SMALL MOLECULE INHIBITORS OF LACTATE DEHYDROGENASE A AS AN ANTICANCER STRATEGY

BY

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DISSERTATION

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Abstract

Exploiting cancer cell metabolism as an anticancer therapeutic strategy has garnered much attention in recent years. As early as the 1920s, German scientist Otto Warburg observed cancer tissues' avid glucose consumption and high rates of aerobic glycolysis, a phenomenon now known as the Warburg effect. Today, we understand the Warburg effect is mediated by a number of complex factors, including overexpression of the insulin-independent glucose transporter GLUT-1 and overexpression of various glycolytic enzymes, including lactate dehydrogenase A (LDH-A). As the terminal enzyme of glycolysis, LDH-A catalyzes the reversible conversion of pyruvate to lactate, and in doing so, oxidizes NADH to NAD⁺. The lactate produced by this reaction is largely excreted into the tumor microenvironment, where it acidifies surrounding tissues and helps the tumor evade destruction by immune cells. The oxidation of NADH to NAD⁺ allows for continued ATP production through glycolysis by replenishing NAD⁺ in the absence, or reduced function, of oxidative metabolism. Cell culture and in vivo studies of LDH-A knockdown (using RNA interference) have been shown to lead to substantial decreases in cell and tumor proliferation, thus providing evidence that LDH-A would be a viable anticancer target. While various in vitro LDH-A inhibitors exist, there is a need for a potent and selective small molecule inhibitor that functions both in cells and in vivo. Here, the development and biological assessment of the N-hydroxyindole class of LDH-A inhibitors, including a series of novel dual-Warburg targeting glucose-conjugated LDH-A inhibitors, developed through a collaboration between the Hergenrother and Minutolo laboratories, is reported. The development of novel assays to assess the relative cell uptake, cell lactate production, and competition with ¹³C glucose for cellular entry, of NHI series compounds are also discussed. Head-to-head cellular assessments of the most promising NHI series compounds alongside literature-reported in vitro inhibitors of LDH-A are reported. Finally, efforts to directly probe the interactions of compounds with LDH-A in cell lysate and whole cells using CETSA and DARTS techniques are discussed.
Acknowledgements

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Chapter 1: Targeting LDH-A and GLUT-1 for anticancer efficacy

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1.1: The Warburg effect

In 1927, German scientist Otto Warburg observed that cancerous tissues consume larger amounts of glucose and have higher rates of aerobic glycolysis than do non-transformed tissues [2, 3]. This phenomenon, now known as the Warburg effect, is recognized as one of the hallmarks of cancer [4]. With the advent of modern genomics, transcriptomics, and metabolomics, it has been discovered that all the enzymes of glycolysis (Figure 1.1), as well as the insulin-independent glucose transporter GLUT-1, are widely overexpressed in human cancers [5, 6].

Targeting the Warburg effect as an anticancer strategy has garnered a great deal of interest in recent years. High expression levels of glycolytic enzymes in tumor biopsy samples have been shown to correlate with poor cancer prognosis, making these metabolic enzymes and proteins attractive therapeutic targets [7-10]. Most efforts have focused on using small molecules to inhibit the function of metabolic enzymes, as comprehensively reviewed elsewhere [11-13].
**Figure 1.1**: The reactions of the glycolytic pathway in humans. Changes in each intermediate are noted in red.

1.2: Lactate dehydrogenase A (LDH-A) as an anticancer target

**Figure 1.2**: Lactate dehydrogenase catalyzes the reversible conversion of pyruvate to L-lactate, oxidizing cofactor NADH to NAD$^+$ in the process.

In exploring metabolic elements that have expression levels that are known to be altered in cancer for their ability to be modulated for anticancer efficacy, lactate dehydrogenase A (LDH-A or LDH-5), has been identified as a potential therapeutic target. LDH performs the reversible conversion of pyruvate to lactate, and in doing so, oxidizes NADH to NAD$^+$ (**Figure 1.2**). Several enzyme active site residues are important for this
catalysis and stabilization of substrate and cofactor. The mechanism of LDH involves deprotonation of the enzyme’s catalytic residue His193 and hydride transfer from NADH to the pyruvate carbonyl carbon (Figures 1.3-1.4). Arg169 forms hydrogen bonds with the carboxylic acid oxygens of pyruvate or lactate to stabilize the substrate in the active site.

Figure 1.3: Computational docking model of pyruvate and NADH situated in the active site of an M monomer unit of LDH. (Adapted with permission from Granchi, et. al. J. Med. Chem. 54: 1599-1612. Figure 6 [14]. Copyright 2011 American Chemical Society.)
LDH is arranged in a tetrameric complex, with each of its four subunits capable of performing the dehydrogenation or hydrogenation independently of the three other subunits. There are two principal types of LDH subunits in humans: LDH-M(ussle), a 331 amino acid, 35 kDa protein produced from the *LDHA* gene on the short arm of chromosome 11, and LDH-H(ear), a 331 amino acid, 35 kDa protein produced from the *LDHB* gene on the short arm of chromosome 12. Thus, there are five possible arrangements of subunits into a tetramer: LDH-H₄ (also known as LDH-B or LDH-1), LDH-H₃M₁, LDH-H₂M₂, LDH-H₁M₃, and LDH-M₄ (also known as LDH-A or LDH-5).

The expression profiles of LDH-A and LDH-B differ among various tissues. LDH-A is known to be expressed in tissues that must accommodate anaerobic metabolism, such as
skeletal muscle. LDH-B is known to be expressed in tissues which primarily perform aerobic metabolism, such as cardiac muscle [15].

While the M subunit of LDH, found as a homotetramer in LDH-A, performs primarily the hydrogenation reaction (conversion of pyruvate to lactate), the H subunit of LDH, found as a homotetramer in LDH-B, performs primarily the dehydrogenation reaction (conversion of lactate to pyruvate) [15]. These opposite activities are due to kinetic and physiological differences between the M and H subunits. The M and H LDH proteins are identical at 250/331 (75%) amino acids in their sequence, including at key active site residues involved in substrate and cofactor binding. However, the $k_{\text{cat}}$ for LDH-M (260 s$^{-1}$) is nearly double that of LDH-H (143 s$^{-1}$) [16, 17]. While both LDH-M and LDH-H have very similar $K_d$ values for NADH and NAD$^+$ (0.62 and 198 µM, respectively, for LDH-M, and 0.53 and 165 µM, respectively, for LDH-H), the $K_M$ value of LDH-M for pyruvate is 3-fold higher than that of LDH-H (158 µM vs. 58 µM) [16, 17]. Further kinetic analysis of LDH-H and LDH-M has revealed the correlation of the protonation state of the catalytic His193 residue on the enzyme’s $K_M$ for pyruvate in the forward reaction. The $K_M$ of LDH-M for pyruvate increases linearly from pH 7 to pH 10, while the $K_M$ of LDH-H for pyruvate increases more gradually from pH 8.5 to pH 10 and is lower than that of LDH-M at all pH values assessed [18]. Thus, LDH-M is considered to be primed for the hydrogenation reaction (pyruvate to lactate) while LDH-H is considered to be primed for the dehydrogenation reaction (lactate to pyruvate).

LDH-A is known to be overexpressed and correlate with poor prognosis in a wide variety of solid tumors, including colorectal, endometrial, gastric, head and neck, non-small cell lung, oral, and pancreatic carcinomas and melanoma (Table 1.1) [19-28]. Increased LDH-A expression in tumor cells is thought to be due to increased transcription of the LDHA
gene, which contains in its proximal promoter sequence binding sequences for hypoxia-inducible factor-1α (HIF-1α) and myelocytomatosis oncogene (c-myc) [29, 30].

Table 1.1: LDH-A overexpression and relation to cancer prognosis in patient biopsy samples

<table>
<thead>
<tr>
<th>Tissue of origin</th>
<th>Fold-overexpression</th>
<th>Assessment method</th>
<th>Association with poor prognosis or survival?</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colorectal</td>
<td>Not quantified</td>
<td>Immunohistochemistry</td>
<td>Yes</td>
<td>[19]</td>
</tr>
<tr>
<td>Endometrial</td>
<td>Not quantified</td>
<td>Immunohistochemistry</td>
<td>Yes</td>
<td>[20]</td>
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<tr>
<td>Gastric</td>
<td>Not quantified</td>
<td>Immunohistochemistry</td>
<td>Yes</td>
<td>[21]</td>
</tr>
<tr>
<td>Head and neck</td>
<td>3-fold</td>
<td>Semiquantitative RT-PCR</td>
<td>Not assessed</td>
<td>[22]</td>
</tr>
<tr>
<td>Head and neck</td>
<td>Not quantified</td>
<td>Immunohistochemistry</td>
<td>Yes</td>
<td>[23]</td>
</tr>
<tr>
<td>Melanoma</td>
<td>Not quantified</td>
<td>Immunohistochemistry</td>
<td>Yes</td>
<td>[24]</td>
</tr>
<tr>
<td>Non-small cell</td>
<td>Not quantified</td>
<td>Immunohistochemistry</td>
<td>Yes</td>
<td>[25, 26]</td>
</tr>
<tr>
<td>lung</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oral</td>
<td>Not quantified</td>
<td>Immunohistochemistry</td>
<td>Yes</td>
<td>[27]</td>
</tr>
<tr>
<td>Pancreatic</td>
<td>3-fold</td>
<td>Semiquantitative RT-PCR</td>
<td>Not assessed</td>
<td>[28]</td>
</tr>
</tbody>
</table>
Elevated LDH-A expression is believed to confer several survival advantages to tumors. First, the conversion of pyruvate to lactate by LDH-A oxidizes NADH to NAD\(^+\), which is required for continued glycolysis in the glyceraldehyde 3-phosphate dehydrogenase reaction (Figure 1.1). This oxidation allows cancer cells to survive in hypoxic regions of tumors, by enabling continued ATP production through glycolysis (Figure 1.5). Second, much of the lactate produced by LDH-A is excreted into the tumor microenvironment through several classes of monocarboxylic acid transporter (MCT) channels also known to be overexpressed in many cancers [31]. High concentrations of lactate in the tumor microenvironment are thought to aid the tumor in evading immune response [32]. Third, lactate excreted from the tumor to surrounding fibroblasts has been shown to stimulate fibroblast production of hyaluronan, a glycosaminoglycan involved in extracellular matrix remodeling, and CD44, a cell surface marker involved in cell migration, which may promote remodeling of the tumor region to facilitate tumor cell migration and metastasis [33]. High (> 40 mM) levels of lactate within tumors and tumor microenvironments at initial diagnosis have been shown to correlate positively with increased metastases, decreased disease-free survival time, and decreased overall survival time in cervical and head and neck carcinomas, compared to low (< 8 mM) levels of lactate at diagnosis [22].
Figure 1.5: Depiction of a tumor microenvironment in which low oxygen conditions in regions distanced from vasculature necessitate anaerobic metabolism to fulfill the tumor cells’ energetic needs. The conversion of pyruvate to lactate by LDH-A oxidizes NADH to NAD\(^+\), which can feed back into an earlier step of glycolysis (the reaction performed by glyceraldehyde 3-phosphate dehydrogenase, which reduces NAD\(^+\) to NADH) to allow for continued ATP production in the absence of oxygen.

In order to validate LDH-A inhibition as a viable anticancer strategy, numerous cell culture and in vivo studies have been performed using cells in which LDH-A expression has been modulated using RNA interference. Leder and colleagues reported in 2006 the cell culture and in vivo tumorigenic properties of Neu4145 mouse mammary gland tumor cells from which LDH-A was stably knocked down using shRNA [34]. LDH-A knockdown cells displayed reduced proliferation and intracellular ATP levels in hypoxia, and increased mitochondrial respiration rates in normoxia, compared to wild type controls. In a syngeneic tumor model in which tumors were generated in murine mammary glands using wild type, LDH-A knockdown, or knockin Neu4145 cells (LDH-A knockdown cells complemented with an LDH-A-expressing vector), tumors generated from LDH-A knockdown cells had the lowest proliferation rate and smallest fraction of Ki67-staining cells (a proliferation marker).
Furthermore, the mice bearing these LDH-A knockdown tumors had the highest overall survival [34].

In a 2011 study by Seth and coworkers, stable knockdown of LDH-A was achieved using shRNA in human A549 non-small cell lung carcinoma cells [35]. Consistent with the results obtained by Leder, Seth and coworkers found that these knockdown cells showed decreased proliferation and ATP production in hypoxia. Seth and coworkers also demonstrated changes in the characteristics of LDH-A knockdown cells in normoxia. Specifically, knockdown cells showed increased rates of oxygen consumption, increased intracellular reactive oxygen species (ROS) formation, and increased apoptosis. In a xenograft animal model in which tumors were generated from wild-type and LDH-A knockdown A549 cells, tumors generated from knockdown cells showed reduced proliferation over 30 days [35].

Wang, Lou, and coworkers reported in 2013 the tumorigenic properties in cell culture and *in vivo* of BXPC-3 human pancreatic adenocarcinoma and MIA PaCa-2 human pancreatic carcinoma cells in which LDH-A was overexpressed (by transfecting cells with a pcDNA3.1-myc-LDHA vector) or silenced (using RNAi) [28]. LDH-A overexpression was found to induce S phase transition and promote tumorigenicity, assessed by colony formation assays on soft agar. RNAi-mediated knockdown of LDH-A expression was found to inhibit colony formation on soft agar, decrease expression of anti-apoptotic proteins XIAP, Bcl-2, and Bcl-XL, increase PARP cleavage, and promote apoptosis. Furthermore, tumors in nude mice generated from LDH-A knockdown BXPC-3 cells showed statistically significantly decreased tumor volume and tumor weight over 25 days compared to tumors generated from wild type cells [28].
Non-small cell lung carcinomas (NSCLCs) are known to be highly glycolytic [36], and thus they represent an excellent model for studying the antitumor efficacy of LDH-A inhibition. Seth and coworkers reported in 2014 an inducible model of LDH-A knockdown and NSCLCs in transgenic Ccsp-rtTa mice [37]. Tumors can be initiated in the lungs of these mice by doxycycline induction of human oncogene K-RAS or mutant human EGFR, and the human LDH-A expressed in these mice can be systemically knocked down by daily intraperitoneal tamoxifen injection for 5 days. Seth and coworkers noted that, upon induction of lung tumors using doxycycline, both mice homozygous and heterozygous for LDH-A knockdown had decreased tumorigenesis and tumor regression. Metabolic profiling of these tumors demonstrated reduced glycolytic flux [37]. These data suggest inhibition of LDH-A would be a tractable anticancer strategy that would reduce cancer cell viability and tumor growth in vivo.

Another important consideration in the identification of anticancer targets is the effect of on-target inhibition in normal, non-cancerous tissues. A common method used to determine these effects is to characterize the phenotype of cellular, tissue, or whole-organism deficiency of the target. For most glycolytic enzymes, one copy of a nonfunctional or functionally impaired enzyme will lead to a moderate to severe phenotype known as glycogen storage disease, often involving chronic hemolytic anemia (given the importance of glycolysis in erythrocytes, which lack mitochondria and thus do not perform citric acid cycle reactions or oxidative phosphorylation). Two copies of a nonfunctional or functionally impaired glycolytic enzyme are generally embryonic lethal [38]. By contrast, an estimated 3 in 2,000,000 humans lack both copies of the LDHA gene (based on an absent or functionally impaired allele frequency of 0.0012) and exhibit only a mild phenotype of exercise intolerance [39-44]. An estimated 12 in 10,000 humans are heterozygous for an
absent or non-functional \textit{LDHA} allele; heterozygotes are not known to present with a phenotype and may go undiagnosed throughout their lifetimes \cite{45}. Thus, on-target inhibition of LDH-A would be predicted to have mild, if any, negative effects on normal tissues.

1.3: \textit{Known classes of LDH-A inhibitors}

Given the cell culture and \textit{in vivo} studies on the impaired tumorigenic properties of LDH-A knockdown cells and tumors \cite{28, 34, 35}, there is a need for a potent, selective small molecule inhibitor of LDH-A to further probe the tractability of LDH-A inhibition in cells and \textit{in vivo}. There have been a number of compounds reported to inhibit LDH-A \textit{in vitro}, with a few reported to have cell culture and \textit{in vivo} efficacy, as detailed below, but all have limitations hindering their use to explore the efficacy of LDH-A inhibition in the clinic. The \textit{in vitro} inhibitory profiles of known LDH-A inhibitors, as well as selectivity data for these inhibitors for LDH-A vs. LDH-B, are listed in \textbf{Table 1.2}. 
Table 1.2: *In vitro* inhibitory properties of published LDH-A inhibitors

<table>
<thead>
<tr>
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<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Gossypol</td>
<td>Kinetic $K_i$ determination</td>
<td>$K_i = 1.9$ µM (vs. NADH)</td>
<td>$K_i = 1.4$ (vs. NADH)</td>
<td>[46]</td>
</tr>
<tr>
<td>FX-11</td>
<td>Kinetic $K_i$ determination</td>
<td>$K_i = 8$ µM (vs. NADH)</td>
<td>Not reported</td>
<td>[47]</td>
</tr>
<tr>
<td>Galloflavin</td>
<td>Kinetic $K_i$ determination</td>
<td>$K_i = 5.46$ µM (vs. pyruvate)</td>
<td>$K_i = 15.1$ µM (vs. pyruvate)</td>
<td>[48]</td>
</tr>
<tr>
<td>AZ 33</td>
<td>Kinetic IC$_{50}$ determination; SPR $K_D$ determination</td>
<td>IC$_{50} = 500$ nM $K_D = 93$ nM</td>
<td>Not reported</td>
<td>[49]</td>
</tr>
<tr>
<td>AZ 35</td>
<td>Kinetic IC$_{50}$ determination</td>
<td>IC$_{50} &gt; 200$ µM</td>
<td>IC$_{50} &gt; 200$ µM</td>
<td>[50]</td>
</tr>
<tr>
<td>ARIAD 9</td>
<td>Kinetic IC$_{50}$ determination; SPR $K_D$ determination</td>
<td>IC$_{50} = 120$ nM $K_D = 19$ nM</td>
<td>Not reported</td>
<td>[51]</td>
</tr>
<tr>
<td>Genentech 22</td>
<td>Kinetic IC$_{50}$ determination; SPR $K_D$ determination</td>
<td>IC$_{50} = 750$ nM $K_D = 5.1$ µM</td>
<td>IC$_{50} = 3.7$ µM</td>
<td>[52]</td>
</tr>
<tr>
<td>Genentech 35</td>
<td>Kinetic IC$_{50}$ determination</td>
<td>IC$_{50} = 500$ nM</td>
<td>IC$_{50} = 2$ µM</td>
<td>[53]</td>
</tr>
<tr>
<td>GSK 1</td>
<td>Kinetic IC$_{50}$ determination</td>
<td>IC$_{50} = 2.6$ nM</td>
<td>IC$_{50} = 43$ nM</td>
<td>[54]</td>
</tr>
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</table>

SPR = surface plasmon resonance
1.3.1: Gossypol and FX-11

**Figure 1.6**: The structures of the natural product gossypol and its derivative FX-11.

Gossypol (Figure 1.6) is a natural product derived from the seeds of cotton plants (genus *Gossypium*). It is a competitive inhibitor of the NADH binding pocket of human LDH-A (Table 1.2) [46], as well as human LDH-C (an isozyme expressed only in male sperm) [55] and the LDH present in *Plasmodium falciparum* (a protozoan parasite which causes malaria) [46]. Gossypol is also reported to inhibit other NADH or NADPH-utilizing enzymes, such as the NADPH-utilizing enzyme human aldose reductase (which catalyzes the conversion of glucose to sorbitol) [56] and the NADH-utilizing enzymes malate dehydrogenase, isocitrate dehydrogenase, and glyceraldehyde 3-phosphate dehydrogenase [55]. Gossypol has also been shown to have additional cellular interactions, including acting as a BH3 mimetic, binding to anti-apoptotic proteins to induce apoptotic cell death [57], binding and non-competitively inhibiting DNA polymerase α [58], and binding human serum albumin [59].

There are several factors that make gossypol an unfavorable compound to pursue further as an LDH-A inhibiting anticancer agent. First, as described previously, gossypol has numerous reported targets and high toxicity in cells. This is likely due to its aldehyde functional group, which can be reactive and modify proteins *in vivo*, as well as its catechol
moiety, which can be metabolized in vivo by cytochrome P450 enzymes and lead to redox cycling and reactive oxygen species (ROS) generation, as has been observed with other catechol-containing compounds [60]. Second, the inherent disadvantage of using LDH-A inhibitors that only interact with the NADH/NAD\(^+\)-binding region of the enzyme is that they may also bind the cofactor binding pockets of other NADH/NAD\(^+\) or NADPH/NADP-utilizing enzymes as well. Indeed, gossypol is known to inhibit several NADH- and NADPH-utilizing enzymes [55], as described above.

In 2010, Dang and Vander Jagt reported the in vitro, cell culture, and in vivo properties of the gossypol derivative FX-11 (Figure 1.6). [47] This compound had been previously synthesized in an effort to obtain more potent, drug-like inhibitors of Plasmodium falciparum LDH. When tested against human LDH-A, FX-11 was found to be a potent, competitive inhibitor of the enzyme’s NADH binding pocket (Table 1.2) [61]. While one of gossypol’s main drawbacks is its lack of selectivity for LDH-A vs. other NADH/NAD\(^+\)-utilizing enzymes, Dang and Vander Jagt demonstrated that FX-11 does not inhibit glyceraldehyde 3-phosphate dehydrogenase at up to 74 µM. While assessing the cell culture efficacy of FX-11, Dang and Vander Jagt found that FX-11 phenocopied siRNA-mediated LDH-A knockdown in P493 human B cells, in that it led to increased ROS generation and reduced cell proliferation in normoxia. Treatment of P493 cells with FX-11 also led to statistically significant reductions in intracellular ATP levels and lactate production. FX-11 treatment also showed efficacy in reducing tumor volume in two xenograft tumor models in mice [47]. In a later study, a murine xenograft study using P493 human B lymphoma cells, treatment with 42 µg (2.1 mg/kg) FX-11 for 6 days led to a reduction in formation of hyperpolarized \(^{13}\)C lactate derived from \(^{13}\)C pyruvate in tumor tissues in vivo, measured by magnetic resonance [62].
However, independent evaluation of **FX-11** by other researchers has suggested that **FX-11** acts as an aggregator. Enzymatic inhibition studies performed on **FX-11** by ARIAD Pharmaceuticals demonstrated that the *in vitro* inhibition of LDH-A by **FX-11** is ablated in the presence of 0.1% Triton detergent, indicative of aggregation. ARIAD researchers also assessed the binding of **FX-11** to immobilized LDH-A by surface plasmon resonance (SPR) and found slow on- and off-rates consistent with aggregation [51].

1.3.2: Galloflavin

![Galloflavin](image)

**Figure 1.7**: The structure of galloflavin.

In 2011, Di Stefano and coworkers reported the *in vitro* inhibition of LDH-A by galloflavin (**Figure 1.7**), a hit from a virtual screen to find novel classes of LDH-A inhibitors [48]. Galloflavin is an oxidized dimer of the natural product gallic acid, a phenolic acid found in the leaves, roots, and barks of many plants. Di Stefano and coworkers found that galloflavin was a moderately potent, competitive inhibitor of LDH-A’s pyruvate binding pocket, with modest residual inhibition of the NADH binding pocket. However, galloflavin showed comparable inhibition of LDH-B (**Table 1.2**) [48]. Galloflavin was shown to have some cell culture efficacy at high concentrations: 100-500 µM concentrations were required to significantly reduce lactate production in PLC/PRF/5 human liver carcinoma cells after 3 hours of treatment. High concentrations of galloflavin also led to modest reductions in intracellular ATP levels and cell death [48].
In 2012, Di Stefano and coworkers published an additional study examining galloflavin’s ability to induce cell death in several variably glycolytic human cancer cell lines [63]. High concentrations (> 100 µM) of galloflavin were shown to induce cell death in MCF-7 human breast ductal carcinoma cells (shown to be weakly glycolytic and most reliant on oxidative metabolism, as assessed by LDH activity and oxygen consumption) concurrent with reduced expression of survival signal estrogen receptor α. In cells that were more reliant on glycolysis, including tamoxifen-resistant MCF-7 cells and MDA-MB-231 human breast adenocarcinoma, high concentrations of galloflavin were shown to induce oxidative stress [63]. Given these different proposed cell death mechanisms, it is unclear whether galloflavin induces cell death exclusively by the downstream effects of LDH-A inhibition or by other mechanisms. In addition, the relatively poor potency of galloflavin in cell culture limits further development of this compound.

1.3.3: Lead compounds from AstraZeneca and ARIAD Pharmaceuticals, derived by fragment-based drug discovery

![Structures of reported AstraZeneca and ARIAD Pharmaceuticals LDH inhibitors, discovered via a fragment-based approach.](image)

**Figure 1.8:** Structures of reported AstraZeneca and ARIAD Pharmaceuticals LDH inhibitors, discovered via a fragment-based approach.
In 2012, AstraZeneca reported a new class of \textit{in vitro} LDH-A inhibitors developed through a fragment-based approach, as exemplified by lead compound \textbf{AZ 33} (Figure 1.8) [49]. These inhibitors were developed by screening in-house libraries for compounds able to bind the adenine and active site binding pockets of LDH-A, both using nuclear magnetic resonance (NMR) and SPR, and then linking the most potent binders of each pocket. This resulted in a small number of inhibitors with nanomolar-range IC$_{50}$ and $K_D$ values against LDH-A \textit{in vitro} (Table 1.2). Treatment of SKBR3 human mammary adenocarcinoma cells with lead compound \textbf{AZ 33} failed to inhibit cellular lactate production [49], likely due to the poor cell permeability of the compound. In order to ameliorate this, AstraZeneca synthesized compound \textbf{AZ 35} (Figure 1.8), the dimethyl ester of \textbf{AZ 33}, for use in future cell culture studies, postulating that it would be converted to the diacid in cells [49]. However, neither cellular data on the efficacy of \textbf{AZ 35}, nor assays querying the \textit{in vitro} or intracellular cleavage of \textbf{AZ 35}, were reported.

In 2013, ARIAD Pharmaceuticals reported a class of \textit{in vitro} LDH-A inhibitors also developed through a fragment-based approach, exemplified by lead compound \textbf{ARIAD 9} (Figure 1.8) [51]. Initial screening was performed by saturation transfer difference NMR (STD-NMR), with follow up testing of hits performed using crystal soaking and SPR. Subsequent \textit{in vitro} assessment of lead compound \textbf{ARIAD 9} showed it to have nanomolar potency against LDH-A (Table 1.2). Several analogs of \textbf{ARIAD 9} were synthesized in order to improve cell permeability, such as derivatives in which the carboxylic acids were removed or esterified, but at the cost of poorer kinetic inhibition of LDH-A (an increase of at least one order of magnitude). Assessment of cellular lactate production inhibition was performed in Ramos human Burkitt’s lymphoma cells following treatment with 200 µM compound for 1
and 4 hours. **ARIA** showed modest inhibition (~40% reduction in lactate production compared to vehicle) at both time points [51].

### 1.3.4: Genentech and GlaxoSmithKline inhibitors

![Representative structure of LDH-A inhibitors reported by Genentech and GSK](image)

**Figure 1.9:** Representative structure of LDH-A inhibitors reported by Genentech and GSK

In 2013, Genentech published two series LDH-A inhibitors discovered through high throughput screening efforts – a 2-thio-6-oxo-1,6-dihydropyrimidine class of inhibitors, as exemplified by **Genentech 22** [52], and a 2-amino-5-aryl-pyrazine class as exemplified by **Genentech 35** (Figure 1.9) [53].

The 2-thio-6-oxo-1,6-dihydropyrimidine class was developed after a high throughput screen of the Genentech and Roche compound libraries [52]. Lead compound **Genentech 22** was found to have kinetic inhibition in the high nanomolar range, with 5-fold enhancement of IC$_{50}$ value for LDH-A vs. LDH-B (Table 1.2). Compounds in this series were tested for their ability to inhibit lactate production in HCC1954 human breast ductal carcinoma cells, but no inhibitory activity was observed for concentrations up to 50 µM. Intriguingly, biophysical assessments of Genentech’s compound series revealed that their binding affinity was greatly reduced (a 63-fold enlargement of $K_D$ value) in the absence of LDH-A’s cofactor NADH [52], suggesting the mode of inhibition of these compounds was not competitive, though further kinetic characterization was not reported [52].
Genentech’s 2-amino-5-aryl-pyrazine LDH-A inhibitors [53] were discovered in the same screen as the 2-thio-6-oxo-1,6-dihydropyrimide class compounds [52]. Through optimization efforts, Genentech 35 (Figure 1.9) was synthesized and explored in cell culture, a membrane permeability study, and a rat pharmacokinetic study. Genentech 35 was reported to have an *in vitro* IC$_{50}$ value of 500 nM against human LDH-A, and was shown to be 4-fold less potent against human LDH-B, 10-fold less potent against human LDH-C, and over 500-fold less potent against structurally-similar human enzymes malate dehydrogenases 1 and 2. In testing Genentech 35’s ability to inhibit cellular lactate production, 50 µM of Genentech 35 led to a 40% reduction in lactate production over 6 hours in MCF-7 human breast ductal carcinoma cells compared to vehicle treatment [53]. To approximate membrane permeabilities of Genentech 35, the Madin–Darby Canine Kidney (MDCK) system was used [64], which showed Genentech 35 was moderately cell-permeable, although aqueous solubility was only modest (81 µM). Rat pharmacokinetics demonstrated that Genentech 35, when dosed orally at 1.5 mg/kg or intravenously at 0.5 mg/kg, was 41% orally bioavailable and had a serum half-life of 2.8 hours [53].

In 2013, GlaxoSmithKline reported the discovery and biological assessment of the quinolone 3-sulfonamide class of LDH-A inhibitors, exemplified by compound GSK 1 (Figure 1.9) [54]. GSK 1 was reported to have an *in vitro* IC$_{50}$ value of 2.6 nM against human LDH-A, compared to 43 nM vs. human LDH-B. Several quinolone 3-sulfonamide class compounds were tested extensively in cell culture. GSK 1, tested at low micromolar concentrations, showed >80% inhibition of lactate production against a panel of cancer cell lines. Metabolic profiling studies using GSK 1 were performed in two different human liver hepatocellular carcinoma cell lines (Snu398 cells, which were demonstrated to have high LDH activity, and HepG2 cells, which were demonstrated to have low LDH activity).
Compared to vehicle treatment, 10 µM GSK 1 treatment was shown to significantly increase levels of NADH as well as several glycolytic intermediates (fructose-biphosphate/glucose-biphosphate and pyruvate), several citric acid cycle intermediates (alpha-ketoglutarate, fumarate, and malate), and several pentose phosphate pathway intermediates (ribose 5-phosphate and ribulose-5-phosphate) in only the Snu398 cells. GSK 1 treatment also increased the splicing of glycolytic enzyme pyruvate kinase to its embryonic M2 isoform [54], which is a less catalytically active species found in many cancer types thought to allow buildup of glycolytic intermediates for use in other cellular pathways [65-68]. While in vitro and cell culture studies demonstrated GSK to be a very potent and promising candidate LDH-A inhibitor, in vivo pharmacokinetic studies in rats demonstrated that it was rapidly cleared, with dosages as high as 100 mg/kg being undetectable in blood 2 hours after intravenous administration [54].

In 2014, Seth and coworkers reported further data on GSK 1 [37]. Treatment of A549 cells with 10 µM GSK 1 led to increased generation of ROS, increased OCR, and decreased ECAR. GSK 1 was also found to inhibit tumorsphere formation of A549 cells [37]. Due to unfavorable pharmacokinetic properties [54], GSK 1 could not be studied in vivo. However, in an ex vivo study, upon being incubated with “Warburg slices” (< 1 mm tissue slices from freshly-resected human NSCLC tumors) and 13C-labeled glucose, slices co-incubated with 10 µM GSK 1 demonstrated reduced 13C-labeled lactate production [37].

1.3.5: Summary and outlook on the development of LDH-A inhibitors

The development of LDH-A inhibitors as anticancer agents is a growing area of interest to both academia and the pharmaceutical industry. Success in discovering LDH-A inhibitors has been reported through screening and optimizing natural products (gossypol, FX-11, and galloflavin), structure-based design (the N-hydroxyindole series, to be discussed
in Chapter 2), fragment-based design (AZ 33 and ARIAD 9), and high-throughput screening (GSK 1 and Genentech 22 and Genentech 35). There are now six classes of inhibitors that show potent (low micromolar or better) in vitro inhibitory profiles against LDH-A and several which show moderate to potent cell culture inhibition of lactate production or potency in killing cancer cells in culture. The remaining challenge is to demonstrate potency and efficacy in vivo, in order to move LDH-A inhibitors into the clinic.

1.4: Glycoconjugation as an anticancer strategy

The strategy of exploiting cancers’ glucose avidity by conjugating a drug to glucose or another GLUT-1 substrate sugar has been reviewed recently [1]. This strategy is inspired by the widespread clinical use of 2-deoxy-2-(18F)fluoro-D-glucose (18F-FDG; Figure 1.10 a), a radiolabeled glucose analog. 18F-FDG has been used to visualize tumors and their metastases due to the tendency of these cancerous tissues to take up glucose at a higher rate than most normal tissues (as shown in Figure 1.10 b and c) [69, 70]. The synthesis and evaluation of glycoconjugated anticancer drugs was first reported in the literature in 1995, and this field has grown markedly in recent years, with the first-in-class conjugate glufosfamide in advanced clinical trials and many others in development.
Figure 1.10: Positron emission tomography (PET) imaging using the radiolabeled glucose analog $^{18}$F-FDG is a widely used clinical tool for the diagnosis and staging of many types of cancer. a) In a healthy patient, $^{18}$F-FDG will be taken up only by tissues that constitutively consume glucose, such as the brain and bladder (grey). In a cancer patient, tumor cells will preferentially uptake $^{18}$F-FDG, allowing clinicians to identify sites of tumors and metastases (red), as well as stage cancer and monitor response to treatment. b) $^{18}$F-FDG PET scan of a patient with metastatic Hodgkin’s lymphoma. c) $^{18}$F-FDG PET scan of a patient with metastatic breast cancer. (Figure 1b and 1c are reprinted by permission of the Society of Nuclear Medicine from: Ben-Haim S and Ell P. $^{18}$F-FDG PET and PET/CT in the Evaluation of Cancer Treatment Response. J Nucl Med. 2009; 50(1): 88-99. Figures 1 and 3[70].)

