

Targeted Anticancer Agents

Dual Targeting of the Warburg Effect with a Glucose-Conjugated Lactate Dehydrogenase Inhibitor**

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Nearly a century ago, German scientist Otto Warburg observed that solid tumors deviate from most normal tissues in their ravenous consumption of glucose and high rates of aerobic glycolysis.^[1] This dysfunctional metabolism has been proposed to convey a survival advantage to tumor cells, allowing them to proliferate in normoxic or hypoxic environments and to evade killing by the immune system.^[2] The molecular mechanisms underlying the Warburg effect have been elucidated, most notably tumor cells' overexpression of the glucose transporter GLUT-1^[3] and the enzymes of glycolysis, including lactate dehydrogenase isoform A (LDH-A).^[4] Targeting dysregulated tumor cell metabolism is emerging as a tantalizing anticancer strategy.^[2a] Herein we report the first demonstration of *dual targeting* the Warburg effect using a glucose-conjugated LDH-A inhibitor, thus

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exploiting both the preferential glucose uptake and increased glycolysis of cancer cells (Figure 1a).

A common clinical application of the selective uptake of glucose into cancerous versus normal tissues is the use of the radiolabeled glucose analog 2-deoxy-2-(¹⁸F)fluoro-D-glucose (¹⁸F-FDG). ¹⁸F-FDG is a ubiquitous imaging tool for diagnosing and staging many types of cancers, including lung, breast, endometrial and colorectal carcinomas, several types of sarcomas, and both Hodgkin's and non-Hodgkin's lymphomas.^[5] In addition, the conjugation of glucose^[6] or similar sugars potentially recognized by GLUT-1 receptors^[7] to anticancer agents offers potential selective targeting of cytotoxic drugs,^[8] with the most clinically advanced glycoconjugate, glufosfamide, reaching phase II and III clinical trials in Europe and the United States.^[9]

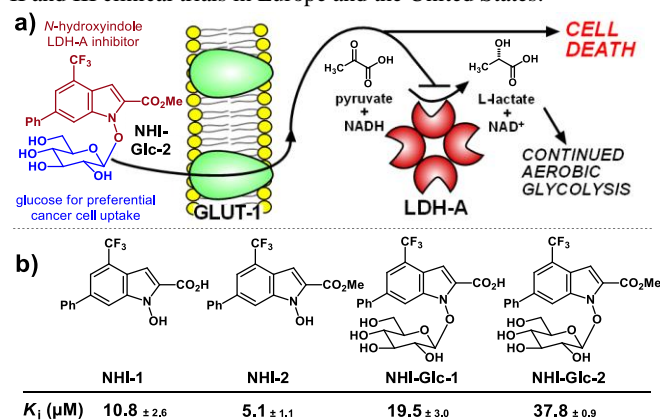


Figure 1. (a) Dual-targeting of the Warburg effect by a glucose-conjugated LDH-A inhibitor. (b) Structures and in vitro K_i values vs. NADH in LDH-A of unconjugated and glucose-conjugated *N*-hydroxyindole (NHI) class compounds. Values are reported as the mean \pm SD of three or more independent experiments.

LDH-A is a key enzyme in glycolysis, catalyzing the reduction of pyruvate to lactate (Figure 1a), generating NAD^+ and thus enabling continued glycolysis and ATP production even in the absence of aerobic oxidation of NADH.^[10] Much of the lactate produced in this reaction is excreted into the tumor microenvironment, acidifying it to limit immune access to tumor tissue.^[11] Overexpression of LDH-A has been noted in numerous solid tumors and has been found to correlate with poor clinical outcome in patients;^[12] these data have been corroborated by a number of studies demonstrating that cancer cells in which LDH-A activity has been attenuated (through RNA interference) are less viable and less tumorigenic.^[13] Importantly, LDH-A inhibition is unlikely to harm normal tissues: LDH-A deficiency is present in the human population at a frequency of 0.0012,^[14] and those individuals heterozygous for LDH-A deficiency have no clinical presentation, while homozygotes present with myoglobinuria only upon extreme exertion.^[15]

