Triton X-100 were obtained from Sigma–Aldrich (St. Louis, MO, USA).

1536-Well kinetic LDHA FDSS assay for primary HTS and IC_{50} confirmation

The HTS screen was conducted on a BioCel 900 automated system outfitted with a Direct Drive Robot (Agilent Automation Solutions, Santa Clara, CA, USA). An FDSS (Functional Drug Screening System) 7000 kinetic fluorescence reader with onboard dispenser (Hamamatsu, Bridgewater, NJ, USA) was integrated with this system, enabling a fully automated workflow, including reagent additions, incubations, and detection.

Prior to the start of the screening campaign, assay-ready plates were prepared using a dedicated plate replication automation system. Using an Echo 555 acoustic dispenser (Labcyte, Sunnyvale, CA, USA), 50 nl per well of 1 mM compound was spotted onto 1536-well low-base, clear-bottom black microplates (Brooks Life Science Systems, Poway, CA, USA) and sealed using an Agilent PlateLoc microplate sealer to prevent evaporation. The sealed plates were then loaded onto the BioCel for processing. The plate seal was removed using an XPeel automated microplate seal removal device (Brooks Life Science Systems), followed by the addition of 6 μl of suspension buffer containing 50 mM Hepes buffer (pH 7.2), 0.01% Triton X-100, and 2 mM dithiothreitol (DTT) using a MultiDrop Combi dispenser (Thermo Scientific, Waltham, MA, USA). Next, 2 μl of 250 μM NADH with 10 nM C-terminally His-tagged full-length LDHA enzyme in reaction buffer A (50 mM Hepes [pH 7.2], 0.01% Triton X-100, and 0.1% BGG) was added using a BioRAPTR dispenser (Beckman Coulter, Indianapolis, IN, USA). The full-length LDHA (A2-F332) was purified as a tetramer from an *Escherichia coli* expression system using nickel–nitrilotriacetic acid (Ni–NTA) and size exclusion chromatography and stored as aliquots in storage buffer (10 mM Tris [pH 8.5], 150 mM NaCl, 45% glycerol, and 0.25 mM TCEP) at −80 °C. For control inhibition, 250 μM oxamate was included in the NADH and LDHA mixture. The plate was then centrifuged using a VSpin Microplate Centrifuge (Agilent Automation Solutions). Following a 10-min incubation at room temperature, the plate was loaded into the FDSS 7000 reader for an initial baseline read of 5 s. The onboard 1536-well pipette head of the FDSS 7000 was then used to transfer 2 μl of 250 μM pyruvate in reaction buffer A from an AutoFill refilling reservoir (Acorn Instruments, South San Francisco, CA, USA) to the assay plates. The final concentration of test compound in the screen was 5 μM. Fluorescence was measured at an excitation wavelength of 340 nm and an emission wavelength of 482 nm, with one read taking place each second for a total of 3 min.

The kinetic traces were analyzed by in a Genedata Screener Kinetic Analyzer (Basel, Switzerland) using the robust slope curve fit. The slopes of the kinetic traces were calculated using a timeframe of 60 to 120 s of the aggregated data. The baseline NADH fluorescence intensity was typically measured at 300 to 400 relative fluorescence units (RFU). Fluorescent compounds with RFU values greater than 500, which was the upper limit of the linear detection range in the FDSS 7000, were excluded. The remaining compounds with more than 50% inhibition were selected for IC_{50} confirmation. A hit rate of less than 0.1% was achieved after removing fluorescent artifacts. Average plate Z' scores were 0.7 (% LDHA activity with enzyme = 99.6 ± 5.8 and without enzyme = 0.3 ± 3.8), with reproducible NADH fluorescence window and oxamate inhibition from run to run.

384-Well IC_{50} confirmation assay using a combination of FDSS 7000 and mass spectrometric methods

IC_{50} follow-up by the mass spectrometric assay was conducted with compounds serially diluted in an 8-point dose response in 384-well clear-bottom black microplates. In a final reaction volume of 50 μl, 0.25 nM LDHA was incubated with 75 μM pyruvate

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