1.4.1: Precedent for cancer-targeting drug conjugates

Conjugation of anticancer agents to molecules that allow for preferential delivery to cancer cells is well-precedented, with several conjugates having clinical efficacy. One approach involves conjugating an established drug to an antibody targeting a specific tumor marker. Brentuximab vedotin (Adcertis; Figure 1.11 a) received US Food and Drug Administration (FDA) approval in August 2011, and European Union approval in October 2012, for the treatment of anaplastic large cell lymphoma and Hodgkin’s lymphoma. Brentuximab vedotin is thought to enter cancer cells via CD30 receptor-mediated endocytosis and subsequently be trafficked to endosomes, where a combination of degradative enzymes and increased acidity lead to cleavage to the active tubulin inhibitor
**Figure 1.11:** Antibody- and folate-conjugated anticancer agents.  

**a)** Brentuximab vedotin (Adcertis) – CD30-targeting monoclonal antibody brentuximab (red) attached to the tubulin polymerization inhibitor monomethyl auristatin E (blue) via a modified peptide linker;  

**b)** Trastuzumab emtansine (Kadcyla) – HER2-targeting monoclonal antibody trastuzumab (red) attached to the tubulin polymerization inhibitor mertansine (blue) via a linker;  

**c)** EC-145 – folate (red) coupled with a vinblastine-derived microtubule polymerization inhibitor (blue) via a modified peptide linker;  

**d)** EC-0225 – folate (red) conjugated to a vinblastine-derived microtubule polymerization inhibitor (blue) and the DNA alkylator mitomycin (green) via a modified peptide linker.
monomethyl auristatin E [71]. Another antibody-drug conjugate, trastuzumab emtansine (Kadcyla; Figure 1.11 b), received US FDA approval in February 2013 for the treatment of HER2-positive breast cancer following the EMILIA clinical trial [72]. While these conjugates have substantial clinical promise, there are significant logistical and economic challenges associated with the use of biologics as therapeutics.

A second, well-precedented approach to drug conjugation for improved cancer selectivity and uptake is folate conjugation. Cancer cells prefer de novo nucleotide synthesis over the salvage pathway that is often favored by normal tissues [73]. This preference of cancer cells means that they must take up large amounts of folate, a cofactor necessary for nucleotide biosynthesis. For this reason, folate receptor α (FR-α) is overexpressed in approximately 40% of human cancers (and expressed only by certain types of epithelial cells in healthy individuals) [74, 75]. This overexpression makes FR-α an excellent drug target. The most clinically-advanced folate-conjugated drug to date is EC-145 (Figure 1.11 c), which is currently in phase III trials (in combination with the established pharmaceutical Doxil) against ovarian cancer in the US. The peptide linker of EC-145 is expected to be cleaved by cellular cathepsins, releasing the cytotoxin intracellularly [76]. Another folate-conjugated drug in development is EC-0225 (Figure 1.11 d). Both the disulfide and peptide bonds are expected to be cleaved in intracellular endosomal compartments [77].

1.4.2: Prevalence of GLUT-1 upregulation in cancer

For glycoconjugation to be effective as a targeted anticancer strategy, glucose transporters must be overexpressed in cancer compared to normal tissues. Similar to FR-α overexpression in cancer, GLUT-1 has been demonstrated to be overexpressed in a large
percentage of cancers from various tissues of origin (Table 1.3, see also a comprehensive review by Medina [6]) [8, 78-86].

Table 1.3: GLUT-1 overexpression and relation to cancer prognosis in patient biopsy samples

<table>
<thead>
<tr>
<th>Tissue of origin</th>
<th>Fold-overexpression</th>
<th>Assessment method</th>
<th>Association with poor prognosis or survival?</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adrenal gland</td>
<td>Not quantified</td>
<td>Immunohistochemistry</td>
<td>Yes</td>
<td>[78]</td>
</tr>
<tr>
<td>Bladder</td>
<td>Not quantified</td>
<td>Immunohistochemistry</td>
<td>Yes</td>
<td>[79]</td>
</tr>
<tr>
<td>Breast</td>
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<td>RT-PCR</td>
<td>Not determined</td>
<td>[80]</td>
</tr>
<tr>
<td>Head and neck</td>
<td>2.1</td>
<td>RT-PCR</td>
<td>Yes</td>
<td>[81]</td>
</tr>
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<td>Hepatocellular</td>
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<td>RT-qPCR</td>
<td>Not determined</td>
<td>[82]</td>
</tr>
<tr>
<td>Oral</td>
<td>Not quantified</td>
<td>Immunohistochemistry</td>
<td>Yes</td>
<td>[8]</td>
</tr>
<tr>
<td>Ovarian</td>
<td>Not quantified</td>
<td>Immunohistochemistry</td>
<td>Yes</td>
<td>[83]</td>
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<td>Prostate</td>
<td>≤ 2</td>
<td>RT-qPCR</td>
<td>Yes</td>
<td>[84]</td>
</tr>
<tr>
<td>Rectal</td>
<td>Not quantified</td>
<td>Immunohistochemistry</td>
<td>Yes</td>
<td>[85]</td>
</tr>
<tr>
<td>Renal</td>
<td>≤ 10</td>
<td>RT-qPCR</td>
<td>Not determined</td>
<td>[86]</td>
</tr>
</tbody>
</table>
A second metric to determine which types of cancers would be viable candidates for glycoconjugate targeting is to examine which cancers are clinically staged using $^{18}$F-FDG PET imaging, summarized in a recent review by Bensinger and Christofk [87]. Currently, lung, breast, colorectal, and endometrial carcinomas, as well as bone and soft tissue sarcomas and Hodgkin’s and non-Hodgkin’s lymphomas, are commonly staged based on their ability to preferentially uptake this radiolabeled glucose analog compared to non-cancerous tissues.

1.4.3: Current progress in developing glycoconjugated anticancer agents

The most salient glucose- or glycoconjugated anticancer agents reported to date are summarized below.

1.4.3.1: Early efforts in evaluating sugar-conjugated drugs

Monosaccharide-conjugated analogs of diverse agents, designed to display improved water solubility, serum stability, and targeting as compared to their aglycones, have been reported in the chemical literature since the early 1990s. In 1991, several glycopeptide analogs of the anti-hypertensive peptide renin, including a mannose and an N-acetylglucosamine linked to renin via a C1 amide, were shown to have increased serum half-life compared to their aglycones [87]. The synthesis of glucose and mannose phosphotriester conjugates of the anti-retroviral nucleotide analog 3’-azido-3’-deoxythymidine (AZT) was also reported in 1991 [88]; these derivatives were shown to have comparable antiviral efficacy and enhanced targeting to the central nervous system (presumably via glucose transporters) than their aglycones [88, 89]. However, none of these conjugates were explicitly queried as anticancer agents.
1.4.3.2: Glufosfamide

**Figure 1.12:** Glufosfamide: 1-β-D-glucose-conjugated ifosfamide mustard

Glufosfamide (Figure 1.12), initially reported by Wiessler and colleagues in 1995 [90], was the first sugar conjugate to be explicitly designed and evaluated as a cancer-targeting cytotoxin. Glufosfamide was developed to decrease the toxicity and increase the cancer selectivity of its parent compound, the DNA alkylating agent ifosfamide. The potency of glufosfamide was comparable to that of its aglycone in cell culture. However, glufosfamide’s anticancer potency was markedly reduced upon co-treatment with 0.1 µM of the GLUT-1 transporter inhibitors phloretin and phlorizin, suggesting that the entry of glufosfamide into cells was at least partially GLUT receptor-mediated. Finally, glufosfamide matched the efficacy of its aglycone in increasing the length of survival of mice in two aggressive tumor models [90].

When glufosfamide and ifosfamide were administered intravenously in mice and rats, glufosfamide was found to have a 4.5-fold greater LD$_{50}$ value (was 4.5-fold less toxic) than ifosfamide in rats (in mg/kg), and a 2.3-fold greater LD$_{50}$ value than ifosfamide in mice (in mg/kg) [90].

A pharmacokinetic and whole-body distribution study of glufosfamide in rats, published by Wiessler and colleagues in 1996, revealed that the drug had a short plasma half-life (32 minutes) with largely renal excretion [91]. Glufosfamide is a prodrug, requiring cleavage of glucose via spontaneous hydrolysis or glycosidase activity [92] to liberate the
active drug. In this study, roughly 64% of total administered radiolabeled glufosfamide was excreted intact within 24 hours, while approximately 18% was excreted as either the liberated ifosfamide or one of two uncharacterized metabolites. When healthy rats were treated with radiolabeled glufosfamide, the drug was distributed among many of the tissues that rely on insulin-independent glucose transporters, including the liver, kidneys, thyroid gland, thymus, and central nervous system. Treatment with glufosfamide generally did not lead to toxicity in healthy tissues, including the liver, kidneys, and brain. Although a small degree of bone marrow toxicity was observed, it was less severe than the bone marrow toxicity induced by ifosfamide. Encouragingly, radiolabeled glufosfamide was shown to localize to the site of a tumor in rats with prostate cancer, with the labeled drug remaining at the site of the tumor for at least 24 hours [91], suggesting that it may be selectively taken up and retained by cancerous cells.

The first human clinical trial on glufosfamide in Europe was initiated in 1997 by Briasoulis and coworkers, with results reported in 2000 [93]. This trial examined 20 patients with solid tumors of various origins treated intravenously with glufosfamide in 0.9% NaCl solution for 6 hours, with patients receiving between two and eight courses of glufosfamide 3 weeks apart. Through this trial, the maximum tolerated dose (MTD) of glufosfamide in humans was established at 6000 mg/m$^2$, or approximately 190 mg/kg. This dose resulted in reversible renal tubular acidosis in 2/6 patients [93], presumably due to toxic ifosfamide metabolites generated during renal processing of the drug [94]. While Briasoulis and coworkers did not include ifosfamide in their study, ifosfamide’s MTD has been reported as 17,000 mg/m$^2$, or approximately 530 mg/kg, when co-administered over 5 days with the adjuvant mesna to counteract adverse renal effects [95]. The plasma concentration of glufosfamide in patients peaked around 420 µM around 10 hours after intravenous
administration of the 6000 mg/m² dose (compared to approximately 500 µM several hours after intravenous infusion of 1000 mg/kg ifosfamide in a separate study [96]), and the median plasma half-life of glufosfamide was 2.3 hours, with only 34% of the total drug administered being excreted via the kidneys [93]. Eight patients showed tumor progression, 10 showed stable disease, and 2 showed objective response to treatment, with one pancreatic cancer patient experiencing complete remission for over 4 years [93].

Human clinical trials of glufosfamide have continued over the past decade, often accompanied with hydration in an attempt to counteract adverse renal effects. Encouraged by the complete remission of one pancreatic cancer patient in the initial trial [93], and bolstered by the observation that pancreatic cancer biopsy samples from patients were found to overexpress GLUT-1 [97], several trials have evaluated the efficacy of glufosfamide against pancreatic tumors. Modest response was seen with glufosfamide alone in one phase II trial (2/35 patients with partial response, 11/35 with stable disease, 18/35 with disease progression) [98]. Combination of glufosfamide with the nucleoside analog gemcitabine – a standard-of-care chemotherapeutic in pancreatic cancer – yielded modest response in two trials on pancreatic adenocarcinoma patients [99, 100]. However, adverse effects remained problematic. In one glufosfamide and gemcitabine study, 4/29 patients treated with 4500 mg/m² (approximately 140 mg/kg) glufosfamide experiencing nonreversible renal failure, 29/29 patients experiencing anemia, 27/29 patients experiencing thrombocytopenia, and 26/29 patients experiencing neutropenia [99]. In a best supportive care phase III trial in terminal pancreatic cancer patients who had experienced disease progression in spite of gemcitabine treatment, treatment with 4500 mg/m² glufosfamide plus best supportive care (analgesics, antibiotics, and other agents with no anti-tumor effects)
led to an 18% increase in survival (a median of 105 days versus 84 days in the best supportive care only arm), which was not statistically significant [101].

European glufosfamide clinical trials in patients with glioblastoma multiforme [102] and non-small cell lung carcinoma [103] have shown glufosfamide to have very modest, if any, efficacy. In the glioblastoma study, researchers did not comment as to whether glufosfamide crossed the blood-brain barrier, although no adverse neurological effects in any of the 31 patients were observed following an intravenous infusion of 1000 mL of a 5000 mg/m² (approximately 156 mg/kg) glufosfamide solution over the course of 60 minutes [102]. In the absence of both glufosfamide efficacy and neurological effects in this study, it is plausible that the glufosfamide administered was not blood-brain barrier permeable.

The results of one Japanese phase I glufosfamide clinical trial in 13 patients with various solid tumors showed some promise (1/13 patients showed partial response, and 8/13 patients showed disease stabilization) [104]. In the United States, 8 clinical trials of glufosfamide in various disease models (pancreatic, ovarian, and lung cancers; glioblastoma multiforme; soft tissue sarcoma) have been registered, with 6 completed and 2 terminated.

While clinical evaluation of glufosfamide is ongoing, its ultimate success may depend on whether administration methods can be devised to allow for delivery of effective therapeutic doses of glufosfamide without dose-limiting renal or hematological toxicity. It seems plausible that the renal adverse effects may be at least partly due to toxic metabolites of ifosfamide generated during renal metabolism [90], but the anemia observed may stem from the fact that erythrocytes express high levels of GLUT-1 [105, 106].
Clinicians evaluating future glycoconjugated drugs should be cognizant of this potential hemolytic phenotype.

1.4.3.3: Glucose-conjugated paclitaxel and other glycoconjugated taxoids

In 2001, Mikuni, Mandai, and coworkers reported the synthesis and cancer cell potency of several glycoconjugates of docetaxel, including conjugates with glucose, galactose, mannose, and xylose. These compounds were shown to have a 3- to 18-fold improvement in activity compared to the aglycone against B16 murine melanoma cells in culture [107], but neither mechanistic nor in vivo data were not reported. A 2008 publication highlighted the in vivo potency of galactose-conjugated docetaxel (Figure 1.13 a) in a syngeneic P388 murine leukemia tumor model compared to docetaxel [108] but did not characterize the mode of entry or potential cleavage products of the conjugate. Further data validating the mechanism of cellular entry and in vivo pharmacokinetics of these glycoconjugates have not been reported.

![Figure 1.13: a) 1-α-D-galactose conjugated to position 12 of docetaxel via a short linker [107, 108]  b) 1-methylglucose conjugated to position 2’ of paclitaxel via a short linker [109, 110].](image-url)
In 2007 and 2008, Chen and coworkers reported the synthesis and cell culture evaluation of several glucose- and glucuronic acid-conjugates of paclitaxel [110] [109]. These key studies provided mechanistic insight into how future glucose-conjugated drugs might be assessed. These glycoconjugates, were designed to improve upon the poor water solubility of paclitaxel, as well as to target it to cancer versus normal cells. Figure 1.13 b depicts the most promising compound reported by Chen and coworkers – 2-D-glucose-conjugated paclitaxel – which is comprised of 1-methylglucose conjugated to paclitaxel at position 2’ using a short linker. Position 2’ of paclitaxel is crucial for the drug’s interaction with microtubules [111], so this compound is presumed to be a prodrug that requires activation in cells (in vitro assays to assess whether glycoconjugates were able to modulate tubulin polymerization were not reported). However, in vitro incubation for up to 24 hours of 2-D-glucose-conjugated paclitaxel with cell culture medium and fetal bovine serum, which contains endogenous β-glucuronidase, failed to show cleavage of the glucose conjugate to the parent aglycone.
Figure 1.14: Fluorescent microscopy images of NPC-TW01 human nasopharyngeal carcinoma cells treated with paclitaxel and 2-D-glucose-conjugated paclitaxel (Figure 1.13 b). Tubulin distribution is visualized above (a-c), and chromosome morphology below (d-f). Following 24 hour treatment with vehicle (a and d), tubulin was well-distributed throughout the cells and nuclear chromatin was diffuse, indicative of healthy cells. However, 24 hour treatment with either 1 µM paclitaxel (b and e) or 1 µM 2-D-glucose-conjugated paclitaxel (c and f) led to tubulin accumulation around the nucleus and nuclear chromatin condensation, suggestive of cells processing through apoptosis. (Reprinted with permission from Lin, et. al. J. Med. Chem. 51: 7428-7441. Figure 5a-c,g-i [109]. Copyright 2008 American Chemical Society.)

To determine whether this glycoconjugate was able to phenocopy paclitaxel’s method of inducing cell death, NPC-TW01 human nasopharyngeal carcinoma cells were treated with vehicle, paclitaxel, or 2-D-glucose-conjugated paclitaxel (Figure 1.13 b) for 24 hours and stained for tubulin distribution and chromosome morphology. While vehicle-treated cells showed diffuse tubulin distribution (Figure 1.14 a) and diffuse chromosomal distribution (Figure 1.14 d), the cells treated with both paclitaxel and glucose-conjugated
paclitaxel showed tubulin accumulation around cell nuclei and nuclear chromatin condensation after 24 hours, suggestive of apoptosis (Figure 1.14 b-c, e-f). However, in time course studies, cells treated with paclitaxel showed a more pronounced and rapid chromatin condensation than cells treated with glucose-conjugated paclitaxel [109].

![m-aminobenzoic acid-labeled 2-D-glucose-conjugated paclitaxel](image)

**Figure 1.15:** a) 2-D-glucose-conjugated paclitaxel appended at position 7 of paclitaxel with the chromophore *m*-aminobenzoic acid (blue) b) Merged confocal microscopy images showing the co-localization of *m*-aminobenzoic acid-labeled 2-D-glucose-conjugated paclitaxel (blue) with tubulin (red) in NPC-TW01 cells. (Figure 1.15 b is reprinted with permission from Lin, et. al. *J. Med. Chem.* 51: 7428-7441. Figure 3c. [109]. Copyright 2008 American Chemical Society.)

Potential colocalization of glucose-paclitaxel with tubulin in cells was further assessed by appending a fluorophore, *m*-aminobenzoic acid, to position 7 of paclitaxel on the glucose-ester conjugate (Figure 1.15 a). Unlike the established unfavorability of substitution at position C2' for microtubule interaction, substitution at position 7 is not known to interfere with paclitaxel's interaction with microtubules [111]. Thus, Chen and coworkers used this compound to study whether the glucose substitution at C2' would impede the compound from colocalizing with tubulin. Confocal microscopy studies examining tubulin...
distribution and compound fluorescence indicated that there was some convergence of tubulin and compound localization (Figure 1.15 b). Additionally, it was found that this colocalization could be inhibited by co-treatment with the GLUT inhibitor phloretin, suggesting that translocation into the cells is at least partially GLUT-mediated [109]. However, it was not determined whether glucose was cleaved prior to tubulin localization in cells.

Consistent with the microscopy study, it was found that the potency of several glycoconjugated paclitaxel compounds, assessed upon 72 hour treatment in seven immortalized cancer cell lines and two immortalized non-cancerous cell lines, was at least 5-fold less than paclitaxel in both cancer and normal cell lines. The conjugate depicted in Figure 1.13 b was the most potent of the all glycoconjugates tested [109, 110]. While no in vivo or further cell-based studies on these paclitaxel glycoconjugates have been reported to date, these initial studies highlight the need for cell-based mechanistic studies to validate future glycoconjugates before proceeding to in vivo studies.

1.4.3.4: Sugar-conjugated chlorambucil and other alkyators

Perhaps inspired by the success of the glucose-conjugated DNA alkylator glufosfamide, several groups have reported the synthesis and preliminary evaluation of glycoconjugated analogs of other DNA alkylators, including chlorambucil (a commonly used agent for the treatment of chronic lymphocytic leukemia) and busulfan (a commonly used agent for the treatment of chronic myeloid leukemia). In 1996, Scherman and coworkers reported the synthesis of a series of glucose conjugates linked directly to chlorambucil via a C6 ester or amide bond. All compounds were able to competitively inhibit the uptake of
radiolabeled glucose by GLUT-1 receptors in human erythrocytes in a dose-dependent manner. One ester-linked compound in the series, 6-D-glucose-conjugated chlorambucil (Figure 1.16 a), was found to be 150-fold more active in inhibiting the entry of radiolabeled glucose than the native substrate, D-glucose [112].

In a related 1997 study, seeking to make glucose-based analogs of the DNA alkylator busulfan, Scherman and coworkers synthesized and assessed a series of mono- and dimesylated glucose conjugates, with methanesulfonyl groups appended via a sulfonate to glucose at either O3, O4, O6, or at two of the three sites. Several conjugates had comparable potency to glucose in inhibiting the cellular uptake of radiolabeled glucose.
in human erythrocytes, suggesting they were substrates for GLUT-1. 6-D-glucose-conjugated methanesulfonate (Figure 1.16 b) was only 3-fold less efficient at inhibiting radiolabeled glucose uptake than D-glucose [113], marking it as a candidate for future study.

In 2008, Weber and coworkers reported the synthesis and preliminary biological assessment of fluorodeoxyglucose-conjugated derivatives of chlorambucil [114], with preliminary in vivo results reported in 2011 [115]. Intriguingly, among the most promising fluorodeoxyglucose conjugates evaluated was a peracetylated glycoconjugate: peracetylated 2-fluorodeoxyglucose conjugated to chlorambucil via a C1 urea linkage; Figure 1.16 c). This glycoconjugate proved to be more cytotoxic than the analog lacking the acyl groups and also showed enhanced cytotoxicity compared to unconjugated chlorambucil in a panel of normal and human cell lines, with up to a 25-fold enhancement in cytotoxicity compared to chlorambucil in human fibroblasts and MCF-7 human breast ductal carcinoma cells. In addition, peracetylated 2-fluorodeoxyglucose-conjugated chlorambucil was shown to demonstrate intracellular ROS production in L929 murine cells at 25 µM [114], suggesting that the compound damages DNA.

In mice, peracetylated 2-fluorodeoxyglucose-conjugated chlorambucil was shown to have a 6-fold increase in MTD on a mg/kg scale, or a 3-fold increase on a molar scale, compared to chlorambucil (90 mg/kg vs. 15 mg/kg). This glycoconjugate was also shown to have efficacy in two different syngeneic mouse models of solid tumors at the 90 mg/kg dosage, while the parent compound chlorambucil was only modestly effective in one trial and ineffective in the second [115].
While peracetylted 2-fluorodeoxyglucose-conjugated chlorambucil was designed to have tumor-selective targeting, no experiments were reported indicating whether it is taken up through glucose transporters, cleaved intracellularly to liberate chlorambucil, or able to alkylate DNA in uncleaved form.

In a 2010 report, Goff and Thorson created a 63-member library of neoglycoside conjugates of chlorambucil, using a variety of amino-substituted monosaccharides common (glucose, mannose) and uncommon (galactose, xylose, gulose, lyxose) in mammalian metabolism. All library compounds were assessed for their ability to induce a decrease in growth proliferation in a panel of immortalized human cancer cell lines. The most promising compound in this assay was D-threoside conjugated to chlorambucil via a methoxyamine linker (Figure 1.16 d), which reduced cell growth 8-fold compared to chlorambucil [116]. Neither the method of cellular uptake of these chlorambucil neoglycoside conjugates, nor the assessment of cleavage to the free alkylator or alkylating activity of the conjugate, was explored in this study.
**Figure 1.17:** a) 8-[O\(^6\)-(4-bromothenyl)-guan-9-yl]-octyl-1-\(\beta\)-D-glucoside – a glucose-conjugated MGMT inhibitor; b) Azomycin with a 2-fluoropropyl linker conjugated via a C6 ether linkage to D-glucose; c) Clioquinol conjugated to \(\beta\)-D-glucose via a C1 ether linkage; d) 8-quinoline conjugated to \(\beta\)-D-glucose via a C1 ether linkage; e) D-lyxose conjugated warfarin; f) D-threo-side-conjugated cyclopamine; g) 2-amino-2-deoxyglucose-conjugated doxorubicin; h) juglone conjugated to 1-\(\alpha\)-methylglucose via a C6 ether linkage.

Wiessler and coworkers published two reports in 2001 [117, 118] on glucose conjugates of the benzylguanine class of inhibitors against the human DNA repair protein.
O\textsuperscript{6}-methylguanine-DNA methyltransferase (MGMT), an enzyme that is involved in DNA repair upon damage induced by alkylating chemotherapeutics. β-D-glucose was linked via a C1 ether linkage to an alkyl chain of varying length attached to benzylguanine, and the glucose-conjugated complexes were assessed for their ability to inhibit MGMT activity \textit{in vitro} and in cells. Most glucose conjugates had poorer inhibition compared to their aglycones, but 8-[O\textsuperscript{6}-(4-bromothenyl)-guan-9-yl]-octyl-1-β-D-glucoside (Figure 1.17 a) was shown to require only 2- to 4-fold higher concentration than its aglycone, O\textsuperscript{6}-(4-bromothenyl)-guanine, to attain the same inhibitory potential both \textit{in vitro} and in cells. Furthermore, this glycoconjugate was shown to have enhanced growth inhibitory potential compared to its aglycone, although it was not cytotoxic at the concentrations required to ablate MGMT activity. Another glucose conjugate of this class was shown to be stable in cell culture medium and pH 7.4 buffer for several days \cite{118}, although stability in serum or cell lysate was not reported. Experiments querying whether this glycoconjugate entered cells via glucose transporters were not reported.

The synthesis and preliminary biological evaluation of a series of glucose-azomycin adducts were recently reported in 2012 by Kumar and coworkers \cite{119}. 2-nitroimidazole compounds have been studied in clinical trials as radiosensitizers. However, these trials failed because the compounds were found to have dose-limiting toxicities at concentrations lower than their therapeutic doses \cite{120}. Kumar and coworkers attempted to overcome this toxicity via glucose-conjugates designed to have improved cancer cell selectivity. Compounds in this series were reported to have low millimolar cytotoxicity in a panel of immortalized murine and human cancer cell lines and to be modestly effective radiosensitizers. To assess whether the uptake of these glucose conjugates was partially GLUT-mediated, glycoconjugates were tested at concentrations ranging from 100 µM to 10
mM for the ability to compete with $^{14}$C glucose uptake in *Xenopus* oocytes, which express GLUT-1. The compound depicted in **Figure 1.17 b** is a diastereomeric mixture of α and β glucose conjugated at the 6 position azomycin via a 3-carbon linker containing a fluorine diagnostic arm. This compound was shown to be the strongest competitor for glucose uptake [119], suggesting that its cellular transport is at least partially GLUT-1 mediated. *In vivo* studies using glucoconjugated azomycins were not reported.

Vecchio, Viale, and coworkers reported in 2012 the synthesis and cell culture evaluation of glucose conjugates of a class of metal-binding compounds, the 8-hydroxyquinolines [121]. The 8-hydroxyquinolones, most notably clioquinol (5-chloro-7-iodo-8-hydroxyquinoline), have shown promise in early stage clinical trials as anticancer agents due to their scavenging of copper(II), a necessary cofactor for tumor growth [122]. 1-O-glucose conjugates of both clioquinol (GluCQ) (**Figure 1.17 c**) and another 8-hydroxyquinoline, 8-quinoline (GluOHQ) (**Figure 1.17 d**), were synthesized to improve targeting of these compounds to tumor cells. The 8-hydroxyquinoline glucose conjugates are prodrugs, unable to efficiently complex with metal ions; intracellular cleavage to free the hydroxyquinoline moiety is necessary for activity. Both glucose conjugates were shown to be substantially cleaved by an almond β-glucosidase within a one hour incubation period; however, cleavage was not assessed in mammalian cells. When the antiproliferative ability of the glucose conjugates and their parent aglycones was assessed, the glucose conjugates were found to be less potent than the aglycones [121], perhaps due to insufficient cleavage of prodrugs to their aglycones in cells.

Thorson and coworkers reported in 2011 the synthesis of a library of 38 glycosylated conjugates of the anticoagulant drug warfarin [123]. As in Thorson’s earlier exploration of neoglycoside-conjugated chlorambucil [116], sugars selected for conjugation ranged from
native mammalian metabolites such as glucose to unnatural sugars such as lyxose and xylose. While most sugar conjugates lost their ability to prevent blood coagulation, some did show cytotoxicity in cancer cells, especially the amino-substituted D- and L-xylosides and lyxosides (D-lyxose-conjugated warfarin, which showed the highest anticancer potency, is depicted in Figure 1.17 e) [123]. In a 2012 report, Goff and Thorson also prepared and evaluated neoglycoside conjugates of the alkaloid cyclopamine, a known teratogen in many species. All neoglycoside conjugates synthesized were shown to have increased growth proliferation inhibition compared to their aglycone in NCI-H460 lung cancer cells, with D-threoside-conjugated cyclopamine (Figure 1.17 f) among the most potent of the set [124]. However, none of the glycoconjugated cyclopamine analogs were assessed for their mutagenic potential or ability to enter cells via glucose transporters.

A 2013 report by Gu and coworkers presented the synthesis and cell culture and in vivo evaluation of a glucosamine conjugate of the topoisomerase II inhibitor doxorubicin (Adriamycin), linked using a succinic acid spacer (Figure 1.17 g) [125]. This doxorubicin glycoconjugate was designed to enhance the selectivity of doxorubicin to cancer cells, and thus to counteract the dose-limiting toxicity of doxorubicin treatment on healthy tissues (notably the heart, liver, and kidneys) [125]. In cell culture, glucosamine-conjugated doxorubicin was found to have similar potency to its aglycone doxorubicin against immortalized cancer cell lines. However, while doxorubicin showed substantial toxicity to human embryonic lung fibroblast (HELF) cells at low nanomolar concentrations after 48 hours, glucosamine-conjugated doxorubicin was not toxic to HELF cells at up to 50 nM for 48 hours [125], though the toxicity of higher concentrations of the glycoconjugate were not tested. The intrinsic fluorescence of doxorubicin was used to monitor the uptake of glucosamine-conjugated doxorubicin in cells by confocal microscopy. Using this technique,
the entry of the glycoconjugate into HepG2 human liver carcinoma cells was shown to be substantially blocked by pretreatment of cells with high concentrations (25 or 50 mM) of the GLUT substrate 2-deoxyglucose [125], suggesting that the glycoconjugate’s cellular uptake was at least partially GLUT-mediated. A xenograft study examining the efficacy of doxorubicin (6 mg/kg) versus glucosamine-conjugated doxorubicin (6 mg/kg) versus saline control in nude mice bearing SKOV3 human ovarian carcinoma tumors demonstrated that both doxorubicin and its glucosamine conjugate had similar efficacy in reducing tumor volume. Post-study histological examination of heart and kidney tissues from mice in all three treatment groups indicated that doxorubicin-treated mice showed heart and kidney damage, while the heart and kidney sections of the glucosamine-conjugated doxorubicin treated mice did not show overt damage and resembled the saline control [125], suggesting that the glycoconjugate may display enhanced tumor targeting or less toxicity to normal tissues. One drawback to this study is that while Gu and coworkers postulated that 2-amino-2-deoxyglucose-conjugated doxorubicin was a prodrug [125], data querying the cleavage of the glycoconjugate in cells, in liver microsomes, or in vivo (pharmacokinetics) was not reported. Additionally, no data were reported on whether the glycoconjugate retained doxorubicin’s ability to inhibit topoisomerase II in vitro or in cells.

In 2014, Anufriev and coworkers reported the synthesis and biological evaluation of a series of 1-methylglucose conjugates of naphtalenes, as exemplified by 1-methylglucose-conjugated juglone (Figure 1.17 h) [126]. These methylglucose conjugates were found to have single micromolar potencies against HeLa cells in culture and were 10- to 30-fold more potent than the aglycone juglone. Compound 1-methylglucose-conjugated juglone was found to inhibit HeLa cell colony formation on soft agar with an IC_{50} value of 1.5 µM. Further study revealed the mode of death induced by these methylglucose conjugates to be
mixed apoptosis and necrosis. However, these glycoconjugates were found to possess considerable toxicity toward non-cancerous JB6 P+ Cl41 murine epidermal cells in culture (48 hr IC_{50} value of 4.9 µM for 1-methylglucose-conjugated juglone) [126], thus limiting their potential for further study.

Several other glucose or other monosaccharide-conjugated anticancer drugs have been reported but are still pending mechanistic evaluation. These include glucose, lactose, and galactose conjugates of the heat shock protein 90 inhibitor geldanamycin [127], glucose conjugates of the natural product cadalene [128], glycoconjugates of the DNA alkylator duocarmycin SA [129, 130], glucose conjugates of the lipooxygenase inhibitor nordihydroguaiaretic acid [131], glucose conjugates of naphthazarin derivatives [132], and glucose-conjugated photocytotoxic iron(III) complexes [133].

1.4.4: Requirements for transport of sugars by GLUT-1

Some information is known regarding the ability and affinity of the GLUT-1 transporter to transport substituted glucose analogs and non-glucose sugars.

1.4.4.1: Glucose substitutions and transport

In order to selectively target cancer cells, glycoconjugates should be substrates for the insulin-independent glucose transporter GLUT-1, which is overexpressed in a wide variety of solid tumors [7, 83, 134-137]. GLUT-1 is a passive, bidirectional glucose uniporter comprised of 12 transmembrane helical domains, 8 of which are assembled in a circular fashion from an exofacial (extracellular top-down) view.
Figure 1.18: Structure-activity relationship of D-glucose as a substrate for the GLUT-1 transporter. Substitutions at C1, C2, and C6 have been most explored to date, demonstrating that some substitutions can be made with the resulting conjugate retaining affinity for GLUT-1. Substitutions at C3 and C4 require further study; to date, no C3- or C4-glucose-conjugated anticancer compounds have been reported.