We recently reported the discovery of *N*-hydroxyindole (NHI)-based LDH-A inhibitors (exemplified by compound **NHI-1**, Figure 1b) as anticancer agents.^[16] While other classes of in vitro LDH-A inhibitors exist,^[17] including the natural product gossypol,

[18] its derivative FX-11,[19] the pyruvate mimetic oxamate,[20] the gallic acid derivative galloflavin,[21] compounds developed in a fragment-based approach by AstraZeneca[22] and by ARIAD Pharmaceuticals,[23] and in screening by Genentech,[24] the NHI inhibitors are attractive candidates due to their facile syntheses, selective toxicity toward cancerous cells, and in vitro and cell culture efficacy.[16a] Thus the NHIs are an outstanding compound class to demonstrate the concept of dually targeting the Warburg effect by linking glucose to a glycolytic enzyme inhibitor.

We previously reported compound **NHI-1** (Figure 1b) as a competitive inhibitor of LDH-A in vitro, with the ability to inhibit the conversion of ^{13}C glucose to ^{13}C lactate in HeLa human cervical carcinoma cells when used at a high concentration (500 μM).[16a] Later, methyl ester **NHI-2** was found to inhibit LDH-A in vitro and kill cancer cells in culture.[16b] Further, **NHI-2** proved to be stable after uptake by cancer cells, suggesting its improved anti-proliferative activity is due improved cell uptake compared to **NHI-1**.[16b] In efforts to enhance the tumor cell selectivity and efficacy of **NHI-1** and **NHI-2**, their glucose conjugates **NHI-Glc-1** and **NHI-Glc-2** (Figure 1b) were synthesized and evaluated (see supporting information for synthetic routes).

Evaluation versus LDH-A in vitro revealed that non-conjugated (**NHI-1** and **NHI-2**)[16b] and glucose-conjugated derivatives (**NHI-Glc-1** and **NHI-Glc-2**) are competitive inhibitors of the NADH binding pocket of LDH-A, with conjugation to the sugar moiety of the NHI derivatives lowering the inhibitory potency of the resulting conjugates by 2- (**NHI-Glc-1**) and 7-fold (**NHI-Glc-2**) (Figure 1b). To rule out inhibition by aggregation, additional assays were conducted in the presence of Triton X detergent and bovine serum albumin (BSA) using conditions described previously.[25] The NHI series, as exemplified by **NHI-1**, **NHI-2**, and **NHI-Glc-2**, retained its inhibitory potency against LDH-A in the presence of both Triton X and BSA (Figure S1).

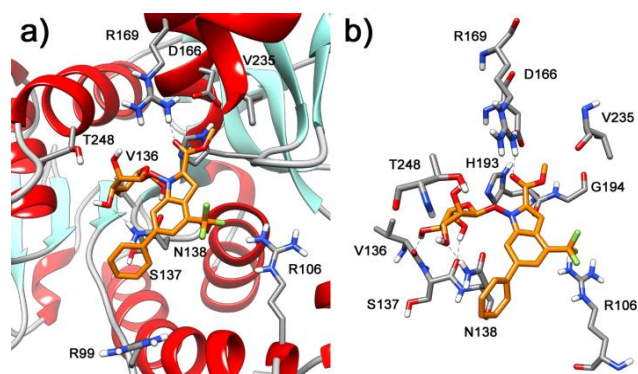


Figure 2. Binding pose resulting from MD simulation of the LDH-A complex with compound **NHI-Glc-2**. (a) disposition of the ligand into the enzyme active site displaying the protein backbone; (b) skewed view of the complex showing the protein residues that are most relevant for interaction with the inhibitor.