The ability of substituted glucose analogues to be substrates for GLUT-1 has been investigated; a summary of findings is presented in Figure 1.18. Kinetic and computational modeling studies using glucose analogs suggest that the hydroxyl groups at positions 1 and 3, and the pyran oxygen 5 in the pyranose (closed) form, are involved in stabilizing hydrogen bonding interactions with amino acid residues within the transporter [138, 139]. Loss of hydrogen bond acceptors at these positions makes glucose analogs poor substrates for GLUT-1: both 1-deoxy-D-glucose and 3-deoxy-D-glucose, which have altered ring conformations, have been determined to have $K_i$ values roughly 10-fold higher than D-glucose in competition assays in GLUT-1 transporters from human erythrocytes [138]. However, substitution of fluorine for the hydroxyl groups at position 1 (in the β
conformation, in which the fluorine is in the equatorial position in the chair form) or position 3 yields $K_i$ values comparable to glucose, which is not surprising given that fluorine has similar dipole properties to oxygen and is unlikely to change glucose’s preferred ring conformation. Interestingly, substitution of fluorine at position 1 in the $\alpha$ conformation results in a poor GLUT-1 substrate [138], suggesting that the $\beta$ conformation is preferred for GLUT-1 transport. However, 2-D-glucose-conjugated paclitaxel (Figure 1.13 b) contains glucose in the $\alpha$ conformation, and this compound was demonstrated to be partially inhibited from entering cells upon treatment with GLUT-1 transporter inhibitor phloretin [109]. Substitutions of C1 oxygen for C1 nitrogen have also been reported in FDG analogs; these analogs have been shown to compete with D-glucose for cellular entry, suggesting their uptake is GLUT-mediated [140].

Compounds with slightly larger substituents at C3, including 3-O-allyl-D-glucose and 3-O-(2',3'-epoxypropyl)-D-glucose, appear to be substrates for GLUT-1 [138], suggesting that this position may tolerate substitution well. In glucose's most thermodynamically stable conformation, the pyranose (closed chair) form, C5 does not contain a hydroxyl substituent, and thus any glycoconjugate candidates substituted at C5 may not assume the chair configuration that is recognized by GLUT receptors.

Thus, these data suggest that for glucose conjugates to remain substrates for GLUT-1, compounds with hydrogen bond acceptors such as nitrogen or oxygen must be retained proximal to carbons 1 and 3, and substitutions at the 1 position may retain higher affinity for GLUT-1 if they are present in an equatorial conformation.

A large fraction of known glucose conjugates discussed previously contain glucose conjugated to the anticancer agent at position 1, with the C1 oxygen intact and locked into
equatorial (β) position; these include glufosfamide (Figure 1.12), peracetylated 2-fluorodeoxyglucose-conjugated chlorambucil (Figure 1.16 c), and glucose-conjugated cliquiniol and quinolinyl (Figure 1.17 c and d). Of these, experiments were performed to indicate that glufosfamide’s cellular transport is at least partially GLUT-mediated, but no such experiments were published regarding the other two classes. No anticancer glycoconjugates that are linked to glucose at the C3 or C5 positions have been reported to date.

The hydroxyl groups of glucose at C2, C4, and C6 are not implicated in hydrogen bonding interactions with the GLUT-1 transporter, and thus addition of bulk to these positions may be tolerated. As previously discussed, 2-18F-fluoro-2-deoxyglucose (18F-FDG) is a widely established clinical tool that is a substrate for GLUT-1 and GLUT-3 transporters [141], and 2-NBDG, in which the C2 hydroxyl is replaced by nitrogen and a bulky aromatic system, is a widely used fluorescent glucose bioprobe which has been shown to be taken into cells via glucose transporters [142, 143]. Of the anticancer glucose conjugates discussed above, glucose-conjugated paclitaxel (Figure 1.13 b) and glucosamine-conjugated doxorubicin (Figure 1.17 g) contain a C2 linkage and have been experimentally shown to enter cells via a GLUT-mediated mechanism [109, 125].

Some substituents at C4 of glucose, such as 4-O-propyl-D-glucose and cellobiose (the disaccharide consisting of 2 glucose molecules connected via a β 1→4 ether linkage), have been shown to have minimal difference compared to D-glucose on $K_i$ in competition; however, lactose, in which glucose is conjugated via C4 to galactose, is not a substrate for GLUT-1 [138]. No anticancer glucose conjugates containing linkages at C4 have been reported to date.
Substituents at C6 of glucose have been reported to be comparable or slightly weaker substrates for GLUT-1 than unsubstituted glucose. 6-deoxy-D-glucose retains the same $K_i$ value as D-glucose, while 6-deoxy-6-fluoro-D-glucose has five times higher inhibitory potential in competition studies than D-glucose [138]. Several reported anticancer glucose conjugates linked at C6 have been reported to date, including glucose-conjugated chlorambucil (Figure 1.16 a), glucose-conjugated methanesulfonate (Figure 1.16 b), and glucose-conjugated azomycin (Figure 1.17 b), all three of which have been implicated as GLUT substrates through experimental evidence.

Importantly, although glucose-conjugated anticancer drugs have been known in the literature for nearly 20 years, there has been no systematic study on the position of substitution for any given anticancer glycoconjugate.

1.4.4.2: GLUT-1 transport studies on non-glucose sugars

As discussed above, currently explored glycoconjugates that have been demonstrated to be transported through the GLUT-1 receptor are limited to substituted glucose conjugates. Neoglycosides – amino-modified sugars – as well as peracetylated glucose conjugates, have been postulated to be taken up via GLUTs [116], but mechanistic studies have yet to be reported. However, it is known that other sugars besides D-glucose can be substrates for GLUTs, and thus can be considered candidates for a GLUT-targeting approach. Specifically, using light diffraction measurement in human erythrocytes that express GLUT-1 as their sole glucose transporter, 2-deoxy-D-glucose, D-mannose, D-galactose, D-mannose, D-xylose, 2-deoxy-D-galactose, L-arabinose, D-ribose, D-fucose, and D-lyxose, in order of decreasing affinity, were all found to be transported into cells in a transporter-mediated fashion. In contrast, D-arabinose, L-fucose, and L-rhamnose were
poor substrates, and the enantiomers of several GLUT substrates, L-glucose and L-xylose, were not GLUT substrates [144]. Thus, it seems that many sugars can be conjugated to anticancer agents to take advantage of GLUT-mediated cellular entry; the choice of which sugar to utilize may depend on the desired cleavage mechanism of the compound. For instance, D-galactose, the C4 epimer of glucose, has been reported to possess an equivalent affinity and uptake rate by GLUT-1 compared to glucose [145]. Galactose-conjugated drugs may be used to selectively target certain types of cancers known to highly express galactosidase enzymes, such as breast and colon cancers [146]. In the absence of substantial tumor galactosidase expression, galactose-conjugated prodrugs can be used in conjunction with tumor-selective monoclonal antibodies linked to galactosidases, which ideally cleave the inactive conjugate to its active form in tumor tissue [145].

1.4.4.3: Validation of glycoconjugates in vitro and in vivo

The glycoconjugates that have been most thoroughly mechanistically evaluated have been highlighted above, to serve as a model for medicinal chemists and chemical biologists in their development of glycoconjugated drugs. Glycoconjugation is an exciting cancer targeting strategy, but caution must be taken before claiming that a glycoconjugate is cancer-selective and cancer-targeted. The following represent important questions that should be examined in the development of glycoconjugates, as well as guidelines for answering these questions based on previous work. Ideally, all assays should compare both the glycoconjugate and its aglycone in parallel.

1.4.4.3.1: Validation of GLUT receptor-mediated entry

A crucial mechanistic question in the development of glycoconjugates is whether their cellular entry is at least partially GLUT-mediated. There are many ways to address
this question, and ideally any conjugate would be evaluated with multiple methods. To examine entry via GLUT-1, the transporter most commonly upregulated in cancer, human erythrocytes are a common model system since GLUT-1 is the only glucose transporter expressed in this cell type. Historically, competition with radiolabeled glucose [138] and changes in erythrocyte volume [144, 147] have been used in this model system.

A second approach is to compare the entry or efficacy of the glycoconjugate in the presence of a GLUT inhibitor, either in erythrocytes or in a cultured cell line. A number of natural products, including the botanical phloretin (a GLUT-1 inhibitor [105]) and the fungally-produced cytochalasin B (a pan-GLUT inhibitor [148, 149]), have been demonstrated to inhibit GLUTs in cell culture. More recently, GLUT inhibitors have been discovered through screening and design studies, such as the GLUT-1 inhibitors fasentin [150] and WZB117 [151]. Assays have been developed to determine whether glycoconjugate cellular entry and activity are lost when cells are co-treated with these inhibitors [109]. The general caveat to using GLUT inhibitors is that it may be difficult to meter the extent of the inhibitor’s interference with GLUT versus its off-target, potentially cytotoxic effects. Cytochalasin B is known to be relatively toxic with several reported targets, including actin [152]. However, newly-developed inhibitors may be more selective.

A third method to probe glycoconjugates’ mode of entry is to stably knock down or knock out GLUT expression. Both of these approaches were employed in a recent study by Anderson and colleagues [153]. In examining the entry of ³H-2-deoxyglucose into cells, it was reported that shRNA-mediated GLUT-1 knockdown in a murine cell line which expressed GLUT-1 as its predominant GLUT, led to only a 25% reduction in ³H-2-deoxyglucose entry compared to control shRNA cells [153], presumably due to an increase in expression of other GLUTs to allow knockdown cells to survive in culture. Anderson and
colleagues also generated a GLUT-1 knockout cell line from a GLUT-1 knockout mouse; these cells showed close to a 50% reduction in entry of $^3$H-2-deoxyglucose compared to control cells [153]. If a GLUT-1 knockout cell line becomes widely available, evaluation of glycoconjugates’ entry, efficacy, and potency in such a cell line may become a valuable metric. Incidentally, humans born with a mutated, functionally-impaired GLUT-1 transporter experience a severe phenotype known as De Vivo disease which results in mental retardation, seizures, and a host of neurological deficits, underscoring the importance of GLUT-1 in supplying glucose to the nervous system [154]. To date, no live births of humans with a completely nonfunctional GLUT-1 transporter have been reported, so efforts to generate a human GLUT-1 knockout cell line must be relegated to the research laboratory.

![2-NBDG](image)

**Figure 1.19**: Structure of the fluorescent glucose bioprobe 2-((N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG).

A fourth approach is the use of fluorescently-labeled glucose bioprobes, as reviewed recently [155]. The fluorescent glucose analog 2-NBDG [142, 143] (Figure 1.19) is perhaps the most well-known member of this class, but a growing interest in this area in recent years has led to the development of many new fluorescent probes which offer increased emission, slower photobleaching, and increased depth penetration to allow for imaging *in vivo* [155-157]. Competition assays can be conducted between glycoconjugates of anticancer drugs and these fluorescent probes, to determine whether increasing concentrations of the drug
glycoconjugates cause decreasing fluorescence in cells, using flow cytometry or fluorescent microscopy as a readout.

1.4.4.3.2: Determination of efficacy in uncleaved vs. cleaved form

Evaluation of a glycoconjugate should begin by discerning whether or not the glycoconjugate retains the activity of its aglycone, using appropriate kinetic evaluation for an enzyme, SPR, or isothermal titration calorimetry (ITC) for binding assessment, or another comparable technique depending on the aglycone’s target. If the glycoconjugate is no longer active against the target and instead is a prodrug, its cleavage to its aglycone in cells or in vivo needs to be examined. For instance, chromatographic techniques can be used to detect the aglycone in cell lysate following treatment with the glycoconjugate, and serum stability or liver microsome analyses may be useful for discerning whether cleavage is likely to occur in vivo. If the glycoconjugate does not appear to be cleaved and no longer retains activity against its target, then the identity of its new target or targets should be examined using various biochemical and molecular biological approaches. Alternately, derivatives that are more likely to be cleaved in cells or in vivo can be synthesized and assessed.

1.4.4.3.3: Considerations for in vivo testing

In vivo testing is a crucial proving ground for glycoconjugates. The extensive in vivo studies of glufosfamide in mice and rats should serve as a template for the testing of future drug conjugates. Particularly, it is important to establish whether the glycoconjugate is serum-stable and to discern its primary modes of metabolism and excretion. It is also critical to determine if the glycoconjugate is preferentially targeted to tumor tissue in animal models, to validate the hypothesis of glucose enabling preferential drug targeted to cancerous tissues. This might be accomplished by isotope labeling of the drug prior to
administration, and whole-animal radiography following administration, as in the glufosfamide evaluation [91]. Appending a fluorophore to the glycoconjugate, such as a near-infrared-emitting fluorophore to provide capability for \textit{in vivo} imaging [158], may be useful, but only if this new construct is demonstrated to still be taken up by GLUTs using the molecular biology methods discussed above.

It is important to assess whether a glycoconjugate has off-target hematological or neurological adverse events, given that GLUT-1 is normally expressed in erythrocytes and in the endothelial cells of the blood-brain barrier. Human, but not mouse or rat, studies of glufosfamide demonstrated anemia due to hemolysis as a common adverse event [99]; for future glycoconjugates, hemolysis should be queried using \textit{in vitro} assays before the initiation of studies in humans or other large mammals. Encouragingly, neurotoxicity has not been observed in the \textit{in vivo} evaluation of any glycoconjugate to date; still, all future glycoconjugates should be evaluated for potential neurological adverse events.

Another potential off-target effect of glycoconjugated drugs is their uptake by healthy tissues expressing GLUT receptors other than GLUT-1. There are at least 12 classes of GLUTs (both insulin-dependent and –independent) that transport glucose or hexoses alone, as well as several in which the transport of glucose is coupled with sodium, as detailed by Medina [6]. While it is conceivable that these transporters expressed in normal tissues may also take up a glycoconjugate, the strongest evidence favoring the preferential targeting of a glycoconjugate to a tumor is the large body of work on \textsuperscript{18}F-FDG preferentially being localized to tumors, as evidenced in Figure 1.10. Most glycoconjugated drugs discussed herein not been subjected to imaging studies to precisely localize the drug’s distribution in a whole organism or whole tumor-bearing organism. Conducting such an experiment, such as the rat imaging study reported by Wiessler with glufosfamide [91], would be useful,
though caution should be taken in labeling the compound in such a way to not alter its ability to be taken up by GLUTs.

1.4.5: *Outlook on the field of glycoconjugated anticancer agents*

Glycoconjugation generally offers improved water solubility and stability and, if the glycoside of choice is a GLUT substrate, the potential for selective targeting to cancer cells. Substantial strides in the field of glycoconjugation have been made, reaching as far as late stage human clinical trials. This field has a great deal of potential and tremendous opportunity for growth, but rigorous mechanistic testing at each stage of the drug development process, and carefully controlled, head-to-head experiments are required to determine the true utility of this strategy.
1.5: References


Chapter 2: Biological validation of the N-hydroxyindole (NHI) class of LDH-A inhibitors

Sections of Chapter 2 have been adapted from two published manuscripts:


All experimental work described in this chapter was performed by E. Calvaresi, unless otherwise specified. The synthesis and kinetic evaluation of all NHI compounds was performed by Dr. Carlotta Granchi and coworkers in the laboratory of Professor Filippo Minutolo at the University of Pisa. All computational modeling was performed by Dr. Tiziano Tuccinardi at the University of Pisa. Dr. Rahul Palchaudhuri, a former graduate student in the Hergenrother laboratory, was instrumental in the design of the $^{13}$C NMR-based assay for cellular lactate detection used to evaluate the first generation NHI compounds [1].

Normoxia and hypoxia experiments on the first generation NHI compounds [1] was performed by Leticia León, Elisa Giovannetti, and Godefridus Peters at the VU University Medical Center in the Netherlands.
2.1: Discovery, in vitro characterization, and cellular evaluation of the NHI class of LDH-A inhibitors

The N-hydroxyindole (NHI) class of LDH-A inhibitors was discovered using a structure-based design approach by Dr. Carlotti Granchi and colleagues in Professor Filippo Minutolo’s laboratory at the University of Pisa. Using the observation that previous classes of small molecules that inhibited LDH-A in vitro contained a hydroxyl group and an adjacent carboxylic acid or aldehyde group on an aromatic scaffold (such as gossypol and FX-11), the NHI was chosen as a query scaffold. The rationale for choosing to pursue NHIs substituted with a carboxylic acid at the 2 position was facility of synthesis (following the methods outlined by Nicolaou and coworkers in the total synthesis of the NHI-containing antibacterial agent nocathiacin I [3, 4]) and improved stability over NHIs with carboxylic acid substitutions at other positions.

Following the synthesis of the first generation of substituted NHIs, simple kinetic assays were performed by Dr. Carlotta Granchi and coworkers in the Minutolo research laboratory to evaluate their ability to inhibit LDH-A in vitro. The structures of these first generation compounds are shown in Figure 2.1. Compounds were screened at 125 µM against human LDH-A in the presence of 2 mM pyruvate and 25 µM NADH, and compounds that decreased the oxidation of NADH to NAD\(^+\) by at least 50% (measured spectroscopically by assessing the disappearance of NADH’s absorption at 340 nm) were selected for more rigorous study. Compounds 1h, 1i, and 1j were found to possess greater than 80% enzyme inhibition in the initial screen, and these compounds were selected for further study [1].
First generation NHIs

Figure 2.1: The structures of the first generation of N-hydroxyindole class compounds [1]

Compounds 1h, 1i, and 1j were then assayed in NADH- and pyruvate-limiting conditions to determine their $K_i$ values for LDH-A inhibition in vitro. All three compounds were found to be competitive inhibitors of the enzyme versus both pyruvate and NADH, with 1j being most potent. To assess the specificity of these NHIs for LDH-A vs. LDH-B, each compound was evaluated against LDH-B and found to have minimal, if any, inhibition of LDH-B (Table 2.1). [1]

Table 2.1: *In vitro* kinetic inhibition of first generation NHIs against LDH-A and LDH-B [1]

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_i$ vs. pyruvate in LDH-A (µM ± StDev, n=3)</th>
<th>$K_i$ vs. NADH in LDH-A (µM ± StDev, n=3)</th>
<th>% inhibition of LDH-B at 125 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1h</td>
<td>15.7 ± 1.5</td>
<td>10.4 ± 1.5</td>
<td>&lt; 3%</td>
</tr>
<tr>
<td>1i</td>
<td>35.4 ± 3.4</td>
<td>19.8 ± 2.2</td>
<td>&lt; 3%</td>
</tr>
<tr>
<td>1j</td>
<td>4.7 ± 0.5</td>
<td>8.9 ± 1.3</td>
<td>11% ± 3%</td>
</tr>
</tbody>
</table>

(Table 2.1 is adapted with permission from Granchi, et. al. *J. Med. Chem.* 54: 1599-1612. Table 1 [1]. Copyright 2011 American Chemical Society.)
Computational analysis of 1j was undertaken by Dr. Tiziano Tuccinardi at the University of Pisa to model the potential interactions of 1j with residues in the active site of LDH-A. The GOLD program was used to dock 1j into LDH-A (Protein Data Bank code 1|10, human muscle LDH-A) for molecular dynamics (MD) calculations to find the most stable conformation of compound and enzyme. In the model generated from this study (Figure 2.2), 1j is shown to interact with several key amino acid residues that span both the substrate and cofactor binding regions of the active site, corroborating kinetic data in which 1j was found to be a competitive inhibitor with respect to both NADH and pyruvate. Specifically, the carboxylic acid group is proposed to form hydrogen bonding interactions with R169, which normally stabilizes the pyruvate carboxylic acid group. Additionally, a water molecule is shown to mediate the interaction between the N-hydroxy group and the catalytic His193 of LDH-A. Further interactions are demonstrated between 1j and Thr248, important in pyruvate recognition, and 1j is found to occupy space in the active site that would normally be occupied by the adenine portion of NADH [1].

Figure 2.2: Computational docking of 1j in the active site of human LDH-A. (Figure 2.2 is adapted with permission from Granchi, et. al. J. Med. Chem. 54: 1599-1612. Figure 5A [1]. Copyright 2011 American Chemical Society.)
To further verify binding of 1\textit{j} to LDH-A, a series of surface plasmon resonance (SPR) experiments were performed. Importantly, the SPR protocol utilized includes a small concentration of detergent in the buffer in which compound “analytes” are evaluated, which can assist in the identification of aggregator compounds which lose inhibitory activity in the presence of detergent [5]. Two analytes of similar structure – 1\textit{j} and 1\textit{g} (which was found to have <3% inhibition of LDH-A at 125 µM [1]) – were chosen for evaluation against lysine-immobilized LDH-A “ligand” on an SPR sensor chip. Maximal response unit changes for each analyte were calculated based on the molecular weight of both the analyte and ligand (Scheme 2.1).

**Scheme 2.1:** Formula for determining the maximum response unit change (\(R_{\text{max}}\)) for an analyte binding a ligand, and solutions to this formula for compounds 1\textit{j} and 1\textit{g}.

\[
R_{\text{max}} = \frac{MW_{\text{analyte}}}{MW_{\text{ligand}}} \times (\Delta RU_{\text{ligand}}) \times \text{stoichiometry}
\]

For 1\textit{j}:

\[
R_{\text{max}} = \frac{321.25 \text{ Da (1j)}}{36,600 \text{ Da (LDH-M monomer)}} \times (16,000 \text{ RU}) \times (4)
\]

\[R_{\text{max}} = 560 \text{ response units}\]

For 1\textit{g}:

\[
R_{\text{max}} = \frac{253.25 \text{ (1g)}}{36,600 \text{ Da (LDH-M monomer)}} \times (16,000 \text{ RU}) \times (4)
\]

\[R_{\text{max}} = 442 \text{ response units}\]
Compounds 1j and 1g were then assessed for their ability to induce dose-dependent response unit changes when flowed over LDH-A. A dose-dependent response was seen with compound 1j, approaching the calculated maximum response (Figure 2.3).

Figure 2.3: Binding of 1j to LDH-A visualized using surface plasmon resonance (SPR)

By contrast, only high concentrations (> 50 µM) of compound 1g led to a substantial change in response units. Even at the highest concentration of 1g assessed (100 µM), the predicted maximal response of 442 units was not achieved (Figure 2.4).

Figure 2.4: Binding of 1g to LDH-A visualized using surface plasmon resonance (SPR)
Recent work by Jessie Peh in the Hergenrother laboratory, in collaboration with Brian Cunningham’s research group at the University of Illinois, revealed a similar pattern of interaction between LDH-A and first generation NHIs [6]. Human LDH-A protein was immobilized in an external cavity laser (ECL) biosensor well, and solutions containing 50 µM concentrations of compound 1j, and two inactive variants, 1g, and 1b were passed over the LDH-A-bound well and a reference well. Only compound 1j led to a lasing wavelength value (LWV) shift in the LDH-A bound well (after subtracted out signals from the reference well; see Figure 2a from [6]). These results are consistent with SPR data, indicating that the kinetically-active compound 1j demonstrates interaction with LDH-A in vitro while structurally-similar but kinetically-inactive NHI variants such as 1g and 1b do not.

Once the in vitro inhibitory and binding profiles of the first generation NHIs were elucidated, further experiments were performed to determine whether these compounds were capable of inhibiting lactate production in cells.

Figure 2.5: Scheme for detection of $^{13}$C glucose and $^{13}$C lactate by $^{13}$C NMR spectroscopy

A $^{13}$C NMR-based assay was used to examine the cellular conversion of $^{13}$C glucose to $^{13}$C lactate (as depicted in Figure 2.5), using medium from cultured cells and an NMR acquisition method that would allow for integration of $^{13}$C peaks. HeLa human cervical carcinoma cells were treated with various compound concentrations for 5 and 10 hours, and medium was sampled at each interval and assessed by NMR. Through this experiment, the
amounts of unconsumed $^{13}$C$_2$-[1,6] glucose remaining in the medium, and $^{13}$C$_1$-[3] lactate produced via glycolysis, were determined. Since high concentrations of lactate are toxic to cells, a large fraction of lactate produced in cells is excreted into the extracellular environment [7, 8]. Representative $^{13}$C NMR spectra generated from this experiment are depicted in Figure 2.6.

This method was used to obtain data confirming that several first generation NHIs showed inhibition of cellular conversion of glucose to lactate at 500 µM concentrations [1]. These data are shown in Figure 2.7. Specifically, compounds 1j, 1i, and 1g, in order of decreasing potency, were able to inhibit lactate production at both 5 and 10 hours relative to vehicle treatment, whereas 1b, 1c, and 1h were ineffective at inhibiting lactate production. The positive control for this assay was treatment with 2-deoxyglucose (2DG), which inhibits hexokinase (an earlier step of glycolysis – see Figure 1.1), and the negative control was the microtubule polymerization inhibitor colchicine, chosen because it is a cytotoxic compound with a mechanism of inducing cell death unrelated to targeting glycolysis. As shown in Figure 2.7, treatment with 2DG led to greater than 50% reductions in lactate production, while treatment with colchicine did not reduce lactate production.
Figure 2.6: Representative $^{13}$C NMR spectra examining the conversion of $^{13}$C$_2$-[1,6] glucose ($^{13}$C-6 peak at 60.95 ppm) to $^{13}$C$_1$-[3] lactate ($^{13}$C-3 peak at 20.28 ppm) using the $^{13}$C-1 DMSO peak at 39 ppm to quantify relative peak ratios. The $^{13}$C NMR spectrum generated from medium from HeLa cells treated with 500 µM 1j for 12 hours shows an ablation of the 20.28 ppm peak corresponding to production of $^{13}$C$_1$-[3] lactate. (Figure 2.6 is adapted with permission from Granchi, et. al. *J. Med. Chem.* 54: 1599-1612. Figure 7C and 7E [1]. Copyright 2011 American Chemical Society.)

Figure 2.7: Lactate production inhibition of the first generation N-hydroxyindole class LDH-A inhibitors, as assessed by $^{13}$C NMR spectroscopy. HeLa human cervical carcinoma cells were treated with indicated compound concentrations or 1% DMSO vehicle, in DMEM supplemented with 10 mM $^{13}$C$_2$-[1,6] glucose. Following 5 or 10 hours, medium aliquots were collected and assessed using a $^{13}$C NMR collection method disabling the Nuclear Overhauser effect (NOE) and allowing for integration of $^{13}$C peaks from $^{13}$C glucose remaining in the medium and resultant $^{13}$C lactate produced and excreted by cells. Bars depict ratio of glucose to lactate produced compared to the vehicle treatment; one biological replicate was performed for each treatment.
Upon additional biological evaluation by researchers at the University of Amsterdam, 1j was shown to have modest potency in killing immortalized cancer cell lines in culture (48 hour IC$_{50}$ values ranging from 10 to 90 µM) and to not induce toxicity in two non-cancerous cell lines in culture (hTERT-HPNE immortalized human ductal pancreatic cells and Hs27 immortalized human skin fibroblasts) at up to 100 µM following 48 hour treatment. Furthermore, 1j was shown to have 16-fold enhanced potency in killing LPC006 human primary pancreatic cancer cell lines in hypoxia compared to normoxia [1], though the mechanisms of death in normoxia versus hypoxia were not determined. However, given the high concentrations of 1j required to inhibit cellular lactate production, efforts were undertaken to develop more potent NHIs for cellular experiments.

2.2: Development and assessment of NHI class LDH-A inhibitors for increased cellular activity

A series of NHI derivatives based on 1j were synthesized by the Minutolo laboratory (Figure 2.8). First, a series of esters of increasing size were made: a methyl ester (4j), an ethyl ester (201), an n-butyl ester (208), and an isopropyl ester (209). The rationale for these modifications is that esters often increase the lipophilicity, and therefore cell membrane permeability of small molecules, while carboxylic acids are negatively charged at physiological pH, and thus are hindered from entering cells [9].

Next, a series of 2,4-dichlorophenyl analogs of 1j and its esters were synthesized by the Minutolo laboratory: dichloro analogs of compounds 1j (147), 4j (154), 201 (202), 208 (216), and 209 (215). Chlorine substitutions on aromatic rings are known to increase lipophilicity [10], thus potentially increasing the cell permeability of these compounds.
**1j ester series**

![Chemical structures](image1)

**Dichloro series**

![Chemical structures](image2)

Figure 2.8: Structures of second generation esters of NHI class LDH-A inhibitors.

In light of the low molecular weights of the NHI series compared to the larger LDH-A inhibitors developed by AstraZeneca [11], ARIAD Pharmaceuticals [12], and Genentech [13], a small series of dimers based on 1j were also synthesized so as to span further into the active site (Figure 2.9).

**Dimer series**

![Chemical structures](image3)

Figure 2.9: Structures of dimeric NHI class LDH-A inhibitors.
The ability of these compounds to inhibit LDH-A in vitro was evaluated by the Minutolo laboratory. Compounds were assessed kinetically to determine $K_i$ values, IC$_{50}$ values, or both. The consumption of NADH was determined by reading fluorescence (excitation 340 nm, emission 460 nm) [2]. The IC$_{50}$ values of selected compounds, as well as the $K_i$ values for others, are presented in Table 2.2.

Table 2.2: In vitro kinetic inhibition of NHI derivatives against LDH-A

<table>
<thead>
<tr>
<th>Compound</th>
<th>Assessment technique</th>
<th>Inhibition (µM ± StDev) vs. pyruvate</th>
<th>Inhibition (µM ± StDev) vs. NADH</th>
</tr>
</thead>
<tbody>
<tr>
<td>4j</td>
<td>$K_i$</td>
<td>NT $^a$</td>
<td>5.1 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>IC$_{50}$</td>
<td>10.5 ± 2.5</td>
<td>14.7 ± 2.1</td>
</tr>
<tr>
<td>201</td>
<td>$K_i$</td>
<td>18.4 ± 0.1</td>
<td>5.3 ± 1.5</td>
</tr>
<tr>
<td>208</td>
<td>$K_i$</td>
<td>NT</td>
<td>1.6 ± 0.3</td>
</tr>
<tr>
<td>209</td>
<td>$K_i$</td>
<td>NT</td>
<td>5.4 ± 1.1</td>
</tr>
<tr>
<td>147</td>
<td>$K_i$</td>
<td>NT</td>
<td>7.7 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>IC$_{50}$</td>
<td>NT</td>
<td>24.3 ± 2.4</td>
</tr>
<tr>
<td>154</td>
<td>$K_i$</td>
<td>NT</td>
<td>5.3 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>IC$_{50}$</td>
<td>NT</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td>202</td>
<td>$K_i$</td>
<td>NT</td>
<td>3.3 ± 1.8</td>
</tr>
<tr>
<td>216</td>
<td>IC$_{50}$</td>
<td>NT</td>
<td>0.31 ± 0.01</td>
</tr>
<tr>
<td>215</td>
<td>$K_i$</td>
<td>NT</td>
<td>40.2 ± 0.5</td>
</tr>
<tr>
<td>14</td>
<td>IC$_{50}$</td>
<td>NT</td>
<td>17.1 ± 0.06</td>
</tr>
<tr>
<td>300</td>
<td>IC$_{50}$</td>
<td>NT</td>
<td>6.3 ± 0.2</td>
</tr>
<tr>
<td>305</td>
<td>IC$_{50}$</td>
<td>NT</td>
<td>1.3 ± 1.0</td>
</tr>
</tbody>
</table>

$^a$ = not tested
To test the hypothesis that the ester analogs of 1j had increased cell permeability compared to 1j, an assay was designed to compare relative rates of cellular uptake of 1j versus its esters. Since esters are often cleaved intracellularly by esterases [14, 15], this assay was designed to be able to detect cleaved species as well. To do this, an LC-MS separation protocol was established to allow for clear resolution of all compounds of interest (Figure 2.10).

Figure 2.10 UV trace at 254 nm and total ion chromatograph (TIC) trace of a methanol sample spiked with 1j, and 4j, and 201, using a method allowing for distinct resolution of each compound.

To correlate compound concentration to integration area of the respective peak in the UV trace or TIC, calibration standards were constructed for each compound by injecting known concentrations of compound over the LC-MS and noting the integration areas in the
UV trace at 254 nm of the resultant peaks (Figure 2.11). Equations were then obtained so the relative concentration of compound in an experimental sample could be calculated from the sample’s UV trace integration. Calibration was also performed relating compound concentration to integration area of the corresponding peak in the total ion chromatograph (TIC), but for consistency and use for compounds with less visibility in the TIC, UV trace integrations were used throughout these studies.

![Figure 2.11: Calibration curve for 1j, 4j, and 201. Integration areas of UV trace peaks at 254 nm were plotted for standards of 1j, 4j, and 201 of known concentrations. An equation for each line was obtained and used to calculate relative compound amount for each compound in experimental samples where concentrations were unknown.](image)

Experiments were performed to assess the relative amounts of cellular uptake and potential intracellular cleavage of the NHIs. Equimolar concentrations of compound were incubated in A549 human non-small cell lung carcinoma cells for various period of time, after which the cells were collected, washed three times, sonicated in methanol, and analyzed by LC-MS to determine the intracellular compound concentration. It was initially
hypothesized that esters of 1j might be cleaved to 1j in cells by cellular esterases, but after 24 hour treatment cellular treatment with either dimethyl ester 4j or ethyl ester 201, no cleavage products matching the retention time and mass spectrum of 1j were observed. A representative spectrum of lysate obtained from A549 cells treated with 4j for 4 hours is shown in Figure 2.12 with no noticeable peak around 14 minutes corresponding to the retention time of 1j using this protocol.

Figure 2.12: Representative UV trace (at 254 nm) of methanol-extracted lysate of HeLa cells treated with 100 µM 4j for 4 hours at 37°C. No appreciable cleavage of 4j to 1j was observed.

Time course experiments were performed examining the relative cell uptake of 1j and its esters 4j and 201 over time. One hundred microliter concentrations of each of the three compounds were incubated in A549 cells for 2, 4, or 8 hours before collecting, washing, and lysing the cellular fraction. Both esters were shown to have increased cellular uptake compared to carboxylic acid 1j. On average, 4j had 4- to 8-fold enhanced uptake and 201 had 3- to 5-fold enhanced compared to 1j, though due to substantial experimental
variation, these differences were largely not statistically significant (p > 0.05). This result also suggested that each compound reaches its maximal amount of uptake within 2 hours, as substantial increases are not observed in the 4 and 8 hour samples (Figure 2.13).