Docking studies followed by molecular dynamic (MD) simulations were carried out to examine the interaction of the glucose conjugates with LDH-A. Starting from the average structure of the minimized LDH-A/**NHI-1** complex that we recently reported,[16a] compound **NHI-Glc-2** was docked in the protein by using GOLD 5.1,[21] and the minimized complex was then subjected to 10 ns of MD simulation using Amber 11.[22] As shown in Figure 2, the ester of **NHI-Glc-2** forms a H-bond with R169, similar to that found by MD simulation of ester-aglycone **NHI-2**.[16b] The indole portion is located in a pocket defined by N138, H193, G194, A238, and Y239, with its 6-phenyl substituent protruding toward the enzyme cavity entrance channel. The glucose moiety establishes strong interactions in the NADH-binding pocket; in particular, H-bonds with Asn138 and with the

backbone of Val136 and Ser137 (Figure 2b and Table S1). These additional interactions largely compensate for the loss of those involving the N-OH group of non-conjugated derivatives. Interestingly, both Val136 and Asn138 were previously found to be similarly involved in crucial interactions with the enzyme cofactor in the X-ray structure of the complex of LDH-A with NADH and oxamate (1I10 PDB code).[23]

Evaluation of these compounds versus a panel of cancer cell lines representing the types of cancers in which LDH-A is commonly shown to be overexpressed[12b, 26] and which highly express GLUT-1[27] demonstrate that, while **NHI-Glc-1** is inactive ($\text{IC}_{50} > 200 \mu\text{M}$ in HeLa cells), **NHI-Glc-2** has 3-5-fold and 6-9-fold enhanced potencies, compared to **NHI-2** and **NHI-1**, respectively (Table 1). All compounds are significantly less potent against non-cancerous mouse embryonic fibroblasts (WT-MEF, Table 1).

Table 1. Cancer cell toxicity of NHI class LDH-A inhibitors [a]

Cancer cell line	Tissue of origin	IC_{50} values (μM)		
		NHI-1	NHI-2	NHI-Glc-2
HeLa	Cervix	43.8 \pm 4.6 [16b]	33.4 \pm 1.0 [16b]	7.2 \pm 0.2
A549	Lung	131.0 \pm 17.6	44.1 \pm 6.2	17.2 \pm 3.0
H1299	Lung	141.0 \pm 11.1	61.1 \pm 11.8	18.0 \pm 1.5
H226	Lung	120.7 \pm 7.8	43.4 \pm 5.3	16.8 \pm 2.8
MCF-7	Breast	124.3 \pm 7.1	64.9 \pm 13.1	16.7 \pm 1.1
BT549	Breast	110.1 \pm 9.5	34.5 \pm 10.0	12.7 \pm 0.4
IGROV-1	Ovary	123.3 \pm 6.8	57.4 \pm 7.3	15.5 \pm 3.0
WT-MEF	Normal fibroblast	245.0 \pm 13.0	80.5 \pm 8.2	32.2 \pm 0.2

[a] All cells were seeded at 5000 cells/well in plates in which vehicle or compound in DMSO was pre-dispensed (1% final concentration DMSO in all wells). Following a 72 hour incubation, biomass was quantified using the Sulforhodamine B (SRB) assay, and IC_{50} values (in μM) were calculated from logistical dose response curves. Averages were obtained from three independent experiments, and error is standard error (n=3).

To assess efficacy in inhibiting lactate production in cells, HeLa cells were treated with varying concentrations of **NHIs** and other reported LDH-A inhibitors including FX-11,[19a] galloflavin,[21] and the AstraZeneca compound AZ 33[22] (structures are depicted in Figure S2). After 8 hours of treatment, the lactate present in cell culture media was quantified by GC-MS. This GC-MS based assay for lactate detection has several-fold increased sensitivity over the ^{13}C NMR-based assay previously employed,[16a] allowing for precise detection of low micromolar lactate (versus the low millimolar detection limit afforded by ^{13}C NMR). As shown in Figure 3a, consistent with the proliferation assay results, **NHI-Glc-1** has only modest effects at 200 μM , similar to its aglycone **NHI-1** (which required 500 μM for substantial efficacy, as previously reported[16a]). On the contrary, treatment with **NHI-Glc-2** leads to significant, dose-dependent reduction in cellular lactate production, and is more potent than its aglycone **NHI-2**. The hexokinase inhibitor 2-deoxyglucose also has a modest effect at very high concentrations (10 mM), whereas negligible effects are observed for LDH-A inhibitors FX-11, galloflavin, and AZ 33 (each tested at 100 μM). Cytotoxic compounds that do not impact glucose metabolism, such as the topoisomerase II inhibitor etoposide, have no effect on lactate production. Furthermore, the reduction in lactate production observed with the **NHIs** precedes the onset of cell death (Figure S3).