Figure 2.13: Intracellular concentration of 1j, 4j, and 201 upon 2, 4, and 8 hour incubation in A549 cells at 100 µM concentrations. Averages are shown, with error bars denoting standard error from three or more independent experiments. Following treatment with compound for the allotted time, cells were washed twice with PBS and sonicated in methanol to disrupt cellular membranes. Soluble fractions were assessed by LC-MS, using the UV trace at 254 nm to quantify relative compound concentration based on calibration curves of each compound. Statistical analysis was performed using a two-sided, unpaired Student’s t test, with p values shown above.

2.3: Development of a GC-MS-based assay for lactate detection to assess cellular activity of NHIs

After assessing the ability of the NHI derivatives to inhibit LDH-A kinetically and be taken up by cells, it was necessary to test their ability to inhibit lactate production in cells. However, the $^{13}$C NMR-based lactate detection method used previously was limited by several factors. First, data collection was extremely low-throughput, with NMR analysis requiring manual locking and shimming of each sample followed by over 5 minutes of
spectral collection on a shared instrument with limited availability, thus limiting the amount of samples that could be realistically assessed in a single experiment. Second, the detection limit for $^{13}\text{C}$ NMR is relatively low; detection of glucose and lactate concentrations below ~ 5 mM could not be detected above the baseline and thus could not be accurately integrated. Third, at least 300-400 µL of medium were required for analysis, increasing the amount of compound that had to be used to achieve up to 500 µM concentrations in that volume. Finally, only the $^{13}\text{C}$-labeled lactate originating from $^{13}\text{C}$-labeled glucose could be detected using $^{13}\text{C}$ NMR, disallowing detection of unlabeled lactate arising from the 1 mM pyruvate, 4 mM glutamine, or other metabolites present in cell culture medium.

Seeking to bypass the limitations of the $^{13}\text{C}$ NMR lactate detection method, a GC-MS assay was designed and optimized to perform this experiment. The sensitivity of GC-MS far outweighs that of $^{13}\text{C}$ NMR, allowing for detection and precise determination of high nanomolar concentrations of compounds. Given this low detection limit, smaller quantities of sample were able to be analyzed, allowing for adaption of the NMR-based assay into a 96-well plate format using only 100 µL of medium and thus requiring less compound.

The GC-MS assay for lactate detection was optimized using the following protocol. Following treatment of compound in cells, medium samples are collected, concentrated, and derivatized in 1.7 mL microfuge tubes using $N$-(tert-butyldimethylsilyl)-$N$-methyltrifluoroacetamide (MTBSTFA) + 1% tert-butyldimethylchlorosilane (TBDMCS) catalyst (Figure 2.14). This derivatization step serves to make lactate and other metabolites volatile enough for GC-MS visualization.
Figure 2.14: Derivatization of lactate to lactate-2-TBDMS.

Lactate in each derivatized sample is calibrated internally using an internal standard, the unnatural amino acid derivative chlorphenylalanine (CPA), spiked in each medium sample at a fixed concentration of 50 mM and derivatized alongside lactate. Lactate concentrations in each sample are thus expressed as a ratio of derivatized lactate to derivatized CPA. This eliminates GC-MS variation in integration area from sample to sample.

It was necessary to ensure that this method led to reproducible data, so pilot experiments were performed to determine the reproducibility of control samples. Aliquots of medium from HeLa cells cultured for 8 hours in the presence of 1% DMSO were obtained, and the relative lactate concentrations were determined using GC-MS. Both biological replicates (samples collected from three different experimental wells) and technical replicates (aliquots of medium taken from the same well but carried through the derivatization process and GC-MS run separately) were assessed, and lactate concentrations were found to be very similar across all samples (Figure 2.15).
Figure 2.15: Assessment of GC-MS lactate detection precision and biological replicate lactate production similarity. Medium was collected from HeLa cells after 8 hours of incubation with DMEM minus phenol red plus 10% dialyzed FBS, 10 mM glucose, 1 mM pyruvate, and 4 mM glutamine, with two aliquots (technical replicates) collected from each of three wells (biological replicates), to which chlorophenylalanine (CPA) internal standard was added. Medium aliquots were dried and derivatized using MTBSTFA + 1% TBDMCS catalyst and analyzed by GC-MS. Ratios of lactate/CPA were determined for all samples, with technical replicate pairs depicted above in the same color (1A and 1B, 2A and 2B, and 3A and 3B) and biological replicates depicted in different colors.

Another advantage to using GC-MS versus $^{13}$C NMR to detect lactate concentrations is that GC-MS can detect lactate from all carbon sources, rather than from only $^{13}$C-labeled carbon sources. In fact, examination of the resulting mass spectra allows for the differentiation of $^{13}$C-labeled versus unlabeled lactate, by examining ratios of certain peaks containing the $^{13}$C label. Thus, by beginning with $^{13}$C-labeled precursors, experiments can be performed examining the conversion of glucose to lactate (Figure 2.16 a), pyruvate to lactate (Figure 2.16 b), and glutamine to lactate (Figure 2.16 c).
Figure 2.16: $^{13}$C-labeled lactate can be produced from (a) $^{13}$C-labeled glucose, (b) $^{13}$C-labeled pyruvate, or (c) $^{13}$C-labeled glutamine.

The ratio of mass spectrum peaks at 261 versus 262 m/z in derivatized lactate’s spectrum (elution time 10.5 minutes) was used to extrapolate the percentage of total lactate that was $^{13}$C-labeled versus unlabeled. In derivatized lactate samples derived from HeLa cells incubated for 8 hours in DMEM (Dulbecco’s Modified Eagle Medium, which contains 10 mM glucose, 1 mM pyruvate, and 4 mM glutamine), the 261/262 mass spectral ratio was determined to be 4.445. However, HeLa cells that were incubated for 8 hours in DMEM made with 10 mM $^{13}$C$_2$-[1,6]-D-glucose, 1 mM $^{13}$C$_1$-[3]-pyruvate, and 4 mM $^{13}$C$_1$-[2]-L-glutamine (each capable of producing $^{13}$C$_1$ lactate), the 261/262 ratio was found to be 0.118 (Figure 2.17). (The m/z=261 peak height was presumably due to any residual unlabeled...
carbon sources present in cells prior to the experiment, or any other unlabeled precursors present in the medium funneled to lactate during the 8 hour incubation.) Thus, the 261/262 ratio of experimental samples could be extrapolated to determine the relative amount of lactate produced from $^{13}$C-labeled versus unlabeled carbon sources.

**Figure 2.17:** The ratio of $^{13}$C-labeled to unlabeled lactate was calculated based on the difference in the m/z = 261 and m/z = 262 peaks at 10.5 minutes (the retention time of lactate-2-TBDMS). The 261/262 ratio for derivatized, unlabeled lactate is approximately 4.445 (a), and the 261/262 ratio for derivatized, $^{13}$C$_1$-labeled lactate is approximately 0.118 (b).

A summary of the $^{13}$C NMR assay compared to the GC-MS assay for lactate detection is presented in **Table 2.3**. The strengths of the $^{13}$C NMR assay are that no sample preparation is required prior to analyzing each sample by NMR, that both $^{13}$C-labeled glucose and $^{13}$C-labeled lactate can be detected using the same acquisition
protocol, and that a spectrum for each sample can be collected in 8 minutes. The strengths of the GC-MS assay are that the detection limit for lactate is several orders of magnitude greater than for $^{13}$C NMR, that both $^{13}$C-labeled and unlabeled metabolites can be detected and differentiated, 3- to 4-fold less sample volume is required for analysis, and that, despite a longer analysis time per sample (22.5 minutes for GC-MS versus 8 minutes for $^{13}$C NMR), sample runs can be automated using an auto-injector.

**Table 2.3:** Comparison of $^{13}$C NMR and GC-MS assays for lactate detection

<table>
<thead>
<tr>
<th>Parameter</th>
<th>$^{13}$C NMR assay</th>
<th>GC-MS assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lower detection limit for lactate</td>
<td>Low millimolar</td>
<td>High nanomolar</td>
</tr>
<tr>
<td>Lower detection limit for glucose</td>
<td>Low millimolar</td>
<td>Not visualized with TBDMS derivatization</td>
</tr>
<tr>
<td>Sample preparation required</td>
<td>None</td>
<td>1-2 hour concentration + 4 hour derivatization</td>
</tr>
<tr>
<td>Volume of sample required</td>
<td>300-400 µL</td>
<td>100 µL</td>
</tr>
<tr>
<td>Time per sample analyzed</td>
<td>8 minutes</td>
<td>22.5 minutes</td>
</tr>
<tr>
<td>Throughput</td>
<td>Low; samples must be run manually</td>
<td>Medium; auto-injector can process samples continuously</td>
</tr>
<tr>
<td>Types of metabolites detected</td>
<td>Only $^{13}$C-labeled</td>
<td>Any, and can differentiate $^{13}$C-labeled from non-labeled metabolites</td>
</tr>
</tbody>
</table>
Various proof-of-concept experiments were performed using this method. First, experiments were conducted to determine the primary precursor of lactate when HeLa cells were incubated with DMEM containing different combinations of $^{13}$C-labeled versus unlabeled glucose (at 10 mM), $^{13}$C-labeled pyruvate (at 1 mM), and $^{13}$C-labeled glutamine (at 4 mM). In the GC-MS protocol used in these studies, lactate-2-TBDMS elutes at 10.5 minutes, pyruvate-2-TBDMS elutes at 11.2 minutes, glutamine elutes at 19.8 and 20.7 minutes, for 3-TBDMS- and 4-TBDMS derivatives, respectively, and glucose is not visualized. These experiments demonstrated that glucose was the predominant metabolic precursor to lactate during 8 hour incubation in normoxia. When HeLa cells were incubated with 10 mM $^{13}$C-labeled glucose, 1 mM unlabeled pyruvate, and 4 mM unlabeled glutamine, 85% of the lactate produced was found to be $^{13}$C-labeled.

A further proof-of-concept experiment demonstrated that, with decreasing concentrations of glucose, cells rely on other carbon sources to produce lactate. HeLa cells were treated for 8 hours in normoxia with either 1% DMSO vehicle, 10 mM hexokinase inhibitor 2-deoxyglucose (2DG), or 10 µM topoisomerase inhibitor etoposide, in DMEM containing 1 mM unlabeled pyruvate and 4 mM unlabeled glutamine. Hexokinase is the first enzyme of glycolysis, and its inhibition by 2DG reduces the amount of glucose that proceeds through glycolysis and can be converted to lactate. Etoposide’s mechanism of action is unrelated to central metabolism and should not impact the conversion of glucose to lactate.

Six different concentrations of $^{13}$C-labeled glucose, ranging from the normal medium concentration of 10 mM to as low as 100 µM, were tested with each treatment. When HeLa cells were fed 10 mM $^{13}$C-labeled glucose, the majority of lactate produced by these cells
Figure 2.18: Percent of lactate derived from $^{13}$C$_2$-[1,6] glucose versus other metabolic precursors present in cell culture medium. HeLa cervical carcinoma cells were incubated for 8 hours in the presence of either 1% DMSO vehicle, 10 mM hexokinase inhibitor 2-deoxyglucose (2DG), or 10 µM topoisomerase inhibitor etoposide, in DMEM without phenol red + 10% dialyzed FBS + 1 % Penstrep + 1 mM unlabeled pyruvate + 4 mM unlabeled glutamine + the indicated concentration of $^{13}$C$_2$-[1,6] glucose, ranging from the normal medium concentration of 10 mM to as low as 100 µM. Medium was then collected, concentrated, derivatized, and assessed by GC-MS. At lower concentrations of glucose, more lactate is derived from non-glucose carbon sources. Furthermore, as concentration of glucose decreases, less lactate is derived from glucose in cells treated with 2DG, consistent with 2DG’s mechanism of inhibiting hexokinase, which converts glucose to glucose 6-phosphate so it can proceed through glycolysis to form pyruvate or lactate. At every concentration tested, the percent of lactate derived from glucose in etoposide-treated samples does not deviate from the percent of lactate derived from glucose in vehicle-treated cells. n=1 for each treatment.

was derived from this labeled glucose, and only a 10% reduction in lactate derived from glucose was observed with 2DG treatment. As the amount of $^{13}$C-labeled glucose fed to cells was reduced, two trends emerged: 1) absolute reduction in the percent of lactate generated from glucose, and 2) up to a 25% reduction in the percent of lactate generated from glucose in 2DG-treated cells compared to vehicle- and etoposide-treated cells (Figure 2.18; since this experiment was only performed once, no statistical conclusions could be
drawn). The first finding suggests that when cells are glucose-starved, cell metabolism shifts so that other metabolites are funneled through glycolysis to produce lactate. The second finding suggests that, as the concentration of 2DG increases compared to the glucose concentration, 2DG’s inhibition of hexokinase further drives cell metabolism toward funneling non-glucose sources through glycolysis to produce lactate.

2.4: Evaluation of cellular lactate production inhibition of NH1 derivatives

After optimizing the GC-MS lactate detection and quantification assay, the ability of the NHIs to inhibit lactate production in cells was determined. HeLa cells were treated for 8 hours with 50, 100, and 200 µM concentrations of compound, after which medium was collected, dried, derivatized, and analyzed by GC-MS for lactate determination. The cellular lactate production inhibition profiles of 1j and its esters are depicted in Figure 2.19. For Figure 2.19 and all further figures in Chapters 2, 3, and 4 depicting lactate production inhibition in cells, the following conventions were followed:

1) Each assay repetition consists of two biological replicates of each treatment, and the lactate amounts produced by the two biological replicates are averaged.

2) The lactate produced by the average of the two vehicle-treated cells in each individual repetition of each assay is defined as 100%.

3) All other lactate values in the same repetition of the assay are represented as percentages of the vehicle lactate value in the same assay. Since lactate produced by vehicle-treated cells varies from assay to assay, this convention allows for standardization of results from each experimental repetition.

4) In pooling data from three or more independent experiments, percentages of lactate relative to the vehicle from each individual assay are averaged to each
other. Since vehicle lactate production is always set to 100% across each assay repetition, error bars are not shown for vehicle treatment, and p values are not generated.

In this assay, while 1j does not substantially reduce lactate production at up to 200 µM, its esters 4j, 201, 208, and 209 reduce lactate production in a dose-dependent manner. The positive control compound in this assay, 2DG, led to a modest reduction in cellular lactate production, while the negative control compound, topoisomerase inhibitor etoposide, did not alter cellular lactate production compared to vehicle treatment (Figure 2.19).

**Figure 2.19**: Lactate production inhibition of the 1j ester series N-hydroxyindole class LDH-A inhibitors. HeLa human cervical carcinoma cells were treated with indicated compound concentrations or 1% DMSO vehicle, in DMEM supplemented with 10 mM unlabeled glucose, 1 mM pyruvate, and 4 mM glutamine. Following 8 hour incubation, medium aliquots were collected, concentrated, derivatized using MTBSTFA + 1% TBDMCS catalyst, and assessed by GC-MS. Lactate was normalized in each sample using the 1 mM chlorophenylalanine (CPA) internal standard, and lactate relative to vehicle is depicted. Averages from three or more independent experiments are depicted, with error bars depicting standard error.
The 2,4-dichlorophenyl derivatives of \textbf{1j} were also assessed for their ability to inhibit lactate production in HeLa cells during an 8 hour incubation (\textbf{Figure 2.20}). All five compounds in this series show dose-dependent lactate production inhibition, with compounds \textbf{202} (ethyl ester) and \textbf{215} (isopropyl ester) showing substantial reductions in lactate production at concentrations as low as 50 \(\mu\text{M}\).

\textbf{Figure 2.20}: Lactate production inhibition of the dichloro ester series of \textit{N}-hydroxyindole class LDH-A inhibitors. HeLa human cervical carcinoma cells were treated with indicated compound concentrations in DMEM supplemented with 10 mM unlabeled glucose, 1 mM pyruvate, and 4 mM glutamine. Following 8 hour incubation, medium aliquots were collected, concentrated, derivatized using MTBSTFA + 1% TBDMCS catalyst, and assessed by GC-MS. Lactate was normalized in each sample using the 1 mM chlorophenylalanine (CPA) internal standard, and lactate relative to vehicle is depicted. Averages from three or more independent experiments are depicted, with error bars depicting standard error.

Finally, the cellular lactate production inhibitory activity of the NHI dimer series was assessed, as compared to the monomer \textbf{1j}. Of this compound series, no compound showed substantial lactate production inhibition at up to 200 \(\mu\text{M}\) treatment for 8 hours in HeLa cells (\textbf{Figure 2.21}), despite inhibition of LDH-A \textit{in vitro}. This lack of cellular activity may be due to poor cell uptake of these polar dicarboxylic acids.
Figure 2.21: Lactate production inhibition of the N-hydroxyindole class dimer LDH-A inhibitors. HeLa human cervical carcinoma cells were treated with indicated compound concentrations in DMEM supplemented with 10 mM unlabeled glucose, 1 mM pyruvate, and 4 mM glutamine. Following 8 hour incubation, medium aliquots were collected, concentrated, derivatized using MTBSTFA + 1% TBDMCS catalyst, and assessed by GC-MS. Lactate was normalized in each sample using the 1 mM chlorophenylalanine (CPA) internal standard, and lactate relative to vehicle is depicted. Averages from three independent experiments are depicted, with error bars depicting standard error.

Based on the ability of GC-MS to differentiate $^{13}$C-labeled lactate versus unlabeled lactate, an additional series of experiments was performed in order to determine the ability of the NHIs to reduce the production of $^{13}$C-labeled lactate derived from $^{13}$C-labeled glucose or $^{13}$C-labeled pyruvate. When treating cells with 100 µM of each compound in medium containing $^{13}$C-labeled glucose, compounds 154, 201, and 202 were able to reduce the amount of $^{13}$C-labeled lactate formed by 60% compared to the vehicle. The other NHIs tested – 1j, 147, and 4j – led to modest (20-30%) reductions in labeled lactate produced compared to vehicle treatment, consistent with previous data collected on the lactate production inhibition of these compounds at 100 µM concentrations. Hexokinase inhibitor 2DG served as a positive control in this experiment, also leading to a 60% reduction in the amount of $^{13}$C-labeled lactate produced compared to the vehicle, while the negative control, etoposide, had no effect (Figure 2.22).
Figure 2.22: Percent of $^{13}$C-labeled lactate production, derived from $^{13}$C-labeled glucose, compared to vehicle treatment. HeLa human cervical carcinoma cells were treated with indicated compound concentrations or 1% DMSO vehicle, in DMEM supplemented with 10 mM $^{12}$C-labeled glucose, 1 mM unlabeled pyruvate, and 4 mM unlabeled glutamine. Following 8 hour incubation, medium aliquots were collected, concentrated, derivatized using MTBSTFA + 1% TBDMCS catalyst, and assessed by GC-MS. Lactate was normalized in each sample using the 1 mM chlorophenylalanine (CPA) internal standard, and lactate relative to vehicle is depicted. Averages from three independent experiments are depicted, with error bars depicting standard error.

A similar assay was performed to evaluate the ability of the NHIs to reduce the production of $^{13}$C-labeled lactate derived from $^{13}$C-labeled pyruvate. All NHIs tested showed only modest (5-15%) reduction in the production of $^{13}$C-labeled lactate compared to vehicle treatment, whereas 2DG treatment led to a 100% increase in lactate production from pyruvate, consistent with its mechanism of inhibiting hexokinase en route to lactate production from glucose (Figure 2.23). The lack of efficacy of the NHIs in inhibiting lactate production from pyruvate in this study is likely due to the small part pyruvate contributes as a metabolic precursor to lactate when cells are fed 10-fold higher concentrations of glucose and 4-fold higher concentrations of glutamine concurrently. This is consistent with previous work implicating glucose as the predominant metabolic precursor to lactate under these culture conditions (Figure 2.18).
Figure 2.23: Percent of $^{13}$C-labeled lactate production, derived from $^{13}$C-labeled pyruvate, compared to vehicle treatment. HeLa human cervical carcinoma cells were treated with indicated compound concentrations or 1% DMSO vehicle, in DMEM supplemented with 10 mM unlabeled glucose, 1 mM $^{13}$C-labeled pyruvate, and 4 mM unlabeled glutamine. Following 8 hour incubation, medium aliquots were collected, concentrated, derivatized using MTBSTFA + 1% TBDMCS catalyst, and assessed by GC-MS. Lactate was normalized in each sample using the 1 mM chlorophenylalanine (CPA) internal standard, and lactate relative to vehicle is depicted. Averages from three independent experiments are depicted, with error bars depicting standard error.

To further study the ability of the NHI class LDH-A inhibitors to directly impact the conversion of pyruvate to lactate, several further conditions, under which the cells would be forced to rely on pyruvate as a lactate precursor, were explored. In the data depicted in Figure 2.23, cells were incubated with 1 mM $^{13}$C-labeled pyruvate but also with 10 mM unlabeled glucose and 4 mM unlabeled lactate, 10% v/v dialyzed fetal bovine serum (FBS), which also contains traces of glucose and other serum metabolites, as well as 200 – 800 μM concentrations of other amino acids (in the standard formulation of DMEM). By eliminating glucose and glutamine entirely and adjusting other conditions, the cells would be induced to use pyruvate to produce lactate using LDH. Three such incubation conditions were explored: 1 mM pyruvate in phosphate-buffered saline (PBS) pH 7.4 (Figure 2.24 a),
Figure 2.24: Lactate production, relative to vehicle, of HeLa cells incubated in 1 mM pyruvate in PBS (a), DMEM + 10% dialyzed FBS (b), and DMEM minus serum (c). Following 4 and 8 hour incubations, medium aliquots were collected, concentrated, derivatized using MTBSTFA + 1% TBDMCS catalyst, and assessed by GC-MS. Lactate was normalized in each sample using the 1 mM chlorophenylalanine (CPA) internal standard, and lactate relative to vehicle is depicted. Averages from two biological replicates are shown, with error bars depicting standard error. Statistical analysis was performed using an unpaired Student’s t test comparing vehicle treatment at each time point to 4j or etoposide treatment.
1 mM pyruvate in DMEM with 10% dialyzed FBS (Figure 2.24 b), and 1 mM pyruvate in DMEM without FBS (Figure 2.24 c).

Medium lactate was assessed at both 4 and 8 hour time points and three treatments: vehicle, 100 µM 4j, and 10 µM etoposide. In the PBS incubation (Figure 2.24 a), a reduction in cell viability was noted via light microscopy at both time points assessed, concurrent with no substantial decrease in lactate production with any treatment. In the DMEM + FBS incubation (Figure 2.24 b), no statistically significant reductions in lactate production upon 4j treatment were observed, perhaps due to other metabolite sources in the FBS superseding pyruvate as lactate precursors. However, in the serum-free DMEM incubation (Figure 2.24 c), there was a 40-50% reduction in lactate production in medium from 4j-treated cells at both 4 and 8 hours; in the 8 hour samples, this reduction was found to be statistically significant (p=0.021). This supports the hypothesis that 4j is directly targeting LDH-A in cells by inhibiting the conversion of pyruvate to lactate.

In 2011, the Minutolo group reported a series of triazole-containing NHI class LDH-A inhibitors [16]. These triazole series inhibitors were designed to occupy additional space in the enzyme’s active site, and they were accessed using azide-alkyne cycloaddition reactions [17]. The structures of these compounds are shown in Figure 2.25. When these inhibitors were tested for their ability to inhibit LDH-A in vitro, their $K_i$ values against both pyruvate and NADH were found to be in the tens of micromolar, with compound 2 being most potent ($K_i$ values of 20 and 37 µM against NADH and pyruvate, respectively), and compound 1b was the least potent ($K_i$ values of 57 and 86 µM against NADH and pyruvate, respectively) [16]. No cellular data were reported on these compounds.
Figure 2.25: Structures of triazole class NHIs.

The results of an assay for cellular lactate production inhibition of these four compounds, incubated 200 or 500 µM for 8 hours in HeLa cells, are shown in Figure 2.26. Treatment with compound 1a showed only modest (20-30%) inhibition of cellular lactate production, while treatment with compounds 2, 1b, and 1g resulted in slight increases or no change in lactate production compared to vehicle treatment.

Figure 2.26: Lactate production inhibition of triazole class LDH-A inhibitors. HeLa human cervical carcinoma cells were treated with indicated compound concentrations or 1% DMSO vehicle, in DMEM supplemented with 10 mM unlabeled glucose, 1 mM pyruvate, and 4 mM glutamine. Following 8 hour incubation, medium aliquots were collected, concentrated, derivatized using MTBSTFA + 1% TBDMCS catalyst, and assessed by GC-MS. Lactate was normalized in each sample using the 1 mM chlorophenylalanine (CPA) internal standard, and lactate relative to vehicle is depicted. Two biological replicates from one independent experiment were averaged to produce the averages shown above; error bars denote standard error.
2.5: Evaluation of potency in cancer cells

After evaluating the ability of the NHIs to enter cells and lead to inhibition of lactate production in cells, the potency of NHIs in killing cancer cells in culture was assessed. For this experiment, various immortalized adherent lung, breast, ovarian, and cervical cancer cell lines were incubated with compound, each prepared in 9 concentrations ranging from 0.0316 µM to 200 µM, for 72 hours in normoxia at 37 °C. After the 72 hour incubation, biomass was quantified in each well using the Sulforhodamine B (SRB) assay [18]. This assay is based on the principle that live adherent cells retain attachment to the collagen-coated plates and flasks in which they are grown, and hence are fixed and stained by SRB dye, but dead cells detach from the plate or flask surface, and thus are not fixed and stained. The potencies of the NHIs are displayed in Table 2.4, which lists an IC$_{50}$ value – the concentration of compound required to affect a 50% reduction in biomass – for each compound and cell line.

All 1j esters and dichloro esters were tested in HeLa cervical carcinoma cells and A549 non-small cell lung carcinoma cells, with the trend being that esters are more potent than their free acid counterparts. A subset of NHIs was tested further in additional cell lines, with ester substitution increasing potency in other cell lines as well, likely due to the increased cell permeability of esters compared to carboxylic acids.
Table 2.4 – 72 hour IC$_{50}$ values of NHIs against a panel of immortalized human cancer cell lines in culture ($\mu$M ± Standard Error, n=3) $^a$

<table>
<thead>
<tr>
<th>Compound</th>
<th>HeLa (cervical carcinoma)</th>
<th>A549 (non-small cell lung carcinoma)</th>
<th>H1299 (non-small cell lung carcinoma)</th>
<th>H226 (non-small cell lung carcinoma)</th>
<th>MCF-7 (breast ductal carcinoma)</th>
<th>IGROV-1 (ovarian carcinoma)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1j</td>
<td>43.8 ± 2.6</td>
<td>131.0 ± 17.6</td>
<td>141.0 ± 11.1</td>
<td>120.7 ± 7.8</td>
<td>124.3 ± 7.1</td>
<td>123.3 ± 6.8</td>
</tr>
<tr>
<td>4j</td>
<td>33.4 ± 1.0</td>
<td>44.1 ± 6.2</td>
<td>61.1 ± 11.8</td>
<td>43.4 ± 5.3</td>
<td>64.9 ± 13.1</td>
<td>57.4 ± 7.3</td>
</tr>
<tr>
<td>201</td>
<td>16.2 ± 0.5</td>
<td>44.3 ± 13.5</td>
<td>38.3 ± 9.2</td>
<td>25.6 ± 3.6</td>
<td>34.9 ± 1.4</td>
<td>30.5 ± 5.0</td>
</tr>
<tr>
<td>208</td>
<td>33.0 ± 0.6</td>
<td>42.7 ± 10.8</td>
<td>NT $^b$</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>209</td>
<td>22.1 ± 0.4</td>
<td>35.8 ± 6.3</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>147</td>
<td>33.1 ± 0.2</td>
<td>56.4 ± 3.2</td>
<td>NT</td>
<td>NT</td>
<td>47.7 ± 3.3</td>
<td>NT</td>
</tr>
<tr>
<td>154</td>
<td>14.6 ± 0.7</td>
<td>25.3 ± 2.5</td>
<td>NT</td>
<td>NT</td>
<td>26.6 ± 2.5</td>
<td>NT</td>
</tr>
<tr>
<td>202</td>
<td>17.4 ± 0.5</td>
<td>23.4 ± 1.6</td>
<td>NT</td>
<td>NT</td>
<td>23.6 ± 0.7</td>
<td>NT</td>
</tr>
<tr>
<td>216</td>
<td>12.2 ± 1.3</td>
<td>23.1 ± 2.9</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>215</td>
<td>9.3 ± 0.8</td>
<td>12.5 ± 1.0</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
</tbody>
</table>

$^a$ Three independent experiments were performed. Remaining biomass after fixing with 10% trichloroacetic acid was quantified by sulforhodamine B staining.

$^b$ NT = Not tested

2.6: Conclusions and future directions

There is a need to develop a class of LDH-A inhibitors that are potent in vitro, active in cells, and efficacious in vivo. The NHI class of inhibitors has the potential to fulfill each of these roles. These inhibitors interact with both the pyruvate and NADH binding pockets of LDH-A, increasing the likelihood that they will be selective for LDH-A versus other NADH-utilizing enzymes found in cells. While the first generation of NHIs, as exemplified by 1j, are poorly active in cells, ester derivatives of 1j show enhanced cellular uptake, enhanced efficacy in inhibiting cellular lactate production, and enhanced potency in killing cancer cells.
Efforts are ongoing to evaluate further NHI class compounds in this triad of assays. To improve upon the cancer targeting and selectivity of the NHIs, glycoconjugates of many NHIs have been synthesized and evaluated in cells, as discussed in Chapter 3. Efforts toward directly comparing the NHIs to other classes of LDH-A inhibitors, both in the preliminary assays described previously and in more intricate approaches such as transcript, metabolic, and cytological profiling, are detailed in Chapter 4. Finally, efforts to directly probe the interaction of NHIs with LDH-A in cells and cell lysate are described in Chapter 5.

2.7: Materials and methods

Surface plasmon resonance (SPR) – The binding of 1j and 1g to LDH-A was queried by SPR using the following method. A CM5 sensor chip (GE Healthcare Life Sciences, Piscataway, NJ) was primed in a Biacore 3000 instrument (GE Healthcare Life Sciences) equipped with Biacore 3000 Central Software (GE Healthcare Life Sciences) for 6 minutes and 20 seconds in deionized water prior to activation of the sensogram. A 1:1 mixture of N-hydroxysuccinimide and ethyl(dimethylaminopropyl) carbodiimide was prepared in 35 µL volume was injected over 7 minutes (flow rate of 5 µL/minute) into two flow cells (reference channel and active channel) to prime carboxyl groups on the chip to react covalently with LDH-A’s lysines. This resulted in a change of 130 reference units (RU). Then 50 µL of LDH-A solution (2:99 dilution of human LDH-A (MyBioSource, San Diego, CA) in Biacore 5.5 acetate solution (10 mM sodium acetate, pH 5.5) was injected over only the active channel at a flow rate of 5 µL/minute, resulting in a positive bulkshift of 16,000 RU. Finally, 35 µL 1 M ethanolamine was injected over both flow cells at a flow rate of 5 µL/minute to covalently bind any carboxyl residues on the chip surface that are not occupied by LDH-A ligand. During the pauses between injections, SPR running buffer (0.01 M 4-(2-
hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 0.15 M sodium chloride, 3 mM ethylenediaminetetraacetic acid (EDTA), and 0.005% surfactant, pH-adjusted to 7.4, degassed and filtered through a 0.22 micron filter) was flowing over both channels at a rate of 5 µL/minute.

Dilutions of 1j and 1g (from 50 mM DMSO stock solutions) were prepared in SPR running buffer, supplementing dilutions with DMSO to keep the concentration of DMSO constant (1%) in all dilutions. These dilutions were of concentrations 0, 5, 10, 25, 50, and 100 µM. These solutions were injected over both channels at a flow rate of 10 µL/minute in 20 µL total volume, in order from lowest concentration to highest concentration of compound. During this time, the aliquot of SPR running buffer flowing over both channels was also supplemented with 1% DMSO to prevent a change in RU due to DMSO alone upon compound injection. The injection series was repeated 3 times, with representative sensograms for each dilution overlaid to examine the differential change in response units for each dilution.

**Intracellular concentration assessment** – A549 human non-small cell lung carcinoma cells (ATCC, Manassas, VA) were grown in T25 flasks prior to being treated with 100 µM compound or vehicle control (prepared in DMSO) in 5 mL total volume (0.2% final concentration DMSO in all treatments). At each time point, cells were rapidly collected by scraping, washed twice with sterile PBS, 37 °C, and disrupted by sonication in methanol, -80 °C, using an XL-2000 Misonix sonicator (Qsonica, Newton, CT). After a 30 minute incubation at 4 °C to facilitate precipitation of proteins, the sonicates were centrifuged, and a portion of the supernatant was analyzed by on a Waters Quattro II quadrupole-hexapole-quadrupole liquid chromatography/mass spectrometry apparatus (Waters, Milford, MA) equipped with an electrospray ionization source. LC separation was achieved using a C18
Waters Xbridge column (2.1 x 20mm, Waters) at 25 °C using a linear gradient of mobile phases: 95% water, 5% acetonitrile, and 0.1% formic acid (A) and 95% acetonitrile, 5% water, and 0.1% formic acid (B). Solution A was initially passed through the column but decreased linearly to 50% of the mobile phase at 10 minutes and 0% of the mobile phase at 25 minutes. The flow rate was 200 µL/min, and the injection volume was 10 µL. The mass spectrometer was operated in negative mode for analysis of all three compounds. The ultraviolet detector was programmed to monitor absorbance at 254 nm, to detect the phenyl ring present in all compounds. Data analysis was performed using MassLynx spectrometry software (Waters). Briefly, UV traces were integrated at 14.1 minutes (1j), 15.8 minutes (4j), and 16.9 minutes (201); the UV trace of vehicle-treated sonicates from both the start and end of the experiment time course contained no peaks in this range. Calibration curves of 1j, 4j, and 201 demonstrated a linear relationship between concentration and UV trace integration area, so a linear equation was generated for each compound to convert integration area to concentration. These values were normalized to each cell pellet’s fresh weight prior to sonication.

**Assessment of lactate production by \(^{13}\text{C}\) NMR spectroscopy** – Confluent HeLa human cervical carcinoma cells (ATCC, Manassas, VA) in a 12 well plate were treated with compound or vehicle control (1% DMSO final concentration in all samples) in phenol red-free DMEM + 10% dialyzed FBS (Gemini Biosciences, Sacramento, CA) + 1% Penstrep, supplemented with 10 mM \(^{13}\text{C}_2\)-[1,6] glucose (Omicron Biochemicals, South Bend, IN), 1 mM pyruvate, and 4 mM glutamine, in a final volume of 1000 µL. Five and ten hours after beginning treatment, 500 µL of cell medium was extracted from each well, centrifuged at 1000 rcf to filter out cellular debris, and stored at -20 °C. Samples were thawed immediately before NMR spectra were collected.
NMR spectra were collected on a Varian Unity 500 instrument (Varian, Palo Alto, CA) with a 5 mm Nalorac QUAD probe and 500 MHz magnet. To collect $^{13}$C NMR spectra that would permit quantitative integration of $^{13}$C peaks, special parameters were used to disable the Nuclear Overhauser effect (NOE)'s enhancement of signals from carbons with multiple hydrogens bound. Briefly, the pulse width was set to 5.5 µs, the pre-acquisition delay was set to 1.2 seconds, and decoupling was only programmed to occur during spectrum acquisition. Data analysis was performed using MestRe-C software (MestreLab Research, Santiago de Compostela, Spain), using the DMSO peak as the internal standard and computing the ratio of C6 glucose at 60.95 ppm to the C3 lactate at 20.28 ppm (Figure 2.2). One sample was performed per treatment.

Assessment of lactate production by GC-MS - Confluent HeLa cells in a 96 well plate were treated with compound or vehicle control (1% DMSO final concentration in all samples) in DMEM minus phenol red + 10% dialyzed FBS + 1% Penstrep, supplemented with 10 mM glucose, 1mM sodium pyruvate and 4mM glutamine, in a final volume of 125 µL per well. Immediately following compound addition, plates were incubated for 4 or 8 hours at 37 °C in a 95% air/5% CO$_2$ atmosphere. Duplicate wells were prepared for each treatment. Following treatment, medium was collected, and 100 µL were added to 2 µL 50 mM chlorophenylalanine (CPA; internal standard for GC-MS analysis). Samples were concentrated, derivatized by a four-hour incubation with MTBSTFA + 1% TBDMCS (Thermo Scientific, Walthman, MA) in acetonitrile at 85 °C, and immediately analyzed using GC-MS (Agilent 6890N GC/5973 MS, equipped with an Agilent DB-5 capillary column, 30 M x 320 µM x 0.25 µM, model number J&W 123-5032, Agilent Technologies, Santa Clara, CA) and an electron impact ionization source. One microliter of each sample was injected using an automated injector, and a solvent delay of 8.20 minutes was implemented. The initial oven
temperature was 120 °C, held for 5 minutes; then the temperature was increased at a rate of 10 °C/minute until a temperature of 250 °C was reached. Temperature was then increased by 40 °C/minute until a final temperature of 310 °C was reached. Total run time per sample was 22.5 minutes.

Compounds were identified using AMDIS Chromatogram software (Amdis, freeware available from amdis.net) and programmed WIST and Niley commercial libraries. The integration area of lactate in each sample was divided by the integration area of CPA in the same sample to achieve a lactate/internal standard ratio. The ratios were averaged for duplicates, and percent lactate production over vehicle was calculated for each independent experiment. The mean lactate production/vehicle was then averaged between three or more independent experiments.

The fraction of $^{13}$C-precursor-driven lactate produced was calculated by collecting peak heights using Agilent Data Analysis software and determining the ratio of m/z=261 and 262 peaks in the mass spectrum of the lactate peak at 10.5 minutes. For proof-of-concept experiments, $^{13}$C$_2$-[1,6] glucose was purchased from Omicron Biochemicals (South Bend, IN); $^{13}$C$_1$-[3] pyruvate was purchased from Santa Cruz Biotechnology (Santa Cruz, CA); $^{13}$C$_1$-[2] glutamine was purchased from Sigma-Aldrich (St. Louis, MO).

**Assessment of cancer cell toxicity** - HeLa human cervical carcinoma, MCF-7 breast ductal carcinoma, A549, H1299, and H226 human non-small cell lung carcinoma, and IGROV-1 human ovarian carcinoma cell lines, grown in RPMI 1640 medium supplemented with 10% FBS and 1% Penicillin/Streptomycin, were added at a density of 5000 cells/well to 96 well plates to which 31.6 nM - 200 µM compound in DMSO was already added (1% final concentration DMSO in all wells; triplicate wells at the same concentration per repetition).
Plates were incubated at 37 °C in a 95% air/5% CO₂ atmosphere for 72 hours. Medium was removed and cells were fixed by the addition of 50 µL 10% trichloroacetic acid in water, 4 °C, to each well. Plates were incubated at 4 °C for at least one hour, and the sulforhodamine B colorimetric assay [18] was performed to assess remaining biomass in each well. Briefly, plates were washed thrice in tap water and dried before the addition of 50 µL sulforhodamine B dye solution (0.057% w/v sulforhodamine B in 1% glacial acetic acid) to each well. Following a 30 minute incubation, unbound dye was removed by washing each plate six times in 1% glacial acetic acid. Two hundred microliters of 10 mM Tris buffer (pH=10.5) was added to each dried well to solubilize the bound dye, and after a 30 minute incubation, absorbance of each well was read at 510 nm in a microplate reader. Cells treated with 1% DMSO were used as the 100% live control for biomass, and wells incubated with medium alone were used as the baseline zero biomass control. IC₅₀ values were calculated using SoftMax Pro software (Molecular Devices, Sunnyvale, CA).
2.8: References


Chapter 3: Glycoconjugated LDH-A inhibitors as targeted and selective anticancer agents

Sections of Chapter 3 have been adapted with permission from one published manuscript:


All experimental work described in this chapter was performed by E. Calvaresi, unless otherwise specified. The synthesis and kinetic evaluation of all NHI compounds was performed by Dr. Carlotta Granchi and coworkers in the laboratory of Professor Filippo Minutolo at the University of Pisa. All computational modeling was performed by Dr. Tiziano Tuccinardi at the University of Pisa.

3.1: Design of glycoconjugated NHIs to dually target the Warburg effect

Vis-à-vis previous work to preferentially target a drug to cancerous tissue by conjugating it to glucose (Section 1.4), we developed a series of glycoconjugated NHIs to dually target the Warburg effect. We envisioned these conjugates to hit two important metabolic targets overexpressed in cancer: the transporter GLUT-1 and the critical glycolytic enzyme LDH-A (Figure 3.1). Selections from this chapter, regarding the biological assessment of Glu-4j (Glc-NHI-2), have been reported recently [1].
Figure 3.1: Depiction of glucose conjugation strategy to dually target the Warburg effect. The glycoconjugated NHI is designed to be selectively taken up into cancer cells overexpressing GLUT-1 transporters. Once inside the cell, it will inhibit LDH-A, upregulated by the same cellular machinery which upregulates GLUT-1, and lead to cell death. Figure adapted with permission from [1], John Wiley and Sons license # 3422021340036.

To explore the tractability of this strategy, glucose conjugates of each of the 1j esters and dichloro 1j esters were prepared, as depicted in Figure 3.2.

**Glucose-conjugated 1j ester series**

Glu-1j  
Glu-4j  
Glu-201  
Glu-208  
Glu-209

**Glucose-conjugated dichloro series**

Glu-154  
Glu-202  
Glu-216  
Glu-215

Figure 3.2: Structures of glucose-conjugated NHI class LDH-A inhibitors
Several other glycoconjugates of 4j were synthesized and assessed in cells. As described previously, anticancer glycoconjugates are not limited to glucose; conjugates containing D-galactose (1-D-galactose-conjugated docetaxel [2, 3]), D-threoside (threoside-conjugated chlorambucil [4] and threoside-conjugated cyclopamine [5]), and D-lyxose (lyxose-conjugated warfarin [6]) have been previously reported. In addition, aminoglycoside-conjugated compounds have also been reported to have anticancer activity [4].

We investigated the use of D-mannose and D-gulose as carbohydrates to append to 4j. D-gulose is a stereoisomer of glucose differing in stereochemistry at the C3 and C4 positions (Figure 3.3). It is used by some archaea but is not known to be found in human serum or used in human metabolism. The ability of D-gulose to be transported by GLUT receptors has not been reported to date. D-mannose is the C2 epimer of D-glucose (Figure 3.3). It is found at concentrations of around 50 µM in human serum, and it is primarily used in the glycosylation of proteins (specifically, mannose-6-phosphate is a localization tag applied to proteins destined for lysosomal import). It is known to be transported into mammalian cells primarily by mannose-specific transporters normally expressed in intestinal cells. However, it has also been reported to be a substrate for GLUT-1, albeit several-fold weaker than D-glucose (K_i of 19 mM compared to 6.3 mM for D-glucose when tested in a competition assay against L-sorbose) [7]. While no mannose-conjugated anticancer compounds have been reported to date, mannose-conjugated compounds to target HIV have been studied in vitro [8]. Interestingly, aberrant mannosidase (an enzyme that cleaves mannose residues) expression has been found in several types of cancer, such that mannosidase inhibition has been proposed as an anticancer strategy [9, 10]. Since mannose is a GLUT-1 substrate, and mannosidase expression is elevated in cancer,
mannose-conjugated anticancer agents have the potential to be effective prodrugs for cancer treatment.

**Epimers and diastereomers of glucose**

Figure 3.3: Structures of D-glucose, its C2 epimer D-mannose, and its diastereomer D-gulose

Aminoglycoside conjugates have also been explored as bioactive agents. Several natural product aminoglycosides, including streptomycin and neomycin, are used in the clinic as antibacterial agents targeting the 30S ribosome subunit in gram-negative bacteria [11]. More recently, aminoglycoside-conjugated anticancer agents have been reported, some of which rely on the ability of aminoglycosides to interact with various forms of RNA [12], and some of which are toxic to cancer cells through an uncharacterized mechanism [4]. To explore the properties of gulose-, mannose-, and aminoglycoside-conjugates of the NHIs, **Man-4j**, **Gul-4j**, and **amino-Glu-4j** were synthesized (Figure 3.4).

**Non-glucose glycoconjugated NHIs**

Figure 3.4: Mannose-, gulose-, and amino-glucose conjugates of 4j

3.2: *In vitro* kinetic testing against LDH-A and computational docking studies
An important initial assessment of these glycoconjugated NHIs was to determine whether they were active LDH-A inhibitors or inactive prodrugs. To address this question, the Minutolo lab performed kinetic assay to assess their ability to inhibit LDH-A. The results of these studies are presented in Table 3.1. Of the compounds in this series, Glu-1j, Glu-4j, Man-4j, and Gul-4j were shown to be moderately active in vitro inhibitors of LDH-A in their own right. Upon characterizing the mode of inhibition of these compounds, Glu-1j, Glu-4j, Man-4j, and Gul-4j were all found to be competitive inhibitors of LDH-A with respect to NADH. Compounds Glu-201, Glu-208, Glu-209, Glu-154, and Glu-202 were found to be active at inhibiting LDH-A at high concentrations, and Glu-216 and Glu-215 were found to be poorly active against LDH-A in vitro.

Table 3.1: *In vitro* kinetic inhibition of glucose-conjugated NHI derivatives against LDH-A

<table>
<thead>
<tr>
<th>Compound</th>
<th>Assessment technique</th>
<th>Inhibition (µM ± StDev) vs. pyruvate</th>
<th>Inhibition (µM ± StDev) vs. NADH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu-1j</td>
<td>$K_i$</td>
<td>NT <em>a</em></td>
<td>19.5 ± 3.0</td>
</tr>
<tr>
<td>Glu-4j</td>
<td>$K_i$</td>
<td>NT</td>
<td>37.8 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>57.2 ± 6.3</td>
<td>115.8 ± 15.3</td>
</tr>
<tr>
<td>Man-4j</td>
<td>$K_i$</td>
<td>NT</td>
<td>34.7 ± 6.8</td>
</tr>
<tr>
<td>Gul-4j</td>
<td>$K_i$</td>
<td>NT</td>
<td>40.2 ± 0.5</td>
</tr>
<tr>
<td>Amino-Glu-4j</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>NT</td>
<td>19.5 ± 0.6</td>
</tr>
<tr>
<td>Glu-201</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>NT</td>
<td>69% at 500 µM</td>
</tr>
<tr>
<td>Glu-208</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>NT</td>
<td>81% at 500 µM</td>
</tr>
<tr>
<td>Glu-209</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>NT</td>
<td>67% at 500 µM</td>
</tr>
<tr>
<td>Glu-154</td>
<td>$K_i$</td>
<td>NT</td>
<td>109.1 ± 9.2</td>
</tr>
<tr>
<td>Glu-202</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>NT</td>
<td>64% at 500 µM</td>
</tr>
<tr>
<td>Glu-216</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>NT</td>
<td>34% at 500 µM</td>
</tr>
<tr>
<td>Glu-215</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>NT</td>
<td>&gt; 200 µM</td>
</tr>
</tbody>
</table>

*a* = not tested

To further investigate the *in vitro* inhibition of LDH-A by many of the glycoconjugated NHIs, molecular dynamic studies using Glu-4j were performed, similar to those described
previously for $1j$. **Glu-4j** was docked into the active site of human LDH-A (Protein Data Bank code 1l10), and simulation was performed to identify the most stable conformation of **Glu-4j** within the region. One view of the result is depicted in **Figure 3.5**, which shows **Glu-4j**, like $1j$, postulated to form hydrogen bonding interactions with Arg169, the amino acid that forms hydrogen bonds with the carboxylic acid group of pyruvate.

**Figure 3.5**: Computational docking of **Glu-4j** (orange) into the active site of human LDH-A.

Congruent with the moderately potent $K_i$ values observed for **Glu-4j** versus NADH in kinetic studies, docking studies indicated that the glucose of **Glu-4j** is purported to occupy space in the NADH binding pocket of LDH-A. As shown in **Figure 3.6**, the glucose hydroxyl groups are predicted to form hydrogen bonding interactions with several residues that
normally interact with NADH, such as Asn138 and Val136. The 2'-hydroxyl is also predicted to be positioned adjacent to the catalytic His193 in the active site.

**Figure 3.6**: Computational docking of Glu-4j (orange) into the active site of human LDH-A.

3.3: Cellular uptake of glycoconjugated NHIs

We hypothesized that the cellular uptake of Glu-4j would be greater than that of its aglycone, 4j, because its import may be GLUT-mediated. This hypothesis was tested in two parts. First, to assess the relative cellular uptake of Glu-4j, this compound was analyzed using the same LC-MS parameters used to successfully resolve 1j and 4j. Glu-4j was found to have a retention time of 12.4 minutes (UV trace) and 12.6 minutes (TIC), such that it would be clearly resolved apart from 1j and 4j using the same acquisition parameters (Figure 3.7). Calibration of Glu-4j, to obtain an equation expressing the relationship of concentration to integration area, was then performed (Figure 3.8).
Figure 3.7: UV trace at 254 nm and TIC trace of a methanol sample spiked with Glu-4j, 1j, and 4j, obtained using the separation method described previously.

Figure 3.8: Calibration of UV trace (254 nm) integration area versus concentration of Glu-4j

Next, the relative cellular uptake of Glu-4j relative to 4j was assessed. In doing so, it could be determined whether Glu-4j was cleaved inside cells to 4j (by glucosidases) or 1j
(by glucosidases and esterases). HeLa cervical carcinoma cells were treated with *Glu-4j* for up to 48 hours, during which time no cleavage to *4j* or *1j* was observed. A representative UV trace from a 4 hour incubation of *Glu-4j* in HeLa cells is shown in Figure 3.9.

**Figure 3.9**: Representative UV trace (at 254 nm) of methanol-extracted lysate of HeLa cells treated with 100 µM *Glu-4j* for 4 hours at 37°C. No appreciable cleavage of *Glu-4j* to *4j* or *1j* was observed.

The relative rates of cell uptake of *4j* and *Glu-4j* were then directly compared by treating HeLa cells with 100 µM *4j* or *Glu-4j* for 4 hours, followed by measurement of the relative concentration of each compound in the cellular fraction. *Glu-4j* was found to have approximately 5-fold enhanced cellular uptake compared to *4j*. The difference between the intracellular concentrations of *4j* and *Glu-4j* was found to be statistically significant (p = 0.0001; **Figure 3.10**).
Figure 3.10: Relative intracellular concentrations of 4j and Glu-4j upon 4 hour incubation in A549 cells at 100 µM concentrations. Following treatment with compound for 4 hours, cells were washed twice with PBS and sonicated in methanol to disrupt cellular membranes. Soluble fractions were assessed by LC-MS, using the UV trace at 254 nm to quantify relative compound concentration based on calibration curves of each compound. Averages are shown, with error bars denoting standard error from four independent experiments. Statistical analysis was performed using an unpaired Student’s t test.

Next, the relative cell uptake levels of 4j versus its three glycoconjugates (Glu-4j, Man-4j, and Gul-4j), and the potential cleavage of Man-4j and Gul-4j in cell culture, was assessed. Calibration standards were prepared for compounds Man-4j and Gul-4j (Figure 3.11 a). A549 cells were treated with 100 µM concentrations of each compound for 4 hours. After washing and lysing the cells, the cellular fraction was assessed by LC-MS. As seen in Figure 3.11 b, all three glycoconjugates had significantly enhanced cell uptake compared to the aglycone 4j. While Glu-4j and Man-4j had similar levels of cell uptake, Gul-4j had slightly lower levels of cell uptake. No cleavage of Gul-4j or Man-4j to 4j or 1j was observed in either the UV trace or TIC of the resultant lysate samples.
Figure 3.11: (a) Standards for Gul-4j and Man-4j to obtain equations to convert compound concentration to integration areas in the UV trace at 254 nm. (b) Results of intracellular concentration comparison study comparing relative uptake of 4j to its three glycoconjugates, Glu-4j, Man-4j, and Gul-4j. All four compounds were incubated at 100 µM for 4 hours in A549 cells prior to washing and lysing the cellular fraction. Following treatment with compound for 4 hours, cells were washed twice with PBS and sonicated in methanol to disrupt cellular membranes. Soluble fractions were assessed by LC-MS, using the UV trace at 254 nm to quantify relative compound concentration based on calibration curves of each compound.

Triplicate data is shown, with error bars denoting standard error. Statistical analysis was performed using an unpaired Student’s t test to compare glycoconjugate intracellular concentrations with 4j intracellular concentration, with * denoting p < 0.05, ** denoting p < 0.01, and *** denoting p < 0.0005.
3.4: Competition of glycoconjugates with glucose for cellular entry

After establishing that Glu-4j had enhanced cellular uptake compared to 4j, the next task was to determine if the cellular import of Glu-4j was at least partially GLUT receptor mediated. We previously developed a GC-MS technique that could differentiate $^{13}$C-labeled lactate from unlabeled lactate using the peak ratios of $m/z = 261$ and $m/z = 262$ (Figure 2.16). One application of this technique was the finding that, as the concentration of $^{13}$C-labeled glucose in the medium was reduced, more of the lactate produced by the cells came from non-glucose precursor metabolites (Figure 2.17).

Based on these findings, experiments were performed in HeLa cells, which are known to overexpress GLUT-1 [13], to determine if other compounds or GLUT transport inhibitors could compete with $^{13}$C-labeled glucose for cellular entry, leading to a reduction in the percent of lactate derived from $^{13}$C-labeled glucose compared to vehicle treatment. The structures of the compounds chosen for this initial experiment are shown in Figure 3.12. Unlabeled glucose was used as a positive control, since it would also be metabolized to lactate, leading to a reduction in the amount of labeled lactate derived from labeled glucose.

Several compounds that are not GLUT substrates were used as negative controls that would not affect the conversion of $^{13}$C-labeled glucose to $^{13}$C-labeled lactate. These negative controls were etoposide, lactose, ribose, and fructose. Etoposide is a topoisomerase inhibitor unrelated to central metabolism. Lactose is a disaccharide, consisting of glucose and galactose linked by a $\beta-1\rightarrow4$ glycosidic linkage, found in breast milk and dairy products. It is cleaved to its two monosaccharide constituents by the enzyme lactase, normally secreted from intestinal cells. Lactase is normally only expressed in and secreted from enterocytes (intestinal absorptive cells) [14] and uncleaved lactose is not a substrate for the GLUT-1 transporter [7]. Ribose, an aldopentose monosaccharide, is a 20-
fold poorer substrate for the GLUT-1 transporter than D-glucose [15]. Its contribution to lactate production is expected to be minimal, as it is preferentially converted to the amino acids tryptophan and histidine or utilized in the pentose phosphate pathway following phosphorylation to ribose 5-phosphate by ribokinase. Fructose, a structural isomer of glucose, is an 150-fold poorer substrate for the GLUT-1 transporter than D-glucose [15]. Instead, its cellular uptake is mediated through GLUT-2, an insulin-dependent glucose transporter expressed by hepatocytes and enterocytes (absorptive cells of the small intestine), and GLUT-5, a fructose transporter expressed by enterocytes [16]. Neither GLUT-2 nor GLUT-5 is known to be overexpressed in cancer cells [17]. Fructose is able to be shuttled into glycolysis in the liver only, through a circuitous mechanism: phosphorylation to fructose-1-phosphate by fructokinase, which is converted by aldolase B into dihydroxyacetone phosphate and glyceraldehyde. These two intermediates can both be converted to the glycolytic intermediate glyceraldehyde 3-phosphate through an additional enzymatic step, by the enzymes triose phosphate isomerase and glyceraldehyde kinase, respectively. The initial enzyme in this fructose utilization pathway, fructokinase, is not known to be overexpressed in cancer [18], so it is unlikely that cancer cells in culture will either be able to uptake fructose or convert it to lactate.

In this experiment, two glucose transporter inhibitors were evaluated for their ability to compete with \(^{13}\)C-labeled glucose for cellular entry (and thus lead to a reduction in \(^{13}\)C-labeled lactate). Cytochalasin B, a natural product isolated from the fungus *Drechslera dematioides*, has been shown to block the entry of glucose into cells through competitive inhibition of GLUT-1 (\(K_i\) value of 0.3 µM) [13]. Phloretin, a natural product derived from apple tree leaves, has also been found to inhibit GLUT-1’s transport of glucose with an IC\(_{50}\) value of 8.7 µM [13], and has been shown to block the entry of glucose [13] and galactose
[19] into cells. Treatment with these two inhibitors should reduce the amount of $^{13}$C-labeled glucose able to enter cells, which will force cells to rely on metabolites other than $^{13}$C-labeled glucose to produce lactate, so the percent of $^{13}$C-labeled lactate will decrease compared to vehicle treatment.

![Structures of compounds used in glucose competition studies](image)

**Figure 3.12:** Structures of compounds used in glucose competition studies

A proof-of-concept experiment was performed by incubating HeLa cells in medium containing only 100 µM $^{13}$C-labeled glucose (instead of the normal 10 mM glucose present in DMEM), because in previous work, treatment with this low concentration of glucose had resulted in only about 50% of lactate produced at the end of an 8 hour incubation being derived from the labeled glucose (Figure 2.16). HeLa cells were co-treated with 100 µM concentrations of alternate sugars (except for D-glucose, which was used at 500 µM), and the percent of $^{13}$C-labeled lactate produced at the end of an 8 hour incubation was determined (using 261/262 ratios obtained from the mass spectra of derivatized lactate peaks). Upon performing statistical workup of the data, it was found that co-treatment with
unlabeled glucose, phloretin, and cytochalasin B led to statistically significant reductions in the percent of $^{13}$C-labeled lactate produced compared to the vehicle, whereas treatment with lactose, ribose, fructose, and etoposide did not significantly change the amount of labeled lactate produced compared to the vehicle (Figure 3.13).

**Figure 3.13:** Compounds which compete with glucose for cellular entry lead to a reduction in the percent of $^{13}$C lactate produced from $^{13}$C glucose. HeLa cells were treated with 100 µM $^{13}$C glucose and either 500 µM unlabeled glucose, equimolar quantities of the disaccharide lactose or the monosaccharides ribose and fructose, which are not known to enter cells via GLUTs, or equimolar quantities of the published GLUT-1 transporter inhibitor phloretin and the non-specific GLUT inhibitor cytochalasin B. Error bars denote standard error (n=3). Statistical analysis performed using Student’s unpaired, two-tailed t test; * denotes p < 0.05 compared to the vehicle; ** denotes p < 0.01 compared to vehicle.

This strategy was then used to discern whether glucose-conjugated compounds also competed with glucose for cellular entry, as assessed by their ability to lead to a statistically significant reduction in the percent of $^{13}$C-labeled lactate from $^{13}$C-labeled glucose compared to vehicle treatment at the end of an 8 hour incubation. As mentioned in Chapter 1, it is crucial that such experiments are performed concurrently with the conjugate’s aglycone to verify that any effect observed is specific.
Glu-PAC-1 and PAC-1 (Figure 3.14) are another set of glycoconjugate and aglycone compounds that have been studied in the Hergenrother group, and so these compounds were used to validate assay conditions. The anticancer agent PAC-1 has been reported by the Hergenrother laboratory to relieve zinc inhibition of procaspase-3 to facilitate its cleavage to caspase-3, an effector of apoptotic cell death [20, 21]. One of the limitations of PAC-1 is its neurotoxicity in vivo [22] when given via intraperitoneal or intravenous injection. Glu-PAC-1 was synthetized to improve the targeting of PAC-1 to cancerous cells overexpressing GLUT-1.

When PAC-1 and Glu-PAC-1 were assessed in the $^{13}$C glucose competition assay in HeLa cells, using 100 µM $^{13}$C-labeled glucose and 30 µM PAC-1 or Glu-PAC-1, it was found that only Glu-PAC-1 led to a statistically significant decrease in $^{13}$C-labeled lactate derived from $^{13}$C-labeled glucose compared to vehicle treatment (Figure 3.15). This suggests that Glu-PAC-1 competes with glucose for cellular entry and is therefore a GLUT substrate.
Figure 3.15: Treatment with Glu-PAC-1, but not its aglycone PAC-1, leads to a statistically significant reduction in the percent of $^{13}$C lactate produced from $^{13}$C glucose. HeLa cells were treated with 100 µM $^{13}$C glucose and varying concentrations of PAC-1, Glu-PAC-1, known GLUT-1 transporter inhibitor phloretin, or non-GLUT substrate etoposide. Error bars denote standard error (n=3). Statistical analysis performed using Student’s unpaired, two-tailed t test; * denotes p < 0.05 compared to the vehicle; ** denotes p < 0.01 compared to vehicle. N.S. = not significant (p > 0.05 compared to vehicle).

Finally, the ability of the glycone/aglycone NHI pair of interest, Glu-4j and 4j, to compete with $^{13}$C glucose for cellular entry using this assay was evaluated. After 8 hour treatment in HeLa cells of 100 µM $^{13}$C-labeled glucose and equimolar 4j or Glu-4j, only Glu-4j was found to significantly reduce the production of $^{13}$C-labeled lactate compared to the vehicle (Figure 3.16), suggesting that Glu-4j is a GLUT substrate.
Figure 3.16: Treatment with Glu-4j, but not 4j, leads to a statistically significant reduction in the percent of $^{13}$C lactate produced from $^{13}$C glucose. HeLa cells were treated with 100 µM $^{13}$C glucose and equimolar quantities of 4j, Glu-4j, known GLUT-1 transporter inhibitor phloretin, or non-GLUT substrate etoposide. Error bars denote standard error (n=3). Statistical analysis performed using Student’s unpaired, two-tailed t test; * denotes p < 0.05 compared to the vehicle; ** denotes p < 0.01 compared to vehicle. N.S. = not significant (p > 0.05 compared to vehicle).

3.5: Cellular lactate production inhibition of glycoconjugated NHIs

Next, the ability of the glycoconjugated NHIs to inhibit lactate production in cells was evaluated using the same technique developed for the compounds described previously. In testing Glu-1j and its esters at 50-200 µM concentrations for their ability to reduce lactate production in HeLa cells following an 8 hour incubation, it was found that Glu-4j led to the most potent, dose-dependent reduction in lactate production of the series. All other compounds showed levels of inhibition (Figure 3.17). Although Glu-1j is the most potent *in vitro* inhibitor in this series (Table 3.1), its lack of cellular activity may be due to poor permeability, as is the case for 1j.
**Figure 3.17**: Lactate production inhibition of glucose-conjugated 1j and its esters. HeLa human cervical carcinoma cells were treated with indicated compound concentrations or 1% DMSO vehicle, in DMEM supplemented with 10 mM unlabeled glucose, 1 mM pyruvate, and 4 mM glutamine. Following 8 hour incubation, medium aliquots were collected, concentrated, derivatized using MTBSTFA + 1% TBDMCS catalyst, and assessed by GC-MS. Lactate was normalized in each sample using the 1 mM chlorophenylalanine (CPA) internal standard, and lactate relative to vehicle is depicted. Averages from three or more independent experiments are depicted, with error bars depicting standard error.

The lactate production inhibition profiles of Glu-154 and its esters were also assessed using the GC-MS-based assay. In this series, only Glu-202 and Glu-215 were found to lead to substantial, dose-dependent reductions in lactate production (Figure 3.18). Glu-202 showed modest activity in the *in vitro* assessment (64% inhibition at 500 µM), while Glu-215 was unable to inhibit LDH-A kinetically at up to 200 µM (Table 3.1). The moderate inhibition of lactate production by Glu-215 in the cell-based assay might be due to cleavage of Glu-215 to compound 215 in cells, which shows modest inhibition of LDH-A *in vitro* ($K_i = 40.2$ µM; Table 2.2) but fairly potent activity in cells (Figure 2.19). Alternately, Glu-215 may be targeting another metabolic enzyme, such as an earlier step of glycolysis, leading to a reduction in lactate production in that manner. Further elucidation of the mechanism of Glu-215 is ongoing.
Figure 3.18: Lactate production inhibition of glucose-conjugated dichloro NHIs. HeLa human cervical carcinoma cells were treated with indicated compound concentrations or 1% DMSO vehicle, in DMEM supplemented with 10 mM unlabeled glucose, 1 mM pyruvate, and 4 mM glutamine. Following 8 hour incubation, medium aliquots were collected, concentrated, derivatized using MTBSTFA + 1% TBDMCS catalyst, and assessed by GC-MS. Lactate was normalized in each sample using the 1 mM chlorophenylalanine (CPA) internal standard, and lactate relative to vehicle is depicted. Averages from three or more independent experiments are depicted, with error bars depicting standard error.

The lactate production inhibition profiles of Man-4j and Gul-4j were assessed and compared to those of 4j and Glu-4j. Both Man-4j and Gul-4j led to a substantial, dose-dependent reduction in lactate production similar to Glu-4j and more potent than 4j (Figure 3.19). This is consistent with the in vitro inhibition of LDH-A observed with both of these compounds (Table 3.1). Finally, the lactate production inhibition profile of Amino-Glu-4j was assessed. No substantial reduction in lactate production was observed at up to 200 µM treatment for 8 hours (Figure 3.20).
Figure 3.19: Lactate production inhibition of glycoconjugates of 4j. HeLa human cervical carcinoma cells were treated with indicated compound concentrations or 1% DMSO vehicle, in DMEM supplemented with 10 mM unlabeled glucose, 1 mM pyruvate, and 4 mM glutamine. Following 8 hour incubation, medium aliquots were collected, concentrated, derivatized using MTBSTFA + 1% TBDMCS catalyst, and assessed by GC-MS. Lactate was normalized in each sample using the 1 mM chlorophenylalanine (CPA) internal standard, and lactate relative to vehicle is depicted. Averages from three or more independent experiments are depicted, with error bars depicting standard error (n ≥3).

Figure 3.20: Lactate production inhibition of amino-Glu-4j. HeLa human cervical carcinoma cells were treated with indicated compound concentrations or 1% DMSO vehicle, in DMEM supplemented with 10 mM unlabeled glucose, 1 mM pyruvate, and 4 mM glutamine. Following 8 hour incubation, medium aliquots were collected, concentrated, derivatized using MTBSTFA + 1% TBDMCS catalyst, and assessed by GC-MS. Lactate was normalized in each sample using the 1 mM chlorophenylalanine (CPA) internal standard, and lactate relative to vehicle is depicted. Averages from three independent experiments are depicted, with error bars depicting standard error (n = 3).
3.6: Cancer cell potency of glycoconjugated NHIs

After assessing the ability of glycoconjugated NHIs to enter cells and inhibit lactate production in cells, their potency in killing a panel of immortalized cancer cell lines was evaluated. The results of these IC\(_{50}\) determinations are presented in Table 3.2. Glu-1j is unable to kill cancer cells at up to 200 µM, consistent with its lack of activity in the cellular lactate production inhibition assay. The remainder of the glycoconjugated NHI compounds were more potent than their respective aglycones. For example, Glu-4j has IC\(_{50}\) values that are 3- to 6-fold lower than that of its aglycone 4j in every cell line tested. Several compounds have fairly low IC\(_{50}\) values (Glu-201, Glu-208, Glu-209, Glu-154) but are relatively inactive in the cellular lactate production inhibition assay. This may suggest that their primary target in cells is not LDH-A, and that they are inducing cell death by an alternate mechanism, which has not yet been elucidated. Glu-216 is inactive in inhibiting lactate production in cells and also has fairly high IC\(_{50}\) values in HeLa and A549 cells, suggesting that it may not be entering cells efficiently.
Table 3.2 – 72 hour IC$_{50}$ values of glycoconjugated NHIs (µM ± standard error, n=3) in a panel of immortalized human cancer cell lines in culture

<table>
<thead>
<tr>
<th>Compound</th>
<th>HeLa (cervical carcinoma)</th>
<th>A549 (non-small cell lung carcinoma)</th>
<th>H1299 (non-small cell lung carcinoma)</th>
<th>H226 (non-small cell lung carcinoma)</th>
<th>MCF-7 (breast ductal carcinoma)</th>
<th>IGROV-1 (ovarian carcinoma)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu-1j</td>
<td>&gt; 200</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Glu-4j</td>
<td>7.2 ± 0.2</td>
<td>17.2 ± 3.0</td>
<td>18.0 ± 1.5</td>
<td>16.8 ± 2.8</td>
<td>16.7 ± 1.1</td>
<td>15.5 ± 3.0</td>
</tr>
<tr>
<td>Man-4j</td>
<td>5.4 ± 1.3</td>
<td>15.2 ± 0.7</td>
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<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Gul-4j</td>
<td>11.8 ± 0.1</td>
<td>24.7 ± 0.9</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Glu-201</td>
<td>11.4 ± 1.1</td>
<td>23.8 ± 3.6</td>
<td>NT</td>
<td>NT</td>
<td>12.5 ± 0.8</td>
<td>NT</td>
</tr>
<tr>
<td>Glu-208</td>
<td>8.8 ± 0.8</td>
<td>12.3 ± 1.3</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Glu-209</td>
<td>7.9 ± 0.6</td>
<td>18.8 ± 2.7</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Glu-154</td>
<td>12.9 ± 0.2</td>
<td>31.2 ± 0.1</td>
<td>NT</td>
<td>NT</td>
<td>21.7 ± 1.8</td>
<td>NT</td>
</tr>
<tr>
<td>Glu-202</td>
<td>14.5 ± 1.6</td>
<td>31.2 ± 0.3</td>
<td>NT</td>
<td>NT</td>
<td>17.1 ± 1.1</td>
<td>NT</td>
</tr>
<tr>
<td>Glu-216</td>
<td>75.2 ± 12.8</td>
<td>173.2 ± 76.0</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Glu-215</td>
<td>24.4 ± 11.6</td>
<td>51.1 ± 9.1</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
</tbody>
</table>

$^a$ Three independent experiments were performed. Remaining biomass after fixing with 10% trichloroacetic acid was quantified by sulforhodamine B staining.