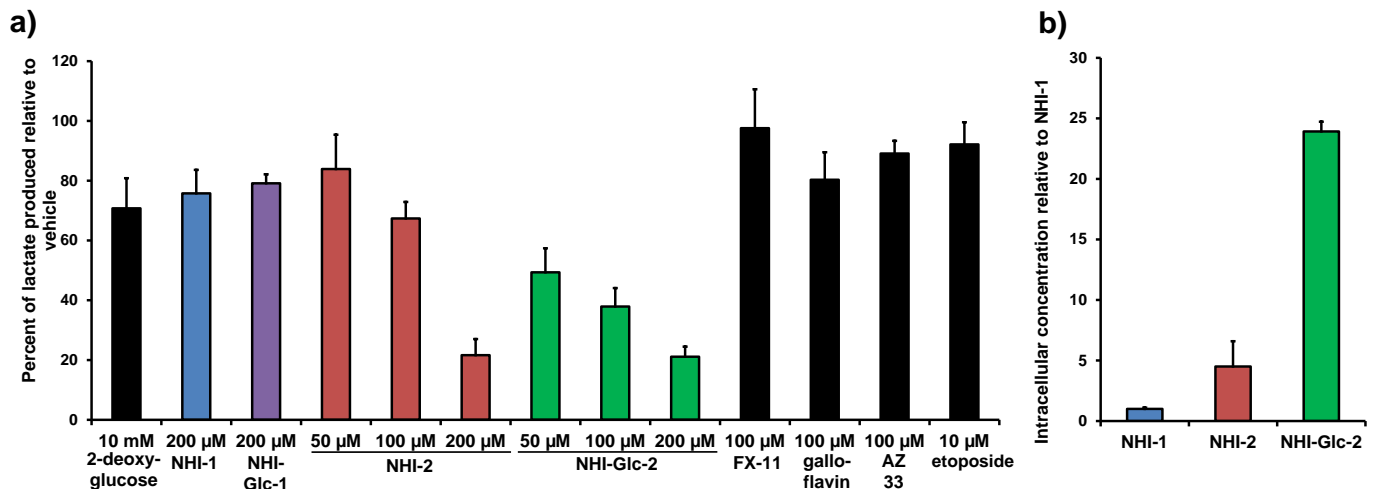


Figure 3. (a) Treatment of HeLa cells with various compounds indicates that **NHI-Glc-2** dose-dependently reduces lactate production, comparing favorably in this regard to **NHI-2**. The effect of **NHI-Glc-2** is significantly more potent than that of **NHI-1**, **NHI-Glc-1**, and other reported LDH-A inhibitors, FX-11, galloflavin, and AZ 33. HeLa cells were treated for 8 hours with compound or DMSO vehicle (1% final concentration DMSO) in DMEM media. To quantify the lactate produced by the cells, derivatized cell culture media was analyzed by GC-MS. Lactate peaks were normalized using an internal standard present in each sample, and are presented as percent of vehicle lactate production. Averages are shown, with error bars denoting standard error of three or more independent experiments. (b) Compound **NHI-Glc-2** is more readily taken up by cancer cells than **NHI-1** or **NHI-2**. A549 cells were treated with compound (100 μ M) or vehicle (0.2% final concentration DMSO). Cells were collected after 4 hours, washed twice in PBS, sonicated in methanol, and analyzed via LC-MS. UV trace integration areas, standardized by sample fresh weights, were converted to relative concentrations using calibration of known concentrations (Figure S4). Relative concentrations are presented as ratios of the concentration of **NHI-1**. Error is standard error of three or more independent experiments.