$^b$ NT = Not tested

3.7: Potency of glycoconjugated NHIs in “normal” cells

The aim of conjugating an anticancer agent to glucose is to be able to target its delivery to cancer cells that ravenously consume glucose and overexpress the insulin-independent glucose transporter GLUT-1, rather than normal tissues that only uptake glucose under insulin stimulation. This selectivity in glucose uptake is evident in $^{18}$F-FDG PET scans; this technique is widely used for the diagnosis and precise staging of many cancers (Chapter 1, Section 1.4.2). Thus, there are several methods to determine whether
a glucose-conjugated drug is capable of selectively being targeted to tumor tissues \textit{in vivo}. First, the drug could be modified with a radioactive label or fluorescent label that is capable of being visualized through tissues, and this labeled compound could then be administered to tumor-bearing animals to examine its distribution pattern, as has been done with glufosfamide [23]. Second, in the course of performing pharmacokinetic and pharmacodynamic studies with the drug in healthy and tumor-bearing animals, samples could be collected from various tissues (tumor, blood, liver, brain, etc.) and analyzed for their drug and drug metabolite contents. This would provide a more complete picture of the whole-organism drug distribution and metabolism, and help to elucidate whether the glycoconjugate might be neurotoxic (as $^{18}$F-FDG is taken up by GLUT-1-expressing neurovasculature and hence is visualized in the brain in PET scans) before administering the drug in human patients.

Such studies have not yet been performed with glycoconjugated NHIs. One surrogate experiment that was performed in cell culture is the evaluation of the potency of \textbf{Glu-4j} in a “normal” cell line, wild type mouse embryonic fibroblast (WT-MEF) cells (murine cells express both LDH-A [24, 25] and GLUT-1 [26]). Since it is well-established that all cell lines, whether they are tumor-derived or immortalized cells of non-tumor origin, undergo a shift to glycolytic metabolism with increasing time in tissue culture [27, 28], the IC$_{50}$ value of \textbf{Glu-4j} in these cells was determined in three consecutive early passages. The IC$_{50}$ value of \textbf{Glu-4j} in WT-MEF cells was found to be statistically significantly higher than the IC$_{50}$ values of \textbf{Glu-4j} in a panel of immortalized cancer cells in culture (Figure 3.21). This experiment suggests that \textbf{Glu-4j} has the potential to be selective to cancers cells.
Figure 3.21: The IC\textsubscript{50} value of Glu-4j is statistically significantly larger in “normal” WT-MEF cells compared to Glu-4j’s IC\textsubscript{50} value in every other cell line examined. Two-tailed, unpaired Student’s t tests were performed comparing the IC\textsubscript{50} value of Glu-4j in each cancer cell line to the IC\textsubscript{50} value of Glu-4j in “normal” WT-MEF cells. Bars depict averages; error bars denote standard error (n=3). ** denotes p values of < 0.01; *** denotes p values of < 0.005.

3.8: \textit{In vivo} efficacy of Glu-4j

An A549 xenograft study in athymic nude mice to assess the \textit{in vivo} efficacy of Glu-4j was conducted. A collaborator, Pankaj Seth, performed the animal work, and we performed the statistical analysis. Subcutaneous tumors were generated in nude mice from A549 cells. After tumors reached 100 mm\textsuperscript{2} in size, vehicle (PEG-400) or 100 mg/kg Glu-4j was administered intraperitoneally each day for the first 8 days, then every other day for days 9-13. All mice were sacrificed at day 15 because excessive weight loss in the Glu-4j-treated cohort prompted cessation of the study.
Figure 3.22: Glu-4j treatment (100 mg/kg in PEG-400 administered intraperitoneally) in a 15-day A549 xenograft model in athymic nude mice led to statistically significant reductions in tumor area (a-b), fold change in tumor growth (c-d), and tumor weight (e). Error bars denote standard error (n=10 for vehicle, n=6 for Glu-4j). Statistical analysis was performed using a two-sided, unpaired Student’s t test. For (b) and (d), the top line represents the 75th percentile, the middle line represents the average, the lower line represents the 25th percentile, and the error bars denote the spread of data.
When tumors were extracted and analyzed, it was found that tumors from mice treated with Glu-4j were significantly smaller in area (Figure 3.22 a-b), had significantly smaller fold change in growth (Figure 3.22 c-d), and weighed significantly less (Figure 3.22 e) than the tumors in vehicle-treated mice. Efforts are ongoing to determine optimal delivery vehicles and administration dosages under which Glu-4j is well-tolerated and demonstrates efficacy against tumors.

3.9: Conclusions and future directions

A series of glycoconjugated NHIs have been synthesized and evaluated in a panel of in vitro and cell culture assays. Glu-4j, the glucose conjugate of 4j, is a moderately potent in vitro inhibitor of LDH-A, has several-fold enhanced cell uptake compared to 4j, displays greater potency in inhibiting cellular lactate production at lower concentrations compared to 4j, and is 3-6-fold more potent than 4j in killing cancer cells in culture. Furthermore, Glu-4j has been shown to compete with glucose for cellular entry, suggesting that its uptake is GLUT-mediated, and it has shown reduced potency in “normal” cells in culture. Encouragingly, Glu-4j has shown modest efficacy in reducing tumor growth in vivo. Glu-4j has also been evaluated head-to-head against other classes of LDH-A inhibitors, as described in Chapter 4. Efforts are ongoing to evaluate Glu-4j in cells and in vivo.

3.10: Materials and methods

Intracellular concentration assessment – This experiment was performed using the same LC-MS instrument, solvent system, and running protocol as described previously, using the UV trace to integrate Glu-4j (elution time: 12.4 minutes in the UV trace, 12.6 minutes in the TIC), Man-4j (elution time: 12.7 minutes in the UV trace, 12.9 minutes in the TIC), and Gul-4j (elution time: 12.9 minutes in the UV trace, 13.1 minutes in the TIC).
$^{13}$C glucose competition assay – These experiments were performed as described previously, except that medium was supplemented with 100 µM $^{13}$C$_2$-[1,6] glucose, no unlabeled glucose, 1 mM sodium pyruvate, and 4 mM glutamine. Dried samples were derivatized using MTBSTFA + 1% TBDMCS (Regis Technologies, Inc., Morton Grove, IL) in acetonitrile at 85 °C for four hours. The fraction of $^{13}$C-glucose-derived $^{13}$C$_1$-[3] lactate produced was calculated by collecting peak heights using Agilent Data Analysis software and determining the ratio of m/z=261 and 262 peaks in the mass spectrum of the lactate peak at 10.5 minutes.

Assessment of potency in “normal” cells – Early passage wild-type mouse embryonic fibroblast (WT-MEF), obtained from ATCC, were cultured in DMEM. Cells were used for IC$_{50}$ determination within as few passages as possible from the date they were thawed from a frozen stock. IC$_{50}$ determination was performed using the SRB assay as previously described.
3.11: References

23. Stüben, J., et al., Pharmacokinetics and whole-body distribution of the new chemotherapeutic agent β-D-glucosylisophosphoramidostearic acid and its effects on the


Chapter 4: Head-to-head assessment of the different classes of LDH-A inhibitors

Sections of Chapter 4 have been adapted with permission from The Royal Society of Chemistry from one published manuscript:


All experimental work described in this chapter was performed by E. Calvaresi, unless otherwise specified. The synthesis and kinetic evaluation of galloflavin, Genentech 35, AZ 33, AZ 35, AZ 35 MME, AZ 33 DEE, 276, and 277 were performed by Dr. Carlotta Granchi, Ilaria Paterni, and coworkers in the laboratory of Professor Filippo Minutolo at the University of Pisa. The synthesis of FX-11 was performed by Dr. Robert Huigens III, a former postdoctoral fellow in the Hergenrother laboratory. All computational modeling was performed by Dr. Tiziano Tuccinardi at the University of Pisa.

4.1: Overview of scope and goals of comparison studies

In performing comparisons between the various classes of published in vitro inhibitors of LDH-A in cells, different inhibitors were evaluated in a sequence of key experiments. Various classes of LDH-A inhibitors were assessed for their ability to be taken up by cells, to reduce cellular lactate production in a dose-dependent manner, and to kill cancer cells in culture. For cytotoxicity assays, several assay conditions were tested: cells grown in normoxia, cells grown in hypoxia, and cells in which LDH-A had been stably knocked down using RNA interference. Then, a global analysis of the effects of inhibitor treatment in cells was performed. To do this, metabolic and cytological profiling studies
were completed, comparing the NHIs and the other classes of LDH-A inhibitors simultaneously. The goals of these head-to-head studies were threefold:

1) To characterize the properties of all compounds in cells, to determine which compounds have a mechanism consistent with lactate dehydrogenase inhibition and which may have other targets;
2) To identify the most promising compound or compounds to be used as a molecular tool to further explore the tractability of LDH-A inhibition as an anticancer strategy in cell culture and *in vivo*; and
3) To identify the most selective, most potent compound or compounds for use in further *in vivo* work to identify a preclinical candidate.

For the purposes of these studies, NHI lead compounds 4j and Glu-4j were chosen for evaluation. The Minutolo research group synthesized and supplied AZ 33 and its dimethyl ester AZ 35 (*Figure 1.8*), galloflavin (*Figure 1.7*), and Genentech 35 (*Figure 1.9*). Gossypol (*Figure 1.6*) was obtained through commercial sources, and FX-11 (*Figure 1.6*) was synthesized by a former Hergenrother laboratory postdoctoral fellow, Dr. Rob Huigens. The LDH-A inhibitors recently reported by ARIAD 9 (*Figure 1.8*) and Genentech 22 (*Figure 1.9*) are currently not commercially available, and given their lack of potent cellular activity [2, 3], they were not included in these studies.

4.2: *Further cellular characterization of AstraZeneca (AZ) LDH-A inhibitors and galloflavin*

Initial characterization studies of the AstraZeneca compounds and galloflavin were required before moving forward with broad comparison studies. Galloflavin has been reported by the DiStefano and Recanatini research groups [4] to have cellular effects at
high (> 100 µM) concentrations [4-6]. It was important to determine before proceeding further with galloflavin if poor cellular uptake would be a mitigating factor in future studies.

Given that AstraZeneca’s LDH-A inhibitors, represented by lead compound AZ 33, are among the most potent inhibitors reported to date [7], it was important to test AstraZeneca’s hypothesis that the dimethyl ester AZ 35 would be cleaved to its diacid, AZ 33, intracellularly, and thus potently inhibit LDH-A in cells. AstraZeneca researchers did not report kinetic evaluation of AZ 35, but the Minutolo research group demonstrated that while AZ 33 potently inhibited LDH-A in vitro (IC$_{50}$ value of 0.54 ± 0.03 µM against NADH and 1.0 ± 0.2 µM against pyruvate), its dimethyl ester AZ 35 did not substantially inhibit LDH-A at concentrations of up to 200 µM.

Compounds AZ 33 (UV trace retention time 8.9 minutes) and AZ 35 (UV trace retention time 11.0 minutes) were resolved using the same LC-MS acquisition parameters that had been used to resolve the NHIs (Figure 4.1 a). Running a set of known concentrations of AZ 33 and AZ 35 on the LC-MS and obtaining the corresponding UV trace integration areas resulted in a linear relationship between concentration and integration area, with linear equations generated to describe these relationships (Figure 4.1 b). The UV trace integrations were used for calibration instead of the TIC integrations for two reasons: 1) to follow the previous precedent of using UV trace to quantify compound concentration and 2) because the AstraZeneca compounds showed strong UV absorbance at 254 nm and comparably poor TIC resolution.
Figure 4.1: (a) UV trace at 254 nm of AZ 33 and AZ 35, resolved by LC-MS using the separation method described previously. (b) Calibration correlating concentration of AZ 33 or AZ 35 with UV trace integration area.

4.2.1: Characterization of AZ 35 cleavage product

HeLa cells were treated with 500 µM of AZ 33 or AZ 35 for 30 minutes. Following treatment, cells were collected, washed to remove the extracellular fraction of compound, and lysed in PBS. Cell lysates were subjected to LC-MS analysis using the resolution parameters described above to determine both 1) the relative concentration of compound able to penetrate the cells and 2) the cellular cleavage products, if any, resulting from compound treatment. In performing this experiment using AZ 35, a new peak at 9.6 minutes appeared in several experimental iterations (Figure 4.2).
The mass spectrum of this peak under negative ionization showed a parent mass of 510 m/z and a significant peak at 466 m/z. We proposed that the identity of the parent species was the monomethyl ester of AZ 35, in which one of the methyl ester groups in the malonate portion of the compound was cleaved to a carboxylic acid. The major peak at 466 m/z is consistent with a decarboxylated species of the monomethyl ester (Figure 4.3).
The formation of the monomethyl ester of AZ 35 was proposed to be due to a cellular esterase attack of the carboxylic acid carbon followed by displacement of methoxide by the esterase’s hydroxyl group, followed by water displacement of the esterase to result in the monomethyl ester species. The subsequent decarboxylation of the molecule would then proceed through a stable six-membered transition state, and the resulting negatively-charged species of molecular weight 466 would be resonance-stabilized (Scheme 4.1). To test this hypothesis, the Minutolo lab synthesized the monomethyl ester of AZ 35 (AZ 35 MME), as well as compounds 276 (single carboxylic acid) and 277 (single monomethyl ester).
Scheme 4.1: Proposed mechanism for the formation of **AZ 35 MME**, and the subsequent decarboxylation occurring to generate the mass spectrum peak of m/z = 467.

Figure 4.4: The structures of AstraZeneca malonate derivatives, **AZ 35 MME**, and related compounds 276 and 277

Standards of the three compounds in Figure 4.4 were analyzed by LC-MS using the same acquisition parameters and solvent system used in the analysis of the previous AstraZeneca compounds, to determine which of these compounds had the retention time of 9.6 minutes in the UV trace and 9.9 minutes in the TIC. Compound **AZ 35 MME** was found
to match both the retention time (Figure 4.5 a) and mass spectrum pattern of the AZ 35 cellular cleavage product, thus confirming this compound as the identity of the cleavage product of AZ 35. Standards of AZ 35 MME were then run to determine a linear equation for the relationship between its concentration and the resulting integration area in the UV trace (Figure 4.5 b).

Figure 4.5: (a) Resolution of AZ 35 MME by LC-MS demonstrates that its retention time (9.6 minutes in this UV trace at 254 nm) matches the AZ 35 cleavage product observed in cell lysate. Furthermore, this LC-MS running protocol can clearly resolve and differentiate AZ 33, AZ 35 MME, and AZ 35. (b) Calibration standard of AZ 35 MME.
4.2.2: NHI and AZ compound stability in medium, PBS, and trypsin

After establishing **AZ 35 MME** as the unknown peak observed in lysate from cells treated with **AZ 35**, it was important to characterize the conditions under which this compound formed. One hypothesis is that the **AZ 35 MME** forms during incubation of **AZ 35** in one or more of the solutions the compound is dissolved in during the course of the experiment: RPMI 1640 + 10% FBS cell culture medium, phosphate-buffered saline (PBS) solution at pH 7.4 used for washing, or trypsin + EDTA solution used to detach the HeLa cells from the bottom of their wells following treatment. Compounds **AZ 35**, the NHI methyl ester **4j**, and another compound synthesized by the Minutolo research group, the diethyl ester derivative of **AZ 33** (**AZ 33 DEE**; **Figure 4.6**), were incubated in each of these three solutions for 30 minutes at 37 °C, mimicking the conditions used in the cell culture experiment.

![AZ 33 DEE](image)

**Figure 4.6**: The structure of **AZ 33 DEE**.

When analyzing the LC-MS data generated from these incubations, it was found that neither incubation in RPMI 1640 medium (**Figure 4.7**), nor PBS (**Figure 4.8**), nor trypsin + EDTA (**Figure 4.9**), led to a formation of any cleavage product of any of these three compounds.
Figure 4.7: Stability of (a) AZ 35, (b) AZ 33 DEE, and (c) 4j in cell culture medium. Compounds were incubated in RPMI 1640 medium + 10 % fetal bovine serum (FBS) and 1% Penstrep for 30 minutes at 37 °C. UV traces at 254 nm of medium samples are shown.

Figure 4.8: Stability of (a) AZ 35, (b) AZ 33 DEE, and (c) 4j in phosphate-buffered saline (PBS) solution, pH 7.4. Compounds were incubated in PBS for 30 minutes at 37 °C. UV traces at 254 nm of PBS solution are shown.
Figure 4.9: Stability of (a) AZ 35, (b) AZ 33 DEE, and (c) 4j in trypsin solution. Compounds were incubated in 0.05% trypsin + 0.53 mM EDTA in HBSS without sodium bicarbonate, calcium, or magnesium for 30 minutes at 37 °C. UV traces at 254 nm of trypsin solutions are shown.

4.2.3: NHI and AZ compound stability and cleavage in cell lysate

The stability of compounds 4j, AZ 35, and AZ 33 DEE in fresh HeLa cell lysate was then assessed. To perform this experiment, HeLa cells were lysed in warmed RPMI 1640 medium. Aliquots of lysate or warmed medium were then added to tubes containing DMSO solutions of compounds. All tubes were incubated at 37 °C, after which 100 µL aliquots from each tube were directly assessed by LC-MS. While 4j and AZ 33 DEE were stable in both medium and lysate, AZ 35 was stable in medium but was cleaved substantially to AZ 35 MME during the 30 minute incubation period (Figure 4.10). This suggests that AZ 35 is cleaved by an esterase or other cellular protein.
AZ 35 is cleaved in HeLa cell lysate but not in cell culture medium, whereas AZ 33 DEE and 4j are stable in both medium and lysate. Compounds were incubated in freshly generated cell lysate in warmed medium, or warmed medium alone, for 30 minutes at 37 °C.

4.2.4: Relative intracellular concentrations of AZ vs. NH1 compounds

Following the characterization of the cellular cleavage product of AZ 35, the relative intracellular concentrations of AZ 33, AZ 35, AZ 35 MME, 1j, and 4j were assessed. This experiment was performed by treating HeLa cells with identical concentrations (500 µM) of each compound for 30 minutes. Consistent with the previous studies in A549 cells, it was found that 4j was taken up by cells at approximately 4-fold higher amounts than 1j. AZ 33 was detected in lysates from treated cells at extremely low concentrations, suggesting that
its cell permeability is poor. **AZ 35** entered cells at approximately 3.5 times the amount of 1j in 30 minutes; where approximately half of it was cleaved in cells to the monomethyl ester species. However, when **AZ 35 MME** was incubated with HeLa cells, the intracellular concentration was below the limit of detection (Figure 4.11).

![Figure 4.11](image)

**Figure 4.11**: Relative intracellular concentrations 1j, 4j, AZ 33, AZ 35, and AZ 35 monomethyl ester (MME) upon 30 minute incubation in HeLa cells at 500 µM concentrations. For **AZ 35**, the orange bar denotes the amount of parent compound present in cell lysate, and the stacked purple bar denotes the amount of **AZ 35 MME** cleavage product present in the lysate. Averages are shown, with error bars denoting standard error from three or more independent experiments. Following treatment with compound for 30 minutes, cells were washed twice with PBS and sonicated in methanol to disrupt cellular membranes. Soluble fractions were assessed by LC-MS, using the UV trace at 254 nm to quantify relative compound concentration based on calibration curves of each compound. This figure has been adapted from the Royal Society of Chemistry from the following manuscript: [1]

4.2.5: **Relative intracellular concentration of galloflavin**

Since galloflavin has been reported by Di Stefano and coworkers to have efficacy (albeit at 100 – 500 µM concentrations) in cells [4-6], the LC-MS-based assay described above was used to determine the relative cell uptake of this compound. Galloflavin was cleanly resolved using the same LC-MS parameters that were used to resolve the NHIs and
AstraZeneca compounds (Figure 4.12 a; UV trace retention time 5.8 minutes) and calibrate its concentration to integration area in the UV trace (Figure 4.12 b). Repeated treatments of 500 µM galloflavin for 30 minutes in A549 cells led to only trace amounts of compound present in the resulting cell lysate (Figure 4.12 c), thus suggesting the cellular uptake of galloflavin was quite low.

![Figure 4.12: Galloflavin is poorly taken up by A549 cells. (a) UV trace at 254 nm of a sample of 100 µM galloflavin in methanol, showing absorbance at over 337,000 absorbance units (AU). (b) Calibration standard and linear equation generated from measuring the UV absorbance of known concentrations of galloflavin. (c) Representative UV trace from a sample of cell lysate in methanol derived from A549 cells treated for 30 minutes with 500 µM galloflavin. The integration area of this absorbance peak is approximately 1100 AU (compared to a standard of 6.25 µM galloflavin (the lowest concentration used to obtain the calibration standard in (b)) having an absorbance peak of 13126 AU). Similar results were observed in triplicate data sets.](image)

4.3: Lactate production inhibition in cell culture

The ability of various in vitro inhibitors of LDH-A to inhibit lactate production in cells was assessed using the GC-MS experiment described previously. Galloflavin, FX-11, and
Genentech 35 were assessed at 50, 100, and 200 µM concentrations in an 8 hour treatment in HeLa cells. Only 200 µM concentrations of FX-11 and Genentech 35 were shown to lead to a substantial (~40%) reduction in lactate formation in this study (Figure 4.13).

![Figure 4.13](image)

**Figure 4.13**: Lactate production inhibition of previously reported kinetic inhibitors of LDH-A. HeLa human cervical carcinoma cells were treated with indicated compound concentrations or 1% DMSO vehicle, in DMEM supplemented with 10 mM unlabeled glucose, 1 mM pyruvate, and 4 mM glutamine. Following 8 hour incubation, medium aliquots were collected, concentrated, derivatized using MTBSTFA + 1% TBDMS catalyst, and assessed by GC-MS. Lactate was normalized in each sample using the 1 mM chlorophenylalanine (CPA) internal standard, and lactate relative to vehicle is depicted. Averages from three or more independent experiments are depicted, with error bars depicting standard error.

The ability of AstraZeneca compounds AZ 33 and AZ 35 to inhibit lactate production in cells was assessed similarly; each compound was testing at concentrations of at 50, 100, and 200 µM for 8 hours in HeLa cells. AZ 33 had minimal effect on lactate production, while 100 and 200 µM AZ 35 treatment led to a substantial (~50%) reduction in lactate production (Figure 4.14). The lack of lactate production inhibition observed for AZ 33 is likely due its poor cell uptake. Interestingly, AZ 35 has an effect on lactate production, though it is cleaved intracellularly to AZ 35 MME, rather than to AZ 33. Subsequent kinetic analysis of AZ 35 MME by the Minutolo research group revealed that this compound is a
moderately potent kinetic inhibitor of LDH-A (*in vitro* IC$_{50}$ values of 4.8 ± 1.0 µM versus NADH and 22.7 ± 3.3 µM versus pyruvate). Thus, we hypothesize that AZ 35 MME, which is formed rapidly in cells, is responsible for the cellular lactate production inhibition observed upon AZ 35 treatment.

**Figure 4.14:** Lactate production inhibition of AstraZeneca’s LDH inhibitors. HeLa human cervical carcinoma cells were treated with indicated compound concentrations or 1% DMSO vehicle, in DMEM supplemented with 10 mM glucose, 1 mM pyruvate, and 4 mM glutamine. Following 8 hour incubation, medium aliquots were collected, concentrated, derivatized using MTBSTFA + 1% TBDMCS catalyst, and assessed by GC-MS. Lactate was normalized in each sample using the 1 mM chlorophenylalanine (CPA) internal standard, and lactate relative to vehicle is depicted. Averages from three or more independent experiments are depicted, with error bars depicting standard error.

The cellular lactate production inhibition profiles of AZ 35 MME, as well as compounds 276 (single carboxylic acid) and 277 (single monomethyl ester), were also determined. Following 8 hour incubation of 50, 100, and 200 µM concentrations of each compound in HeLa cells, lactate production in all samples remained relatively unchanged (Figure 4.15). Compound AZ 35 MME was previously demonstrated to be poorly cell
permeable (Figure 4.11), and, similar to AZ 33, its inability to enter cells likely prevents cellular activity of this kinetic inhibitor of LDH-A. While the cell uptake of compounds 276 and 277 have not been assessed, kinetic analysis by the Minutolo research group revealed that neither compound is a potent \textit{in vitro} inhibitor of LDH-A.

![Graph showing lactate production inhibition](image)

\textbf{Figure 4.15:} Lactate production inhibition of compounds derived from the AstraZeneca LDH inhibitors. HeLa human cervical carcinoma cells were treated with indicated compound concentrations or 1% DMSO vehicle, in DMEM supplemented with 10 mM glucose, 1 mM pyruvate, and 4 mM glutamine. Following 8 hour incubation, medium aliquots were collected, concentrated, derivatized using MTBSTFA + 1% TBDMCS catalyst, and assessed by GC-MS. Lactate was normalized in each sample using the 1 mM chlorophenylalanine (CPA) internal standard, and lactate relative to vehicle is depicted. Averages from three or more independent experiments are depicted, with error bars depicting standard error (n=3).

Similar to previous cell lactate production inhibition experiments in which the ability of inhibitors to reduce the conversion of a specifically $^{13}$C-labeled precursor metabolite to lactate was assessed, the abilities of FX-11 and its methyl ester (FX-11 ME; a synthetic precursor, Figure 4.16), as well as NHIs 4j and Glu-4j, to reduce the formation of labeled lactate from labeled glucose or labeled pyruvate were tested.
Figure 4.16: The structure of the methyl ester of FX-11

All compounds were incubated at 100 µM concentrations in HeLa cells and fed 10 mM $^{13}$C-labeled glucose and standard concentrations of unlabeled pyruvate, glutamine, and amino acids, for 8 hours. It was determined that treatment with 100 µM concentrations of Glu-4j, but not 4j, FX-11, or FX-11 ME, led to a reduction in labeled lactate derived from labeled glucose (Figure 4.17). Since glucose is the primary source of lactate under these conditions (Figure 2.17), this result is consistent with the amount of lactate produced upon treatment with these concentrations of compounds in studies using unlabeled metabolites. Compound 4j does not substantially reduce lactate production.

Figure 4.17: Percent of $^{13}$C-labeled lactate production, derived from $^{13}$C-labeled glucose, compared to vehicle treatment. HeLa human cervical carcinoma cells were treated with indicated compound concentrations or 1% DMSO vehicle, in DMEM supplemented with 10 mM $^{13}$C-labeled glucose, 1 mM unlabeled pyruvate, and 4 mM unlabeled glutamine. Following 8 hour incubation, medium aliquots were collected, concentrated, derivatized using MTBSTFA + 1% TBDMCS catalyst, and assessed by GC-MS. Lactate was normalized in each sample using the 1 mM chlorophenylalanine (CPA) internal standard, and lactate relative to vehicle is depicted. Averages from three independent experiments are depicted, with error bars depicting standard error.
at 100 µM concentrations for 8 hours (Figure 2.18), while Glu-4j treatment shows a marked reduction in lactate production at this concentration and time point (Figure 3.17), and FX-11 is relatively inactive at this concentration and time point (Figure 4.13).

The abilities of 4j, Glu-4j, FX-11 and FX-11 ME to affect the transformation of $^{13}$C-labeled pyruvate to $^{13}$C-labeled lactate under these conditions (in which cells were also fed their preferential lactate source, 10 mM glucose, as well as other potential lactate sources including glutamine (at 4 mM) and other amino acids (at 200 - 800 µM)) were assessed. The results of this experiment are shown in Figure 4.18.

![Bar chart](image-url)

**Figure 4.18**: Percent of $^{13}$C-labeled lactate production, derived from $^{13}$C-labeled pyruvate, compared to vehicle treatment. HeLa human cervical carcinoma cells were treated with indicated compound concentrations or 1% DMSO vehicle, in DMEM supplemented with 10 mM unlabeled glucose, 1 mM $^{13}$C-labeled pyruvate, and 4 mM unlabeled glutamine. Following 8 hour incubation, medium aliquots were collected, concentrated, derivatized using MTBSTFA + 1% TBDMCS catalyst, and assessed by GC-MS. Lactate was normalized in each sample using the 1 mM chlorophenylalanine (CPA) internal standard, and lactate relative to vehicle is depicted. Averages from three independent experiments are depicted, with error bars depicting standard error.
Consistent with previous data obtained with these conditions (Figure 2.21), treatment with 10 mM 2DG led to an increase in lactate production derived from pyruvate (since 2DG blocks the conversion of glucose to lactate by inhibiting hexokinase), and etoposide treatment had no effect on lactate production derived from pyruvate. Also consistent with the previous result, neither 4j nor Glu-4j led to a reduction in labeled lactate production. (In a previous study, 4j led to a reduction in the conversion of labeled pyruvate to labeled lactate only when other potential sources of lactate were excluded. This experiment was performed with 1 mM labeled pyruvate in DMEM – which contains 200 µM – 800 µM concentrations of unlabeled amino acids – without serum, glucose, or glutamine, with results shown in Chapter 2, Figure 2.22). Intriguingly, in this study both FX-11 and FX-11 ME led to a moderate increase (136% and 141%, respectively) in the labeled lactate production derived from labeled pyruvate compared to the vehicle, though the overall lactate production was only slightly increased over vehicle treatment (115% and 119%, respectively), suggesting that these compounds do not inhibit LDH-A in cells.

4.4: Comparative cancer cell potency

An important preliminary assessment of the various in vitro LDH-A inhibitors is their potency in killing cancer cells in culture. Given that 4j, Glu-4j, and AZ 35 inhibit lactate production in cells, but galloflavin, FX-11, AZ 33, and AZ 35 MME do not substantially inhibit lactate production at concentrations of up to 200 µM, it is likely that the cytotoxicity of these latter compounds arises from mechanisms other than LDH-A inhibition. The 72 hour IC₅₀ value of each compound in HeLa cells was determined (Table 4.1). Gossypol was also included in this analysis for reference, and had the lowest IC₅₀ value (highest potency) in the series, consistent with its many reported targets and mechanisms of cytotoxicity in cells [8-11]. FX-11 was also shown to be reasonably potent (IC₅₀ value of 23 µM), despite its
inactivity against LDH-A, suggesting that it is targeting proteins or macromolecules besides LDH-A in cells. Indeed, enzymatic inhibition studies performed on FX-11 by ARIAD Pharmaceuticals demonstrated that the in vitro inhibition of LDH-A by FX-11 is ablated in the presence of 0.1% Triton detergent, indicative of aggregation. ARIAD researchers also assessed the binding of FX-11 to immobilized LDH-A by surface plasmon resonance (SPR) and found slow on- and off-rates consistent with aggregation [2]. Galloflavin was relatively inactive, and neither AZ 33 nor AZ 35 MME induced 50% cell death at concentrations up to 500 µM or 200 µM, respectively. Compound AZ 35, which inhibited lactate production in cells, was found to have an IC$_{50}$ value of 75.4 µM, 2-fold lower than that of 4j (Table 2.3) and 10-fold lower than that of Glu-4j (Table 3.2).

Table 4.1 – 72 hour IC$_{50}$ values of reported LDH-A inhibitors (µM ± standard error, n=3) in HeLa human cervical carcinoma cells in culture $^a$

<table>
<thead>
<tr>
<th>Compound</th>
<th>72 hr IC$_{50}$ value in HeLa human cervical carcinoma cells (Average ± StErr, µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>gossypol</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td>FX-11</td>
<td>23.3 ± 1.1</td>
</tr>
<tr>
<td>galloflavin</td>
<td>131.4 ± 20.4</td>
</tr>
<tr>
<td>AZ 33</td>
<td>&gt; 500</td>
</tr>
<tr>
<td>AZ 35</td>
<td>75.4 ± 11.9</td>
</tr>
<tr>
<td>AZ 35 MME</td>
<td>&gt; 200</td>
</tr>
</tbody>
</table>

$^a$ Three independent experiments were performed. Remaining biomass after fixing with 10% trichloroacetic acid was quantified by sulforhodamine B staining.

4.5: Potency in LDH-A knockdown cells

The potency of several classes of in vitro LDH-A inhibitors was assessed in A549 cells in which LDH-A was stably knocked down using shRNA. This shRNA is maintained in the cells using puromycin selection, as the plasmid containing the shRNA also codes for a
resistance marker to the eukaryotic poison puromycin, which is added to the culture at all times. This cell line was obtained from Dr. Pankaj Seth, who generated and characterized these cells [12].

When following the literature protocol for culturing A549 + pLKOΔLDHA knockdown cells (specifically using the minimal Ham’s F12 medium [12]), both the A549 + pLKOΔLDHA knockdown cells and the A549 + pLKO vector control cells grew very poorly. Both sets of cells became vacuolated and went into senescence after about 5 passages in culture. To culture cells such that they would be viable for growth and experiments, the growth medium was switched to DMEM, a richer medium which contains roughly four-fold the amounts of nutrients as Ham’s F12 medium.

4.5.1: Assessment of total LDH activity in knockdown cells

It was previously determined by Western blot [12] that the expression of LDH-A in the A549 + pLKOΔLDHA knockdown cells was markedly reduced, but the expression of LDH-B remained the same in LDH-A knockdown versus vector control cells. To complement this data, and to evaluate the utility of these cell lines in moving forward with further studies, total LDH activity of both cell lines, as well as wild type A549 cells, was determined. An assay was performed using a commercially available kit to measure lactate dehydrogenase activity using a coupled tetrazolium salt reduction reaction with a colorimetric readout (Scheme 4.2). Importantly, this assay does not distinguish between LDH-A and LDH-B activity, but instead provides a readout of total LDH activity. The assay protocol was modified to detect LDH activity in whole cells by using the kit’s lysis reagent, normally used to lyse the dead control cells, to lyse all cells at the beginning of the study.
Scheme 4.2: Colorimetric assay used to calculate total LDH activity of cells. Cells are lysed using conditions that allow for the release and continued activity of LDH enzymes. L-lactate, NAD\textsuperscript{+}, the tetrazolium salt INT (2-(4-iodophenyl)-3-(4-nitrophenoxy)-5-phenyl-2H-tetrazolium), and a diaphorase catalyst are added. Upon incubation, the LDH present in lysed cells will convert lactate to pyruvate, and the NADH generated from this reaction will allow the tetrazolium salt to be reduced to a red formazan dye. Following quenching of the reaction, the absorbance of the produced formazan dye at 490 nm is proportional to the total LDH activity present in the cell sample.

Using this modified protocol, the total LDH activity in A549 cells, A549 + pLKO vector control cells, and A549 + pLKΔLDHA cells was determined. While the total LDH activity of the A549 + pLKO vector control cells is not significantly different from that of the A549 cells, LDHA knockdown led to a statistically significant reduction in total LDH activity (Figure 4.19). A large portion of total LDH activity measured using this assay must be derived from LDH-B, which was shown to be present in these cells [12].
Figure 4.19: Total LDH activity of A549, A549 + pLKO vector control cells. LDH activity was determined in freshly-lysed cells using a colorimetric detection system involving tetrazolium salt reduction. Averages are shown with error bars denoting standard error (n=3). Statistical analysis was performed using a two-tailed Student’s t test, with * denoting p < 0.05.

4.5.2: IC\textsubscript{50} determination of compounds in knockdown cells

The ability of the NHI class and other classes of in vitro LDH-A inhibitors to induce cell death in LDH-A knockdown cells was determined. To do this, the 72 hour IC\textsubscript{50} values of several NHIs (1\textit{j}, its methyl ester 4\textit{j}, its ethyl ester 20\textit{1}, its dichloro analog 14\textit{7}, and the methyl ester of the dichloro analog 15\textit{4}, as well as Glu-4\textit{j}) and other classes of LDH-A inhibitors (gossypol and FX-11) were assessed in all three cell lines (wild type A549 cells, A549 cells containing the pLKO vector control, and A549 cells containing the pLKOΔLDHA vector). The findings from these studies are shown in Table 4.2. Only 1\textit{j} was less potent (had a higher IC\textsubscript{50} value) in the LDH-A knockdown cells than in the vector control and wild type cells. Compounds 4\textit{j}, Glu-4\textit{j}, 14\textit{7}, and gossypol had relatively similar IC\textsubscript{50} values in
Table 4.2 – 72 hour IC\textsubscript{50} values of reported LDH-A inhibitors (µM ± standard error, n=3) in A549, A549 + pLKO vector, and pLKOΔLDH-A knockdown cells in culture \textsuperscript{a}

<table>
<thead>
<tr>
<th>Compound</th>
<th>A549</th>
<th>A549 + pLKO</th>
<th>A549 + pLKOΔLDH-A</th>
</tr>
</thead>
<tbody>
<tr>
<td>1j</td>
<td>122.7 ± 15.3</td>
<td>137.0 ± 18.2</td>
<td>226.7 ± 33.5</td>
</tr>
<tr>
<td>4j</td>
<td>46.2 ± 2.1</td>
<td>41.7 ± 3.4</td>
<td>40.5 ± 2.3</td>
</tr>
<tr>
<td>Glu-4j</td>
<td>24.0 ± 4.1</td>
<td>12.3 ± 1.0</td>
<td>15.8 ± 2.6</td>
</tr>
<tr>
<td>201</td>
<td>36.7 ± 3.2</td>
<td>23.0 ± 1.0</td>
<td>24.3 ± 3.8</td>
</tr>
<tr>
<td>147</td>
<td>53.1 ± 4.1</td>
<td>68.8 ± 11.9</td>
<td>49.3 ± 8.2</td>
</tr>
<tr>
<td>154</td>
<td>32.3 ± 8.6</td>
<td>25.8 ± 2.0</td>
<td>19.8 ± 0.3</td>
</tr>
<tr>
<td>gossypol</td>
<td>8.2 ± 1.9</td>
<td>9.5 ± 0.8</td>
<td>9.0 ± 1.6</td>
</tr>
<tr>
<td>FX-11</td>
<td>82.4 ± 6.2</td>
<td>64.5 ± 12.5</td>
<td>47.0 ± 0.7</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Three independent experiments were performed. Remaining biomass after fixing with 10% trichloroacetic acid was quantified by sulforhodamine B staining.

The implications of these results are not immediately clear. Would a \textit{bona fide} LDH-A inhibitor be more or less potent in an LDH-A knockdown cell line compared to the wild type cell line? GlaxoSmithKline scientists reported that treatment with potent LDH-A inhibitor \textit{GSK 1} in HCC1937 breast ductal carcinoma cells in which LDH-A had been knocked down led to no difference in extracellular acidification rate (ECAR) compared to control cells, though treatment with \textit{GSK 1} did lead to reduction of ECAR in HCC1937 cells in which LDH-B was knocked down [13]. However, the majority of literature characterizing the biological activity of other classes of LDH-A inhibitors [4-6, 14] does not report the relative potencies of inhibitors in LDH-A knockdown cells. Perhaps reduced potency in the
LDH-A knockdown cell line compared to the control cell line signifies that in the absence of the target (LDH-A), the compound is non-toxic. Perhaps enhanced potency in the LDH-A knockdown cell line compared to the control cell line signifies that with less LDH-A protein in the cell, lower concentrations of inhibitor are required to fully inhibit LDH-A and induce cell death, or that off-target effects contribute to cytotoxicity.

4.6: Potency of LDH-A inhibitors in normoxia versus hypoxia

A common hypothesis in using a drug targeting a glycolytic enzyme is that this drug will be especially potent in hypoxia, since in normoxia cells can overcome the drug’s inhibition and evade cell death by shunting metabolites toward oxidative metabolism to produce ATP. Since these alternate pathways for ATP generation are not accessible in the absence of O₂, inhibiting an enzyme such as LDH-A in hypoxia would then induce cell death at lower concentrations than those required in normoxia. Indeed, we have previously reported that first generation NHIs 1h, 1i, and 1j are significantly more potent in inducing cell death in hypoxia (48 hour IC₅₀ values of less than 1 µM) than in normoxia (48 hour IC₅₀ values of 14-18 µM) in LPC006 primary pancreatic cancer cells [15]. Hexokinase II inhibitor 3-bromopyruvate was also shown to be selectively potent in hypoxia in HCT116 human colorectal cancer cells and Raji human Burkitt’s lymphoma cells, while other chemotherapeutics such as doxorubicin and ara-C (1-β-D-arabinofuranosylcytosine, an antimetabolite) have been shown to be less potent in hypoxia compared to normoxia [16].

Based on this hypothesis, a series of experiments comparing the potencies of various LDH-A inhibitors in normoxia versus hypoxia were performed. Tirapazamine and deoxyxynboquinone (DNQ) were used as controls for selective potency in hypoxia and normoxia, respectively. Tirapazamine (Figure 4.20) is bioreductively activated to a toxic
free radical species in low oxygen conditions, and is currently in clinical trials in the United States for various solid cancers [17]. DNQ (Figure 4.21) is a substrate of the enzyme NQO1 (NAD(P)H:quinone oxidoreductase 1) that induces redox cycling and ROS generation [18-20], and is thus selectively toxic in the presence of oxygen.

Figure 4.20: The structures of Tirapazamine (selectively potent in hypoxia) and DNQ (selectively potent in normoxia)

The 72 hour IC$_{50}$ values of tirapazamine, DNQ, the hexokinase inhibitor 2DG, the NHIs 4j and Glu-4j, the nonselective LDH-A inhibitor gossypol, and gossypol derivative FX-11 were determined in HeLa cells in both normoxic (20% oxygen) and hypoxic (1% oxygen) conditions (Table 4.3). While tirapazamine was approximately 5-fold more potent in hypoxia compared to normoxia, and DNQ was approximately 3-fold more potent in normoxia compared to hypoxia, mixed results were seen with the other compounds assessed. Compound 2DG and 4j were approximately equipotent in normoxia compared to hypoxia. Compounds Glu-4j and FX-11 were approximately 2-fold less potent in hypoxia compared to normoxia, and gossypol was roughly 5-fold less potent in normoxia compared to hypoxia.
Table 4.3 – 72 hour IC$_{50}$ values of reported LDH-A inhibitors (µM ± standard error, n=3) in normoxia versus hypoxia in HeLa human cervical carcinoma cells $^a$

<table>
<thead>
<tr>
<th>Compound</th>
<th>Normoxia (Average ± StErr, µM)</th>
<th>Hypoxia (Average ± StErr, µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tirapazamine</td>
<td>24.2 ± 1.0</td>
<td>4.8 ± 0.5</td>
</tr>
<tr>
<td>DNQ</td>
<td>0.030 ± 0.013</td>
<td>0.084 ± 0.017</td>
</tr>
<tr>
<td>2DG $^b$</td>
<td>1100</td>
<td>1350</td>
</tr>
<tr>
<td>4j</td>
<td>27.1 ± 3.2</td>
<td>35.1 ± 2.9</td>
</tr>
<tr>
<td>Glu-4j</td>
<td>9.0 ± 1.2</td>
<td>20.1 ± 1.3</td>
</tr>
<tr>
<td>FX-11</td>
<td>23.3 ± 1.1</td>
<td>48.6 ± 5.3</td>
</tr>
<tr>
<td>gossypol</td>
<td>1.7 ± 0.1</td>
<td>8.8 ± 0.6</td>
</tr>
</tbody>
</table>

$^a$ Three independent experiments were performed. Remaining biomass after fixing with 10% trichloroacetic acid was quantified by sulforhodamine B staining.

$^b$ n=1

Since gossypol is known to have multiple mechanisms in cells, including acting as a BH3 mimetic, binding to anti-apoptotic proteins to induce apoptotic cell death [10], binding and non-competitively inhibiting DNA polymerase α [11], and binding human serum albumin [21], its single-digit micromolar potency in both hypoxia and normoxia is unsurprising and likely reflects multiple modes of inducing cell death. For the remainder of compounds assessed, a possible reason for the results obtained are that immortalized cancer cells adapted to long-term culture tend to be highly glycolytic even under normal oxygen tension [22], and so a temporary shift into hypoxic conditions may not allow for a marked difference in potency for compounds that modulate tumor glycolysis. It is unclear whether different results may have been seen by allowing cells to acclimate to hypoxia for longer than 6 hours.

The hypoxia 72 hour IC$_{50}$ values of several compounds in both normoxia and hypoxia were also determined in several other cell lines, including 3T3 mouse embryonic
fibroblast cells and CHO-K1 Chinese hamster ovary cells. However, though tirapazamine continued be selectively potent in hypoxia and DNQ continued to be selectively potent in normoxia in these cell lines, no substantial difference was observed between the potency of LDH-A inhibitors in normoxia versus hypoxia.

4.7: Metabolic profiling analysis

A series of metabolic profiling studies were performed in A549 cells to assess the changes in glucose and amino acid metabolism induced by treatment with in vitro LDH-A inhibitors. Compounds chosen for inclusion in this study were: two representative NHI class compounds (4j and Glu-4j) non-selective inhibitor gossypol and its derivative FX-11, and cell-permeable prodrug AZ 35. (In vitro LDH-A inhibitor galloflavin was excluded from metabolic profiling studies due to its poor cell uptake and poor cellular activity.) The hexokinase inhibitor 2-deoxyglucose (2DG) was also included in these metabolic profiling studies for comparison between the metabolic profiles of LDH-A inhibition and the inhibition of an earlier glycolytic step.

In order to treat with a concentration of compound that would show cellular effects during the 6 hour sampling interval chosen, the 24 hour $IC_{50}$ value of each compound was determined in A549 cells. This concentration (or, in the case of 2DG, a high concentration at which efficacy is seen in cellular lactate production inhibition studies) was used for the 6 hour cell treatment. These treatment concentrations are listed in Table 4.4.
### Table 4.4 – Concentrations used for metabolic profiling studies in A549 cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration used for metabolic profiling studies (µM) (24 hr IC₅₀ value, in µM, in A549 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4j</td>
<td>75</td>
</tr>
<tr>
<td>Glu-4j</td>
<td>35</td>
</tr>
<tr>
<td>FX-11</td>
<td>115</td>
</tr>
<tr>
<td>AZ 35</td>
<td>34</td>
</tr>
<tr>
<td>Gossypol</td>
<td>10,000</td>
</tr>
<tr>
<td>2DG</td>
<td></td>
</tr>
</tbody>
</table>

For metabolic profiling studies, confluent T150 flasks of A549 cells in consecutive passages were treated with the indicated concentrations of compounds to generate a total of five biological replicates per treatment. Following treatment, cells were washed twice in sterile PBS, and cell pellets in methanol were stored at -80 °C until all samples were collected. Pellets were then sonicated, and metabolites were extracted from sonicates in a 4-step process to obtain both polar and nonpolar metabolites. Sonicates were treated with pure methanol, then a 1:1 methanol:water mixture, then pure water, and finally chloroform. Soluble fractions at each step were pooled together and dried, with the addition of internal standard near the end of the drying step. Dried extracts were then chemically derivatized in two steps (Scheme 4.3). Methoximation was performed first, so that carbonyl groups could be differentiated by mass spectrometry from the alcohols that would be silylated by MSTFA in the second step. Derivatized samples were then analyzed by GC-MS. A total of 35 samples (5 replicates of 7 treatment groups) were subjected to extraction, derivatization, and analysis by GC-MS.
1. Methoximation

\[
\begin{align*}
R_1 & \quad R_2 \\
\text{O} & + \quad \text{NH}_2 \\
\text{OH} & \quad \text{MeO} \cdot \text{HCl} \\
\text{methoxyamine HCl} & \quad \text{pyridine, 50°C} \\
1 \text{ hour} & \quad R_1 \quad R_2 \\
\text{NOMe} & \quad \text{NO}_2 \\
\text{NOMe} & \quad \text{NOMe}
\end{align*}
\]

2. Silylation

\[
\begin{align*}
R_1 & \quad R_2 \\
\text{NOMe} & + \quad \text{Me}_3\text{Si} - \text{N} - \text{Me} \\
\text{OH} & \quad \text{F}_3\text{C} \\
\text{MSTFA} & \quad 70°C \\
2 \text{ hours} & \quad R_1 \quad R_2 \\
\text{NO}_2 & \quad \text{Me}_3\text{Si} - \text{Me} \\
\text{NO}_2 & \quad \text{Me}_3\text{Si} - \text{Me}
\end{align*}
\]

MSTFA = N-methyl-N-(trimethylsilyl) trifluoroacetamide

**Scheme 4.3:** The two-step derivatization process used to methoximate and silylate amino acids, sugars, and other cellular metabolites for GC-MS detection. In the first step, methoximation, metabolites are reacted with 80 µL 20 mg/mL methoxyamine HCl in pyridine at 50 °C for 1 hour. This step serves to modify carbonyl groups so they are not derivatized by the silylating reagent. In the second step, silylation, metabolites are reacted with MSTFA for 2 hours at 70 °C to append trimethylsilyl (TMS) groups to primary and secondary alcohols and amines.

Following completion of GC-MS analysis, metabolites were identified using AMDIS Chromatograph software, using custom-built libraries of spectra combining the W8N08 and NIST08 commercial libraries [23]. Relative concentrations of metabolites were then calculated based on the peak intensity of the known concentration of internal standard added to each standard. Each of the 35 samples' lists of metabolites (ranging from 300-400 metabolites) was then manually curated. This curation served to 1) combine different peaks derived from the same metabolite (i.e. alanine which had been silylated on one versus two positions), and 2) to discard metabolites present in fewer than 2 samples.
After this process, statistical analysis of the data was performed using R software [24]. Statistical testing for differences between treatments was performed by first fitting a mixed linear model to estimate the level of each of the seven treatment groups using the limma package [25], which uses an empirical Bayes correction [26] that helps to improve power by borrowing information across metabolites; the model also adjusted for the correlation due to differences in the days on which samples were obtained [27]. Pairwise contrasts between the vehicle control and the other 6 groups were then extracted from the model, as well as the equivalent of a 1-way ANOVA between the seven groups. Correction for multiple hypothesis testing using the false discovery rate (FDR) method [28] was formed for the 1-way ANOVA. A cumulative data table was compiled for all treatment groups, listing the metabolites found to be present at significantly different concentrations in compound-treated versus vehicle-treated cells (Table 4.5).

Upon treatment with 4j, levels of early glycolytic intermediates, such as glucose, glucose 6-phosphate, glyceraldehyde 3-phosphate, and 3-phosphoylcerate were increased several-fold compared to vehicle treatment (Table 4.5), as would be consistent with inhibition of a later glycolytic step. Pyruvate was present at concentrations almost 6-fold less than vehicle (Table 4.5), suggesting that the pyruvate produced was rapidly shuttled into alternate metabolic pathways, such as the citric acid cycle. In addition, levels of both sedoheptulose and ribose 5-phosphate, intermediates of the pentose phosphate pathway, were increased upon 4j treatment compared to vehicle treatment (Table 4.5), suggesting diversion of built-up early glycolytic intermediates into this alternate pathway. The pentose phosphate pathway (PPP) allows for the synthesis of nucleic acid precursors as well as NADPH, which is crucial in defending cells against reactive oxygen species (ROS) by allowing for the recycling of reduced glutathione. The PPP has been shown to be
**Table 4.5** – Fold-changes of metabolic intermediates upon treatment of A549 cells with various agents targeting cellular metabolism $^a$

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Metabolite</th>
<th>4j</th>
<th>Glu-4j</th>
<th>FX-11</th>
<th>gossypol</th>
<th>AZ 35</th>
<th>2D G</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycolysis</td>
<td>Glucose</td>
<td>7.9</td>
<td>3.3</td>
<td>8.9</td>
<td>12</td>
<td>5.5</td>
<td>8.5</td>
</tr>
<tr>
<td></td>
<td>Glucose 6-phosphate</td>
<td>8.5</td>
<td>15</td>
<td>12</td>
<td>31</td>
<td>2.9</td>
<td>6.2</td>
</tr>
<tr>
<td></td>
<td>Fructose</td>
<td>3.2</td>
<td>2.6</td>
<td>3.6</td>
<td>-</td>
<td>-</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>Glyceraldehyde</td>
<td>-</td>
<td>26</td>
<td>-</td>
<td>18</td>
<td>2.7</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Glyceraldehyde 3-phosphate</td>
<td>6.2</td>
<td>49</td>
<td>18</td>
<td>33</td>
<td>8.6</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>3-phosphoglycerate</td>
<td>25</td>
<td>3.9</td>
<td>34</td>
<td>84</td>
<td>10</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>2-phosphoglycerate</td>
<td>-</td>
<td>-4.8</td>
<td>-</td>
<td>137</td>
<td>-2.7</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>Glycric acid</td>
<td>29</td>
<td>-</td>
<td>118</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Pyruvate</td>
<td>5.7</td>
<td>-19</td>
<td>-</td>
<td>-</td>
<td>-4.2</td>
<td>-</td>
</tr>
<tr>
<td>Citric acid cycle</td>
<td>Citrate</td>
<td>-</td>
<td>-</td>
<td>3.5</td>
<td>-28</td>
<td>-</td>
<td>-2.7</td>
</tr>
<tr>
<td></td>
<td>Fumarate</td>
<td>2.4</td>
<td>2.1</td>
<td>3.1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Malate</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3.1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pentose phosphate pathway</td>
<td>Seduheptulose</td>
<td>15</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2.6</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>Ribose 5-phosphate</td>
<td>2.0</td>
<td>-</td>
<td>3.0</td>
<td>-</td>
<td>3.8</td>
<td></td>
</tr>
<tr>
<td>Amino acid metabolism</td>
<td>Isoleucine</td>
<td>65</td>
<td>39</td>
<td>55</td>
<td>-</td>
<td>48</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>Glutamine</td>
<td>19</td>
<td>7.3</td>
<td>11</td>
<td>14</td>
<td>-</td>
<td>3.4</td>
</tr>
<tr>
<td></td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>Aspartate</td>
<td>2.4</td>
<td>-</td>
<td>-</td>
<td>2.8</td>
<td>-2.6</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Arginine</td>
<td>-</td>
<td>4.5</td>
<td>-</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Serine</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2.9</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Asparagine</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>14</td>
</tr>
</tbody>
</table>

$^a$ values listed are fold-changes of metabolites found to be significantly increased in compound treatment versus vehicle. Table entries with dashes ("-") denote metabolites in treatment groups that were found not to be significantly changed compared to vehicle treatment. Each fold-change is an average of five biological replicates collected over three independent experiments.
upregulated in some cancer cells as a stress response against ROS [29]. Accordingly, this upregulation of PPP intermediates upon 4j treatment may indicate a response to ROS generated by 4j. Finally, treatment with 4j led to increased cellular levels of several amino acids, notably glutamine (Table 4.5). Glutaminolysis – the process of converting glutamine to glutamate, which can then be converted to α-ketoglutarate and additional metabolic intermediates and cellular building blocks – has been proposed as a major metabolic pathway in cancer cells [30, 31]. Since the cell culture medium in which cells were grown during the metabolic profiling study – RPMI 1640 – contains 1 mM glutamine, the cells may have increased their uptake of glutamine from the medium in order to serve various metabolic purposes upon 4j treatment.

The metabolic profile of Glu-4j was found to be in many ways similar to the metabolic profile of 4j. Metabolites which were present at several-fold or greater concentrations in Glu-4j treated cells compared to vehicle-treated cells included glycolytic intermediates (glucose, glucose 6- and glucose 1-phosphate, fructose, glyceraldehyde 3-phosphate, and 3-phosphoglycerate), citric acid cycle intermediates (fumarate), and amino acids (glutamine, isoleucine) (Table 4.5). Intriguingly, both pyruvate and 2-phosphoglycerate (the product of the seventh enzyme of glycolysis, phosphoglycerate mutase, which acts on 3-phosphoglycerate) were present in several-fold decreased amounts in Glu-4j treated compared to vehicle-treated cells, suggesting that earlier intermediates in glycolysis were being funneled toward alternate cellular pathways.

Upon treatment with FX-11, cells were found to have increased levels of early glycolytic intermediates compared to vehicle treatment, including glucose, glucose 6-phosphate, glyceraldehyde 3-phosphate, and 3-phosphoglycerate (Table 4.5). Moderately increased levels of citric acid cycle intermediates such as citrate and fumarate, and pentose
phosphate pathway intermediates such as ribose 5-phosphate, compared to vehicle-treated cells, were also observed. This suggests funneling of metabolic products toward pathways other than glycolysis. The substantial (55-fold) increase in the branched chain amino acid isoleucine noted in cells treated with **FX-11** is intriguing; isoleucine can be converted to citric acid cycle intermediates succinyl-CoA and oxaloacetate, perhaps providing an alternate mechanism for **FX-11**-treated cells to gain energy through oxidative metabolism upon glycolysis inhibition. Indeed, **FX-11** treatment has been shown to increase cellular oxygen consumption while decreasing lactate production and extracellular acidification rate [14].

Metabolites present in higher amounts in gossypol-treated cells relative to vehicle-treated cells included early glycolytic intermediates glucose, glucose 6-phosphate, glyceraldehyde 3-phosphate, 3-phosphoglycerate (notably 87-fold higher), and 2-phosphoglycerate (notably 137-fold higher; Table 4.5). Interestingly, gossypol treatment reduced the concentration of the citric acid cycle intermediate citrate by 28-fold and increased glutamine concentration by 14-fold compared to vehicle treated cells. These findings seem to suggest a blockage in later steps of glycolysis, use of glutamine as an alternate carbon source, and perhaps reduction in citric acid cycle metabolism to shunt carbon backbones toward other metabolic pathways.

A549 cells treated with **AZ 35** had increased levels of early glycolytic intermediates such as glucose, glucose 6-phosphate, glyceraldehyde 3-phosphate, and 3-phosphoglycerate, but decreased levels of later glycolytic intermediates 2-phosphoglycerate and pyruvate, compared to vehicle-treated cells (Table 4.5). Two intermediates of the pentose phosphate pathway (ribose 5-phosphate and sedoheptulose) were also found to be slightly increased in **AZ 35**-treated cells compared to vehicle-treated cells. These results
would suggest downregulation of terminal glycolytic reactions and funneling of metabolites toward other cellular pathways, including the pentose phosphate pathway to potentially neutralize any reactive oxygen species generated by AZ 35. To date, no other mechanistic studies of AZ 35 have been reported, so it is unclear whether AZ 35 induces ROS production or leads to increased oxygen consumption, which would further support these data.

Finally, metabolic profiling of A549 cells treated with 2DG demonstrated increased levels of glycolytic intermediates glucose, glucose 6-phosphate, fructose, 3-phosphoglycerate, and 2-phosphoglycerate compared to levels found in vehicle-treated cells (Table 4.5). The glucose analog 2DG is known to competitively inhibit phosphoglucoisomerase (which converts glucose 6-phosphate to fructose 6-phosphate) as well as to noncompetitively inhibit hexokinase (which converts glucose to glucose 6-phosphate) [32]. Glucose 6-phosphate levels in 2DG-treated cells were found to be modestly elevated compared to vehicle-treated cells, and fructose 6-phosphate levels were not significantly altered. The pentose phosphate pathway intermediate ribose 5-phosphate, found in modestly-increased levels in 2DG-treated cells, can be converted to fructose 6-phosphate, potentially overcoming the inhibition of 2DG in these cells to account for the increased levels of other glycolytic intermediates.

In summary, these metabolic profiling results demonstrate that treatment of A549 cells with various classes of LDH-A inhibitors or 2DG lead to perturbations in levels of many glycolytic intermediates as well as a few intermediates of the pentose phosphate pathway, the citric acid cycle, and amino acid metabolism. While one goal of metabolic profiling studies was to elucidate the metabolic profile of LDH-A inhibition, there does not appear to be a ‘consensus’ phenotype from compound treatment in this data set. One comparison
that was not performed was comparing the metabolic profiles of LDH-A knockdown cells with the metabolic profiles of wild type cells treated with \textit{in vitro} LDH-A inhibitors. However, several factors precluded the performance of such an experiment, including: 1) the difficulty of culturing sufficient quantities of viable A549 + pLKOΔLDHA knockdown cells before the cells would undergo senescence, 2) the mixed results obtained when determining the potencies of compounds in LDH-A knockdown cells (\textbf{Table 4.2}), and 3) the utility in comparing the metabolic profiles of cells in which LDH-A had been permanently knocked down, causing the cells to undergo permanent metabolic rerouting, versus the metabolic profiling of cells in which LDH-A activity was transiently, and perhaps only partially, inhibited, for only a short time period.

\textbf{4.8: Cytological profiling analysis}

Cytological profiling is an emerging technique to characterize and classify changes in cytoplasmic and nuclear marker levels, and overall cell and nuclear morphology, in both mammalian [33-35] and bacterial [36] cells, induced by treatment with various bioactive compounds. In mammalian cells, cytological profiling studies have been performed to screen compounds with unknown modes of action alongside compounds with known modes of action. Similarities in changes in nuclear and cytoplasmic marker staining induced by compound treatment led to both elucidation of unknown compounds’ modes of action and the development of a clustering scheme of compounds with similar modes of action [34]. For instance, under this classification scheme, Cluster A compounds were defined as those resulting in cell cycle arrest due to inhibition of DNA replication. Phenotypically, these cells had enlarged nuclei and decreased staining with the fluorescent markers 5-ethynyl-2’-deoxyuridine (EdU), which is normally added to replicating DNA strands in S phase, and antiphospho-histone H3 (pH3), an antibody binding histones in cells undergoing mitosis.
Cluster A compounds identified using cytological profiling included agents known to affect DNA synthesis or transcription, including DNA crosslinker mitomycin C, topoisomerase I inhibitor camptothecin, and transcription inhibitor actinomycin D [34]. Cluster B compounds included the pan-kinase inhibitor staurosporine; this compound cluster was characterized as inducing a larger-than-average nuclear morphology in treated cells. In contrast, cluster C compounds were defined as those that led to cell cycle arrest, but with the smaller, condensed nuclei of cells undergoing apoptosis. Anthracycline, which is believed to intercalate DNA and inhibit topoisomerase II, and its derivatives, were found to populate cluster C. Cluster D compounds included the v-ATPase inhibitor bafilomycin A1 and compounds with similar modes of action; treatment of these compounds led to cells with decreased nuclear size and decreased DNA replication. Cluster E compounds were defined as having a weak phenotype, with cytological profiling markers in compound-treated cells that deviated little from untreated cells [34].

The goal of cytological profiling studies performed with in vitro LDH-A inhibitors was to determine if cellular treatment with various classes of LDH-A inhibitors led to similar or different cytological profiles. Cytological profiling experiments were performed at the Chemical Screening Center at the University of California Santa Cruz by Professor Scott Lokey’s research group. Five in vitro LDH-A inhibitors evaluated: 4j, Glu-4j, FX-11, gossypol, and AZ 35. The cytological profiling experiment (cell culture treatment and microscopy) was performed following the Lokey group’s published protocol [34]. Briefly, HeLa cells cultured in 384-well plates were treated with 10 concentrations of each compound for 24 hours at 37 °C. Two identical plates were prepared for all compounds, one of which was stained for nuclear markers and the other for cytoplasmic markers. Nuclear stains were EdU, pH3, and Hoechst. Cytoplasmic stains were α-tubulin, actin, pH3,
and Hoechst. Resulting microscopy images were quantified to measure and score different elements of cell morphology and staining. Representative fluorescent microscopy images from the cytological profiling experiment are shown in Figure 4.21.

Data were analyzed using Java TreeView software [37], and compounds were assigned to clusters based on the criteria outlined by the Lokey and Schulze research groups [34]. Heat maps generated this analysis are shown for 4j (Figure 4.22), Glu-4j (Figure 4.23), FX-11 (Figure 4.24), gossypol (Figure 4.25), and AZ 35 (Figure 4.26). Based on the heat maps and the previous criteria outlined for assigning compounds to phenotypic clusters [34], the five compounds analyzed were assigned to clusters. Given that the potency of all compounds tested (with the exception of gossypol) was fairly low, only the highest few concentrations of each compound likely resulted in phenotypic changes consistent with the compound’s mode of action. Thus, cluster assignments were made, in collaboration with Walter Bray at the University of California Santa Cruz, based largely on the phenotypes generated from treatment with the higher concentrations of each compound.

Treatment of HeLa cells with 16.7 and 52.7 µM concentrations of 4j led to positive deviations in nuclear intensity and both nuclear and cytosolic pH3, with strongly negative deviations in nuclear morphology, tubulin, actin, and EdU compared to control cells (Figure 4.22), indicative of the Cluster D phenotype of cells undergoing apoptosis. Treatment with 16.7 and 52.7 µM concentrations of Glu-4j also led to negative deviations in nuclear morphology, tubulin, actin, and EdU, with only slight positive deviations in nuclear intensity compared to control cells (Figure 4.23). Interestingly, Glu-4j treatment also led to decreases in nuclear and cytosolic pH3 and an increase in multinucleated cells at the highest concentration (52.7 µM). These data indicate that Glu-4j induces apoptosis similar
Figure 4.2: Confocal microscopy images of HeLa cells treated for 24 hours with the indicated concentrations of compound. In the cytoplasmic staining panels, blue staining represents total DNA (Hoechst), red staining represents actin, green staining represents tubulin, and cyan staining represents phosphorylated histone H3. In the nuclear staining panels, blue staining represents total DNA (Hoechst), cyan staining represents EdU (newly synthesized DNA), and magenta staining represents phosphorylated histone H3.
but the increase in multinucleated cells and decrease in mitotic markers (cytosolic to 4j and other Cluster D compounds, and nuclear pH3) observed in cells treated with Glu-4j may have been due to incomplete or defective mitosis occurring in these cells.

In assessing the cytological profiles of other classes of reported in vitro LDH-A inhibitors, FX-11 treatment was similar to 4j treatment, with positive deviations in nuclear intensity and both nuclear and cytosolic pH3, and with strongly negative deviations in nuclear morphology, tubulin, actin, and EdU compared to control cells (Figure 4.24). Interestingly, treatment of cells with 16.7 µM, but not 52.7 µM, FX-11 led to a marked increase in the number of multinucleated cells present. These data seem to indicate that FX-11 induces a similar cell death mechanism as both 4j and Glu-4j, which perhaps matches the Cluster D apoptotic mechanism.

In assessing the cytological profile of cells treated with gossypol, all concentrations assessed led to marked increases in nuclear pH3, smaller increases in cytoplasmic pH3, larger nuclear morphology, and markedly decreased actin, tubulin, and DNA replication (EdU staining; Figure 4.25). This phenotype is similar to that described by Lokey and coworkers as cluster A, populated by compounds that directly interfere with DNA replication or transcription [34].

Finally, treatment of HeLa cells with AstraZeneca inhibitor AZ 35 led to only slight deviations in all parameters measured compared to control treatment (Figure 4.26). Thus, at concentrations of up to 52.7 µM, AZ 35 best fits with Cluster E (weak phenotype) compounds. Treatment of cells with higher concentrations of this compound may be useful to further elucidate its mode of cell death.