To test if the enhanced cancer cell toxicity and lactate production inhibition by **NHI-2** and **NHI-Glc-2** was due to enhanced cell uptake, the ability of these compounds to penetrate A549 cells was evaluated. To compare the relative intracellular concentrations, A549 cells were treated with equimolar compounds or vehicle for 4 hours, and cell lysates were subjected to LC-MS analysis using calibrations of known concentrations generated using the same LC-MS protocol (Figure S4). In A549 cells, **NHI-2** was present in approximately 4.5-fold higher concentrations in the lysate of samples compared to **NHI-1**, and **NHI-Glc-2** was present at approximately 24-fold higher concentrations in the lysate samples (Figure 3b). **NHI-Glc-2** does not appear to be appreciably cleaved to **NHI-2** or **NHI-Glc-1** inside the cell (representative UV traces are shown in Figure S5).

To examine whether **NHI-Glc-2** is entering cells via GLUT transporters, a competition assay was performed between **NHI-Glc-2** and GB2-Cy3, a fluorescent Cy3-linked glucose bioprobe recently developed by Park and coworkers.^[28] While a number of glucose bioprobes are known,^[29] GB2-Cy3 has been shown to possess an enhanced fluorescent signal in live cells and an enhanced ability to compete with glucose for uptake in cultured cells compared to the known 2-deoxyglucose analog 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-amino]-2-deoxy-D-glucose (2-

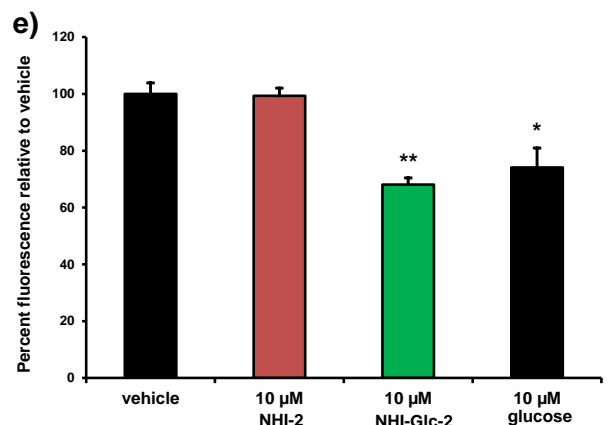
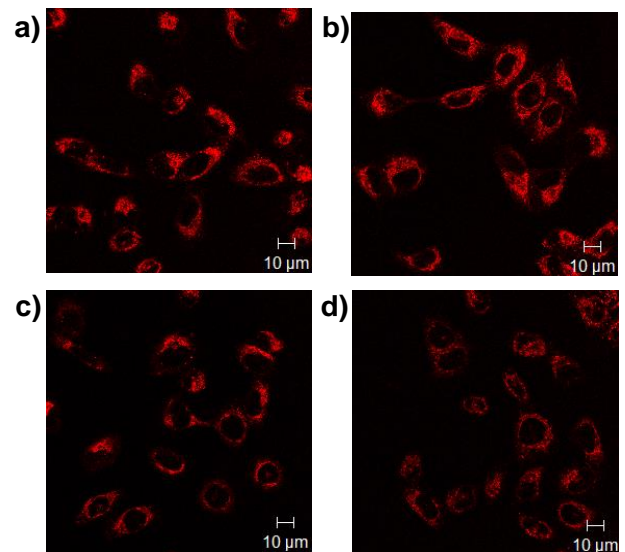


Figure 4. A549 cells were grown in RPMI 1640 growth media on cover glass bottom dishes (60,000 cells/dish). When cells reached 70% confluence, they were treated with GB2-Cy3 (2.5 μ M) in the absence (a) or the presence of **NHI-2** (10 μ M) (b), **NHI-Glc-2** (10 μ M) (c), or glucose (10 μ M) (d) for 30 minutes at 37 $^{\circ}$ C. Cellular fluorescence was observed using a Zeiss LSM700 confocal microscope, using a photomultiplier gain of 844 and a laser power of 555 nm (representative images are shown). (e) The intracellular fluorescence of **NHI-Glc-2**- and glucose-treated cells is statistically significantly less than that of vehicle-treated cells, indicating that the uptake of the fluorescent GB2-Cy3 probe in these cells was inhibited by treatment with **NHI-Glc-2** and glucose. The mean fluorescence intensities of each sample were calculated by averaging the fluorescence intensities of 40-60 cells per treatment over three independent experiments. Error bars denote standard error (n=3); statistical analysis was performed using an unpaired, two-tailed Student's t test. * denotes $p < 0.05$; ** denotes $p < 0.01$.

NBDG), with 5 μM GB2-Cy3 more potently inhibiting glucose uptake than 50 μM 2-NBDG.^[30] For our purposes, cellular fluorescence was used as a readout for whether the cellular uptake of GB2-Cy3 was hindered by co-incubation with compound. A549 cells, which highly express GLUT-1^[27b], were treated for 30 minutes with 2.5 μM GB2-Cy3 in the presence of either vehicle or 10 μM **NHI-2**, **NHI-Glc-2**, or glucose. Cells were then imaged by confocal laser scanning microscopy, and fluorescence was quantified and averaged over 40-60 cells per treatment. Treatment with **NHI-Glc-2** and glucose caused a statistically significant decrease in fluorescence compared to vehicle, whereas treatment with **NHI-2** did not (Figure 4), thus suggesting that **NHI-Glc-2** and glucose are competing with GB2-Cy3 for cellular entry through GLUT transporters. To confirm that loss of cell viability was not skewing this result, 30 minute toxicity assays were performed for both **NHI-2** and **NHI-Glc-2**; the results show that no appreciable loss of viability is observed at compound concentrations up to 200 μM at 30 minute treatment times.

In summary, **NHI-Glc-2** has been designed as the first compound aimed at dual targeting of the Warburg effect, created to exploit a) the enhancement in glucose uptake, and b) the increased glycolysis that characterizes many aggressive tumors. **NHI-Glc-2** has improved potency against cancer cells and increased cell permeability compared to its aglycone, showing a modest reduction in its inhibition potency on isolated enzyme that is highly compensated by its improved cell uptake via GLUT transporters. This compound will be an outstanding tool to fully probe the tractability of LDH-A inhibition in advanced mammalian tumor models. In addition, these results suggest application of this dual targeting strategy to inhibitors of the various other enzymes involved in glycolysis.

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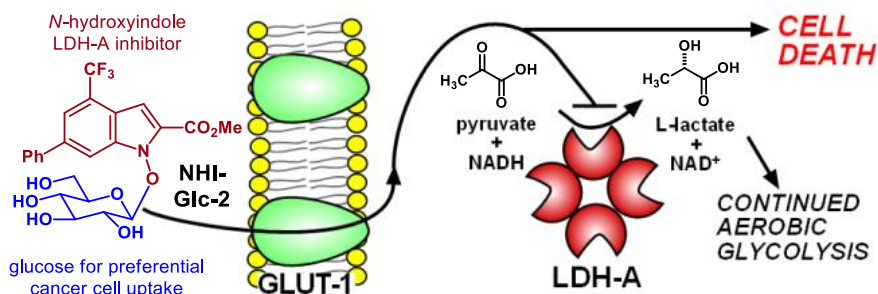
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Targeted Anticancer Agents

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Dual Targeting of the Warburg Effect
with a Glucose-Conjugated Lactate
Dehydrogenase Inhibitor



Glucose transporters and the glycolysis enzyme lactate dehydrogenase A (LDH-A) are both overexpressed in cancer cells, two proliferation tactics that underlie the phenomenon known as the Warburg effect. Herein we report the development and activity of a glucose-conjugated LDH-A inhibitor designed to target both of these tumor-promoting facets. In addition to the promise of this conjugate, dual targeting of the Warburg effect using glycoconjugation as an anticancer strategy could be applied to inhibitors of many of the enzymes involved in glycolysis or tumor metabolism.