Overall, cytological profiling results demonstrated that the mode of cell death induced by gossypol treatment is different from that induced by 4j, Glu-4j, and FX-11. While cytological profiling studies may have utility in characterizing modes of action of
compounds that directly interact with the markers assessed (DNA replication, actin, microtubules), the utility of these studies in compounds that we believe act primarily on glycolytic enzymes is limited.
Figure 4.22: Heat map visualized using Java TreeView software of the cytological changes resulting from various concentrations of 4j treatment (displayed in rows) in HeLa cells. Each column represents a particular queried feature, which have been grouped here by general class (PH3 in cytosol, nuclear morphology, etc.) Increasingly-saturated red rectangles indicate positive deviations compared to vehicle treatment. Increasingly-saturated blue rectangles indicate negative deviations compared to vehicle treatment.
Figure 4.23: Heat map visualized using Java TreeView software of the cytological changes resulting from various concentrations of **Glu-4j** treatment (displayed in rows) in HeLa cells. Each column represents a particular queried feature, which have been grouped here by general class (PH3 in cytosol, nuclear morphology, etc.) Increasingly-saturated red rectangles indicate positive deviations compared to vehicle treatment. Increasingly-saturated blue rectangles indicate negative deviations compared to vehicle treatment.
Figure 4.24: Heat map visualized using Java TreeView software of the cytological changes resulting from various concentrations of FX-11 treatment (displayed in rows) in HeLa cells. Each column represents a particular queried feature, which have been grouped here by general class (PH3 in cytosol, nuclear morphology, etc.) Increasingly-saturated red rectangles indicate positive deviations compared to vehicle treatment. Increasingly-saturated blue rectangles indicate negative deviations compared to vehicle treatment.
Figure 4.25: Heat map visualized using Java TreeView software of the cytological changes resulting from various concentrations of gossypol treatment (displayed in rows) in HeLa cells. Each column represents a particular queried feature, which have been grouped here by general class (PH3 in cytosol, nuclear morphology, etc.) Increasingly-saturated red rectangles indicate positive deviations compared to vehicle treatment. Increasingly-saturated blue rectangles indicate negative deviations compared to vehicle treatment.
Figure 4.26: Heat map visualized using Java TreeView software of the cytological changes resulting from various concentrations of AZ 35 treatment (displayed in rows) in HeLa cells. Each column represents a particular queried feature, which have been grouped here by general class (PH3 in cytosol, nuclear morphology, etc.) Increasingly-saturated red rectangles indicate positive deviations compared to vehicle treatment. Increasingly-saturated blue rectangles indicate negative deviations compared to vehicle treatment.
4.9: Summary and conclusions

In summary, head-to-head evaluation of several different classes of LDH-A inhibitors was performed using several cell-based assays. Among the AstraZeneca inhibitors, diacid AZ 33 was confirmed to be a very potent kinetic inhibitor of LDH-A in vitro, but this compound was found to have poor cellular uptake, no ability to reduce cellular lactate production, and no cytotoxicity against cancer cell lines in culture. The AstraZeneca dimethyl ester, AZ 35, was shown to be inactive in inhibiting LDH-A in vitro, but in cells and cell lysate, this compound was demonstrated to be cleaved to a monomethyl ester species. This cleavage product, when independently synthesized and evaluated, was found to be a moderately potent in vitro inhibitor of LDH-A. Evaluation of AZ 35 in cells demonstrated that it was taken up by cells in similar concentration to NHI class inhibitor 4j, and it was moderately potent in reducing cellular lactate production and inducing cancer cell death.

In evaluating other classes of LDH-A inhibitors using the same methodology established for the NHIs, galloflavin was found to be poorly uptaken by cells, unable to reduce cancer cell lactate production, and poorly potent in killing cancer cells in culture. While the cell uptake levels of FX-11 and Genentech 35 were not assessed, these compounds were found to have only modest ability to reduce cancer cell lactate production.

Various classes of LDH-A inhibitors were assessed to determine their potencies in LDH-A knockdown cells and in hypoxia, as well as their phenotypes in several whole-cell approaches, including metabolic and cytological profiling. The goals of these studies were: 1) to be able to define a cellular phenotype of small molecule inhibition of LDH-A, 2) to determine how similarly or how differently compounds behaved in the same set of experiments, and 3) to identify the most promising inhibitor(s) with which to move forward to in vivo models. Such a comparison of the different classes of LDH-A inhibitors has not
been reported in the literature to date, as most evaluations of LDH-A focus on a single class of compounds [3-7, 13, 38]. In general, the data generated from these studies demonstrated similar results for most classes of inhibitors tested. However, neither a single individual experiment nor a combination of multiple experiments was able to define a phenotype for small molecule inhibition of LDH-A, underscoring the high degree of difficulty in this task. Also, none of these experiments were able to directly probe whether any of these small molecules were able to directly engage LDH-A in cells. Thus, following completion of these broad studies, target engagement of these inhibitors in cells was evaluated through the methods detailed in Chapter 5.

4.10: Materials and methods

Intracellular concentration and cleavage assessment – This experiment was performed using the same LC-MS instrument, solvent system, and running protocol as described previously, using the UV trace to integrate galloflavin (elution time: 5.8 minutes in the UV trace, 6.1 minutes in the TIC), AZ 33 (elution time: 8.9 minutes in the UV trace, 9.2 minutes in the TIC), AZ 35 (elution time: 11.0 minutes in the UV trace, 11.3 minutes in the TIC), and AZ 35 MME (elution time: 9.6 minutes in the UV trace, 9.9 minutes in the TIC).

Media, PBS, and trypsin stability study – One hundred micromolar concentrations of compounds 4j, AZ 35, and the diethyl ester of AZ 33 were incubated at 37 °C for 30 minutes in 1.7 mL microfuge tubes in the presence of warmed RPMI 1640 medium (+10% FBS, +1% Penstrep), warmed phosphate-buffered saline solution (PBS), pH 7.4, or warmed 0.05% trypsin + 0.53 mM EDTA in HBSS without sodium bicarbonate, calcium, or magnesium. Immediately following incubation, 100 µL aliquots of each aliquot (6 aliquots in
total) were collected and analyzed by LC-MS (with concurrent UV trace at 254 nm) using the solvent system and running protocol described previously.

**Lysate and media cleavage study** – HeLa cells were collected from a T75 flask and counted. The cell pellet was stored on ice until immediately before sonication, at which point 0.8 mL RPMI 1640 medium + 10% FBS + 1% Penstrep, pre-warmed to 37 °C, was added. Two hundred microliter lysate aliquots were immediately added to microfuge tube to which 2 µL of a 10 mM DMSO stock solutions of 4j, AZ 35, AZ 33, or 2 µL DMSO, had already been added (for final concentrations of 100 µM compound). Simultaneously, 200 µL of warmed RPMI 1640 medium + 10% FBS + 1% Penstrep were added to four identical microfuge tubes containing compound or DMSO. All tubes were vortexed briefly and incubated on a heating block warmed to 37 °C for 30 minutes. One hundred microliter volumes of each sample were submitted for LC-MS analysis using the same acquisition parameters and solvent system as described previously. Lysate and medium DMSO-only samples were also analyzed by LC-MS to subtract out from compound peaks the integration areas of any peaks inherently present in medium or cell lysate only.

**Total LDH activity assessment in A549 knockdown and control cells** – Total LDH activity was queried using the Cytotoxicity (LDH) Detection Kit Plus (Roche Applied Sciences, Indianapolis, IN, kit catalog #04 744 926 001). Briefly, the reaction mixture was prepared by adding 1 mL MilliQ water to the catalyst solution, and allowed to sit for 10 minutes at room temperature before thorough mixing and aliquoting the reconstituted solution (solution A) at -20 °C (stable for up to 3 months when frozen). Immediately before performing each assay, 50 µL of reconstituted solution A was added to 2.5 mL solution B (containing the lactate, NAD⁺, and INT salt) to create the “reaction mixture.”
To query the total LDH activity of the cells, A549, A549 + pLKO control vector, and A549 + pLKOΔLDHA knockdown cells were seeded at 25,000 cells/well in DMEM + 10% FBS + 1% Penstrep in quadruplicate in a 96 well plate and allowed to adhere for 4 hours at 37 °C in normoxia. After four hours, one hundred microliters of cell culture medium was removed from all wells to be analyzed separately for LDH activity (serving as a blank control to subtract out LDH or nonspecific activity in the medium). Then, 5 µL of lysis solution (solution C) was added to each well. Plates were shaken briefly and incubated at 37 °C for 15 minutes. One hundred microliters of freshly-prepared reaction mixture was added to each well, and the plates were incubated in the dark at room temperature for 15-30 minutes. Once a color change was noted, indicating conversion of the INT salt to the reduced product, 50 µL of stop solution (solution D) were added to each well. Plates were shaken for 10 seconds in the plate reader before absorption readings at 492 nm and 692 nm were taken. LDH activity was determined by subtracting $\text{Abs}_{692\text{ nm}}$ from $\text{Abs}_{492\text{ nm}}$, and subtracting the average resulting absorbance in the four medium control wells from the average resulting absorbance in the four lysed cell wells of each cell line. This experiment was performed in triplicate using three separate passages of each cell line.

**Normoxia/hypoxia potency** – This protocol was adapted after the method previously published by the Hergenrother research group on IC$_{50}$ determination in normoxia versus hypoxia [19]. All HeLa cells used for each repetition of the experiment were grown in a common batch of T75 flasks in normoxia (20% oxygen) in RPMI 1640 medium + 10% FBS + 1% Penstrep. On the day of the experiment, HeLa cells in all T75 flasks were pooled and divided into two aliquots. The first aliquot was returned to culture in normoxia in one T75 flask, and the second aliquot was placed in hypoxia (1% oxygen). After 6 hours of acclimation to these culturing conditions, cells were detached, and a viable cell count was
taken (counting cells which excluded trypan blue as live). Five-thousand viable cells were seeded in 198 µL volumes into 96 well plates to which nine concentrations of each compound, with each concentration present in three wells (biological replicates), prepared in DMSO solutions had already been added. The final DMSO concentration of each well was 1%.

Tested concentrations for each compound:

- **Tirapazamine**: 100, 31.6, 10, 3.16, 1, 0.316, 0.1, 0.0316, 0.01 µM
- **DNQ**: 10, 3.16, 1, 0.316, 0.1, 0.0316, 0.01, 0.00316, 0.001 µM
- **Gossypol, FX-11, 4j, Glu-4j**: 200, 100, 31.6, 10, 3.16, 1, 0.316, 1, 0.316 µM
- **2DG**: 5, 2.5, 1, 0.316, 0.1, 0.0316, 0.01, 0.00316, 0.001 mM

Note: for tirapazamine and DNQ, repeated freeze-thaw cycles were known to lead to loss of compound integrity. To ensure compound integrity, stock solutions from solid stock and dilution plates were remade for each repetition of every experiment.

Cells were returned to either normoxic or hypoxic conditions and incubated for 72 hours continuously before plates were fixed with 10% trichloroacetic acid and assessed by Sulforhodamine B assay. Three independent replicates were performed, with the IC50 value obtained from each individual replicate being averaged at the end to determine the cumulative IC50 value and standard error for each condition and treatment.

**Metabolic profiling studies** – A549 cells were grown to confluence in T150 flasks (approximately 20 million cells per flask). Upon reaching confluence, cells were treated for 6 hours with the concentration of compound required to kill 50% of A549 cells treated for 24 hours (the 24 hour IC50 value; Table 4.4), to ensure that enough compound was present to have an effect on cells. Each compound was diluted into 15 mL RPMI 1640 medium from a
DMSO stock, such that the final concentration of DMSO in each sample was 1%. These 15 mL solutions were added to confluent flasks of cells in which the growth medium had been removed.

Following the 6 hour treatment time, cells were collected from each flask by scraping and washing twice in sterile PBS, pH 7.4. Pellet fresh weights were collected after the final wash step, prior to 0.75 mL cold pure methanol being added to each pellet. All pellets were stored at -80 °C until all repetitions had been performed (~2 weeks). A total of five biological replicates over three experiment days were collected for each treatment (30 samples total). One replicate was collected on experiment day 1, and two replicates of each treatment were collected on experiment days 2 and 3.

To prepare samples for GC-MS analysis, a four step extraction was used to obtain a variety of polar and nonpolar metabolites. These four steps involved subjecting cell sonicates to:

1) Pure methanol – for extraction of hydrophilic, polar metabolites
2) 1:1 methanol:water – for continued extraction of hydrophilic, polar metabolites
3) Pure water – for continued extraction of hydrophilic, polar metabolites
4) Chloroform – for extraction of lipophilic, nonpolar metabolites

In the extraction process, pellets in 0.75 mL methanol were sonicated for 60 seconds using an XL-2000 series QSonica LLC Misonix Sonicator. Sonicates samples were vortexed for 10 seconds before being centrifuged at top speed for 3 minutes. The supernatants, containing the polar metabolites from each lysate sample, were placed in clean tubes and concentrated to allow liquid to evaporate for several hours. After this fraction had dried, the second extraction step was initiated by adding 0.75 mL 50%
methanol prepared in MilliQ water to the tubes containing the sonicated pellets. Pellets were vortexed, centrifuged, and the soluble fraction was added to the corresponding tube containing the dried supernatant from the previous extraction. The tubes were concentrated again. In the third extraction step, 0.75 mL MilliQ water were added to each sonicated pellet, vortexed, and centrifuged. The soluble fraction was again added to the corresponding tube of dried supernatants from the previous two extractions. Finally, after this extracted supernatant had dried, the fourth and final extraction step was initiated by adding 30 µL methanol and 750 µL chloroform to each sonicated pellet. The purpose of adding methanol was so that it would bind the remaining water, since water and chloroform are not miscible.

When the supernatant from the last extraction was almost finished being concentration, 10 µL 10 mg/mL hentriacontanoic acid (the internal standard; a long-chain fatty acid not found in mammalian cells) prepared in pyridine (and heated before adding to allow for thorough dissolution) was added to each aliquot and allowed to dry completely.

After completion of the extraction and drying steps, metabolites were derivatized using a two-step process. First, 80 µL 20 mg/mL methoxyamine HCl (prepared fresh from a solid stock in pyridine and vortexed vigorously) was added to each sample to react with carbonyl groups to prevent them from getting silylated. This derivatization reaction was performed at 50 °C for 1 hour. Immediately after the first derivatization reaction had finished, 80 µL of N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA derivatization reagent, Fluka product #69479) was added to each sample, and samples were directly transferred to a heating block pre-set to 70 °C for 2 hours. This served to append trimethylsilyl (TMS) groups on hydroxyl groups (especially those on sugars) and amines, so as to make these metabolites volatile enough to be detected by GC-MS.
Following derivatization, samples were vortexed and centrifuged at top speed for 3 minutes. Fifty microliters of supernatant were transferred to a GC-MS vial equipped with a glass insert. Samples were run within 36 hours on a 7890A GC system (Agilent Technologies, Palo Alto, CA) equipped with an autosampler and a 5975C inert XL EI/Cl mass selective detector (MSD; Agilent Technologies) and 7683B autosampler series injector (Agilent Technologies). Gas chromatography was performed on an HP-5MS (60 m × 0.25 mm I.D. and 0.25 µm film thickness) capillary column (Agilent Technologies). The inlet and MS interface temperatures were 250 °C, and the ion source temperature was adjusted to 230 °C. A 1 µL aliquot of each sample was injected with the split ratio of 40:1. The helium carrier gas was kept at a constant flow rate of 1.5 milliliters per minute. The temperature program was: 5 minute isothermal heating at 70 °C, followed by an oven temperature increase of 5 °C per minute to 310 °C, and a final 10 minute hold at 310 °C. This running program was detailed in the file “Profiling HP-SMS.M.” The mass spectrometer was operated in positive electron impact mode (EI) at 69.9 eV ionization energy in m/z 30-800 scan range. The solvent used to rinse the needle and syringe in between injections was pyridine.

Once all samples had been run on the GC-MS, metabolites detected in each sample were determined by using AMDIS Chromatogram software. The spectra of all chromatogram peaks were compared with electron impact mass spectrum libraries of known compounds, consisting of a fusion of NIST08 (NIST, MD, USA) and W8N08 (Palisade Corporation, NY, USA) libraries, and a custom-built library. The targets within these libraries were accessible through “C:\NIST02\AMDIS32\LIB\MAIN2006.MSL,” and the AMDIS software’s “Analyze GC/MS Data” was used to a perform analysis of the data, exporting detected metabolite identities, their integration areas, and other data to create one
text file for each sample. Metabolite concentrations are reported as “(analyte concentration relative to hentriacontanoic acid internal standard) per fresh weight” (relative concentration), i.e., as target compound peak area divided by the internal standard (IS) peak area (since IS concentration is the same in all samples). Relative concentration is an accepted way to compare the same metabolite between different samples but does not allow comparisons between different metabolites within a sample due to different MSD responses to various compounds.

The text files were then manually edited to delete duplicate metabolites, collate integration areas for metabolites that were derivatized into several distinct species (i.e. 2-TMS, 3-TMS, and so forth, each with a different retention time and mass spectrum profile).

Statistical Analysis of Metabolic Profiling Data – All quality control assessments, data processing and statistical analyses were done in R [24] (version 2.15.2) using packages from the Bioconductor project [39] as indicated below. After data pre-processing, there were 421 metabolites. Any metabolite that was not detected in at least 2/5 samples of at least one of the seven treatment groups was filtered out; 289 metabolites remained after filtering. Normalization was done by dividing all metabolites in a given sample by the value of the internal standard peak in the same sample. All values were then multiplied by 1,000,000 to aid data visualization. To reduce the heteroscedacity of the data, the values were then transformed to the log2 scale after adding 5 (i.e., ~half the smallest non-zero value).

Statistical testing for differences between treatments was done by first fitting a mixed linear model to estimate the level of each of the seven treatment groups using the limma package [25], which uses an empirical Bayes correction [26] that helps to improve power by borrowing information across metabolites; the model also adjusted for the correlation due to
which day the samples were run [27]. Then pairwise contrasts between the vehicle control and the other 6 groups were pulled from the model, as well as the equivalent of a 1-way ANOVA between the seven groups. Correction for multiple hypothesis testing using the FDR method [28] was done for the 1-way ANOVA. Forty-four metabolites had FDR p-values < 0.2 for the one-way ANOVA test, which is the equivalent to a raw p-value <= 0.03. A heatmap was made for these 44 metabolites using values that have been adjusted for the day correlations and then scaled each metabolite to have a mean of zero and standard deviation of one.

**Cytological profiling studies** – Cytological profiling experiments were performed at the Chemical Screening Center at the University of California Santa Cruz by Professor Scott Lokey’s research group. The Hergenrother group shipped a total of 35 compounds with various anticancer activity, prepared in DMSO stocks with ten concentrations per compound. Included in this set were five *in vitro* LDH-A inhibitors: 4j, Glu-4j, FX-11, gossypol, and AZ 35 (galloflavin was also prepared but not analyzed to plate size limitations). The method and equipment used are as described in a previous publication by this group [34]. Briefly, HeLa cells cultured in DMEM were seeded at 2500 cells per well in a 384-well plate were treated with each concentration of each compound for 24 hours at 37 °C. Two identical plates were prepared for all compounds, one of which was stained for nuclear markers and the other for cytoplasmic markers. Nuclear stains were 5-ethynyl-20-deoxyuridine (EdU), histone H3, and Hoechst. Cytoplasmic stains were α-tubulin, actin, histone H3, and Hoechst. Imaging was performed using an ImageXpress Micro epifluorescent microscope (Molecular Devices, Sunnyvale, CA) with a 103 Nikon objective lens using four images per wavelength used. To quantify relative staining, MetaXpress software (Molecular Devices) was used to measure and score different elements of cell
morphology and staining. Clustering was performed using Cluster 3.0 software [37], and images and clustered files were sent to the Hergenrother group by Walter Bray in the Lokey group. Data were analyzed using Java TreeView software [37], and compounds were assigned to clusters based on the criteria outlined in a previous publication by the Lokey and Schulze research groups [34].
4.11: References


Chapter 5: Assessment of intracellular target engagement of LDH-A inhibitors

5.1: Overview of label-free techniques to detect protein-small molecule interactions (PSMIs)

The ability to detect a small molecule engaging its target within a cell or cell lysate is critical to the fields of chemical biology and drug development. A limited number of label-free techniques for elucidating protein-small molecule interactions (PSMIs) have been developed, as recently reviewed [1]. In order to examine the interaction of LDH-A with small molecule inhibitors, as well as to examine the interaction of GLUT-1 with GLUT substrates or inhibitors, two label-free techniques were explored: the Cellular Thermal Shift Assay (CETSA), reported in 2013 by Nordlund and coworkers [2], and the Drug Affinity Responsive Target Stability (DARTS) technique, reported by Huang and coworkers in 2009 [3].

The concept underlying CETSA is that a protein bound by a small molecule will have enhanced thermal stability compared to the unbound protein [2]. In CETSA, cell lysates or whole cells are treated with a small molecule or vehicle control before being subjected to heating at a range of temperatures that will denature unbound proteins. The protein of interest is visualized by Western blotting, and the band intensity (representing the amount of intact, non-thermally-denatured protein) is compared between vehicle-treated and compound-treated samples. To date, CETSA has been used to validate the binding of small molecules to their target proteins in cell lysate, whole cells, and in in vivo samples [2, 4]. However, results published to date have shown enhancement in proteins’ thermal stability only when the small molecules bind to their targets with better than 100 nM affinity [2, 4, 5]. One study compared the ability of a pair of enantiomers – (R)-crizotinib and (S)-crizotinib – to engage the nucleotide pool sanitizing enzyme human mutT homologue 1
(MTH1) \textit{in vitro} and in cell culture. Isothermal titration calorimetry experiments demonstrated that (\textit{R})-crizotinib had a $K_d$ of 781 nM, while (\textit{S})-crizotinib had a $K_d$ of 48 nM against MTH-1. When each compound was incubated at 5 \(\mu\)M in KRASV12-expressing BJ human fibroblast cells and CETSA was performed, only MTH-1 from (\textit{S})-crizotinib-treated cells had increased thermal stability. A negative CETSA result (no target stabilization compared to vehicle control) was obtained when treating lysate from human cholangiocarcinoma Mz-ChA-1 cells with 50 \(\mu\)M of the X-linked inhibitor of apoptosis protein (XIAP) inhibitor embelin [5], which was shown to have an IC$_{50}$ value of 2.8 \(\mu\)M against XIAP \textit{in vitro} [6]. Thus, while CETSA is a powerful technique for examining target engagement in cells of small molecules with high affinities for their protein targets, the utility of CETSA in studying lower affinity (100 nM or poorer) PSMIs is uncertain.

Another label-free technique to probe the interactions of proteins with small molecules in whole cells or in cell lysate is DARTS [3, 7]. The underlying principle is that proteins bound to small molecules will have decreased susceptibility to protease degradation compared to unbound proteins. The DARTS assay has been used in several dozen published studies to date, with applications including target validation [3, 8-10], target identification [3, 11-13], and detection of proteins that have been covalently modified [14]. While current data suggest that compounds must bind to targets with high affinity to be detected using CETSA, DARTS has been able to confirm binding of small molecules with micromolar target affinity [3].

5.2: CETSA proof-of-concept work with MetAP2 and TNP-470

In order to validate CETSA and develop a protocol for testing of LDH-A and GLUT-1, a proof-of-concept experiment was performed based on a result reported by Nordlund and
coworkers [2]. Compound TNP-470 (Figure 5.1 a) is a covalent inhibitor of the enzyme methionine aminopeptidase 2, with a $K_i$ value of 1 nM [15]. Nordlund and coworkers demonstrated using CETSA that treatment of A549 cell lysate with 1 µM TNP-470 led to a 10 °C increase in the $T_m$ of MetAP2 (from 67 °C to 77 °C) compared to vehicle treatment [2]. Using an identical protocol, it was found that 10 minute treatment of A549 cell lysate with 1 µM TNP-470 followed by 3-minute heating of sample aliquots at 3 °C intervals from 55 °C to 88 °C led to an increase in the $T_m$ of MetAP2 from 69 °C to 85 °C, as shown in Western blots for MetAP2 from each fraction (Figure 5.1 b) and quantified by densitometry (Figure 5.1 c).

**Figure 5.1**: Proof-of-concept CETSA experiment examining the interaction between MetAP2 and its covalent inhibitor TNP-470. a) the structure of covalent MetAP2 inhibitor TNP-470; b) Western blots comparing band intensity of MetAP2 in vehicle- and TNP-470-treated A549 cell lysate subjected to CETSA. Twenty micrograms of protein were loaded per well, and the membrane exposure time during visualization was 120 seconds for both blots. c) graphical depiction of MetAP2 relative band intensity (obtained using densitometry with ImageJ software) versus temperature in vehicle-treated versus 1 µM TNP-470-treated A549 lysate
5.3: Using CETSA to investigate LDH-A interactions in cell lysate

When the same CETSA protocol was adapted to determine the $T_m$ of LDH-A in the absence of inhibitors in in A549 cell lysate, no melting of LDH-A was observed during a 10 minute incubation in temperatures at up to 80 °C (Figure 5.2 a). This finding is contrary to literature reports that define the $T_m$ of LDH-A as 61°C [16]. In a further effort to achieve melting of LDH-A, a CETSA experiment was performed by incubating A549 cell lysate aliquots for temperatures up to 96 °C, but no melting of LDH-A was achieved (Figure 5.2 b). Since elevating the temperature further would result in boiling and loss of cell membrane integrity (see SI of [2]), protein-small molecule interactions of LDH-A were investigated using a complementary strategy, DARTS.

![Figure 5.2: CETSA with LDH-A. a) Western blot of LDH-A from A549 cell lysate aliquots heated for 10 minutes at 60-80 °C; b) Western blots of LDH-A from A549 cell lysate aliquots heated for 10 minutes at 75-96 °C. Ten micrograms of protein were loaded per well, and the membrane exposure time during visualization was 1 second for both blots.](image)
5.4: Using CETSA to investigate GLUT-1 interactions in cell lysate

GLUT-1 in A549 cell lysate was also assessed by CETSA (Figure 5.3 a), and its $T_m$ was found to be $\sim 73 \, ^\circ C$ by densitometry analysis (Figure 5.3 b). This value is similar to that reported in the literature for GLUT-1 (a $T_m$ of 68 °C was determined using differential scanning calorimetry [17]).

![GLUT-1 Western blot](image)

**Figure 5.3**: CETSA with GLUT-1. **a)** Western blot of GLUT-1 from A549 cell lysate aliquots heated for 10 minutes at 60-80 °C. Five micrograms of protein were loaded per well, and the membrane exposure time during visualization was 1 second. **b)** Graphical depiction of GLUT-1 relative band intensity (obtained using densitometry with ImageJ software) versus temperature.

A previous study also demonstrated that the thermal stability of GLUT-1 was increased by 4 °C when the transporter was incubated in the presence of 500 µM concentrations of its substrate glucose [17]. Based on this finding, the thermal stability of GLUT-1 in the presence of Glu-4j or its aglycone 4j was assessed using CETSA (Figure...
5.4 a). It was found that treatment of A549 cell lysate with 500 µM concentrations of Glu-4j led to a slight elevation in the Tₘ of GLUT-1, from ~ 73 °C in the 4j-treated lysate to ~ 75 °C in the Glu-4j-treated lysate (Figure 5.4 b). Attempts to determine a dose-dependence of this response, or to assess GLUT-1 thermal stability in the presence of high concentrations of glucose or GLUT inhibitors such as phloretin and cytochalasin B, were unsuccessful.

Figure 5.4: CETSA with GLUT-1. a) Western blots of GLUT-1 from A549 cell lysate aliquots treated with 500 µM 4j or Glu-4j before being heated for 10 minutes at 60-80 °C. Five micrograms of protein were loaded per well, and the membrane exposure time during visualization was 1 second. b) Graphical depiction of GLUT-1 relative band intensity (obtained using densitometry with ImageJ software) versus temperature in both treatment groups.
5.5: Using DARTS to query LDH-A interactions in cell lysate and whole cells

The DARTS technique relies on compound-bound proteins being less susceptible to protease degradation than their unbound counterparts. DARTS was employed to test the ability of LDH-A inhibitors and GLUT substrates to protect LDH-A and GLUT-1, respectively, from protease digestion. Following protocols outlined by Huang and coworkers [3, 7] in which proteases with broad substrate specificity were used, several candidate proteases for DARTS were explored. These included the heat-stable thermolysin from Geobacillus stearothermophilus (which cleaves peptide bonds adjacent to bulky and aromatic amino acid residues [18] and is predicted to have 110 cleavage sites in human LDH-A [19]), subtilisin A from Bacillus licheniformis (which cleaves peptide bonds adjacent to bulky, uncharged amino acid residues), and pronase (a mixture of at least three proteases isolated from Streptomyces griseus that together cleave a wide variety of peptide bonds).

Initial screening of thermolysin, subtilisin A, and pronase were performed to assess their abilities to digest LDH-A and GLUT-1 in the presence or absence of a protease inhibitor. The initial DARTS experiments performed by Huang and coworkers did include the use of protease inhibitor in each protease digest, with the rationale being that small quantities of protease inhibitor would neutralize the effects of endogenous proteases but not substantially affect the digestion of the comparably larger quantities of exogenous protease [3, 7]. In all of these initial experiments, 25 µL of A549 cell lysate (~ 35 µg of protein) were incubated with various concentrations of protease for 30 minutes at 37 °C in the absence or presence of a commercially-available protease inhibitor cocktail without the metal chelator EDTA (since EDTA inhibits thermolysin). Concentrations of protease chosen in the initial screening were adapted from published DARTS protocols [7].
In the initial digestion with thermolysin, 5 – 20 µM concentrations of protease were found to completely digest LDH-A and GLUT-1 in 30 minutes in the presence of protease inhibitor, while 5 µM thermolysin incompletely digested GLUT-1 in the absence of protease inhibitor (Figure 5.5). All concentrations of subtilisin assessed (5 – 30 µM) completely digested both LDH-A (Figure 5.6 a) and GLUT-1 (Figure 5.6 b) in the presence and absence of protease inhibitor. Treatment with pronase led to partial digestion of LDH-A at the lowest ratio used (1:3000 pronase:lysate; Figure 5.7 a), and partial digestion of GLUT-1 at all ratios used (Figure 5.7 b).

**Figure 5.5:** DARTS with thermolysin. Western blots of LDH-A (a) and GLUT-1 (b) from A549 cell lysate aliquots digested with 0 – 20 µM thermolysin in the presence or absence of 1% EDTA-free protease inhibitor cocktail. Each digestion contained 25 µL lysate (~35 µg of protein) and digests were quenched by adding 5 µL 0.5 mM EDTA, pH 8.0, and immediately boiling for 10 minutes. Twelve microliters of each digest (~ 10 µg of protein) were loaded into each well. Exposure times for LDH-A and GLUT-1 were 60 and 30 seconds, respectively.
Figure 5.6: DARTS with subtilisin A. Western blots of LDH-A (a) and GLUT-1 (b) from A549 cell lysate aliquots digested with 0 – 30 µM subtilisin A in the presence or absence of 1% EDTA-free protease inhibitor cocktail. Each digestion contained 25 µL lysate (~35 µg of protein) and digests were quenched by adding 5 µL 0.5 mM EDTA, pH 8.0, and immediately boiling for 10 minutes. Twelve microliters of each digest (~10 µg of protein) were loaded into each well. Exposure times for LDH-A and GLUT-1 were 60 and 30 seconds, respectively.

Figure 5.7: DARTS with pronase. Western blots of LDH-A (a) and GLUT-1 (b) from A549 cell lysate aliquots digested with 1:3000 – 1:300 ratios of pronase:lysate in the presence or absence of 1% EDTA-free protease inhibitor cocktail. Each digestion contained 25 µL lysate (~35 µg of protein) and digests were quenched by adding 5 µL 0.5 mM EDTA, pH 8.0, and immediately boiling for 10 minutes. Twelve microliters of each digest (~10 µg of protein) were loaded into each well. Exposure times for LDH-A and GLUT-1 were 60 and 30 seconds, respectively.
The ideal protease to be used in DARTS experiments would be one that would yield
dose-dependent digestion of the protein of interest. Probing the protection of LDH-A from
protease digestion became the primary objective, so further optimizations of GLUT-1
digests were not performed. Further work performed using pronase failed to yield
consistent digestion patterns of LDH-A, so pronase was excluded from further study.
Attempts to optimize subtilisin digest by lowering enzyme concentrations to as low as 1.25
µM failed to yield dose-dependent digestion of LDH-A; only complete digestion of LDH-A
was observed.

In performing concentration optimizations studies of thermolysin, it was found that
lowering the thermolysin concentration range to 1.25 – 15 µM protease yielded partial
protection of LDH-A in A549 cell lysate treated with 250 and 500 µM concentrations of AZ
33 and 250 µM Glu-4j (Figure 5.8 a). Densitometry analysis indicated that up to a 30%
protection of LDH-A proteolysis was observed during digest of lysate with 2.5 µM
thermolysin in the presence of 250 µM AZ 33 (Figure 5.8 b). Since AZ 33 is among the
most potent LDH-A inhibitors known (K_D of 93 nM [20]), and since this assay was performed
in cell lysate, which mitigates the poor cell permeability of AZ 33, this result was promising
and merited further assay optimization. Upon further investigation of the use of thermolysin
in DARTS, quenching conditions were optimized from 0.5 mM EDTA to 0.5 M EDTA, which
led to a substantial increase in undigested LDH-A bands present upon an immediate
quench (Figure 5.9).
Figure 5.8: DARTS for LDH-A in A549 cell lysate with thermolysin. a) Western blots of LDH-A from A549 cell lysate aliquots treated for 30 minutes at room temperature with DMSO vehicle or 500 µM compound before being digested with 0 – 15 µM thermolysin in the presence of 1% EDTA-free protease inhibitor cocktail. Each digestion contained 25 µL lysate (~35 µg of protein) and digests were quenched by adding 5 µL 0.5 mM EDTA, pH 8.0, and immediately boiling for 10 minutes. Twelve microliters of each digest (~ 10 µg of protein) were loaded into each well. Exposure time for all blots was 8 seconds. b) Graphical depiction of LDH-A relative band intensity (obtained using densitometry with ImageJ software) versus temperature in all treatment groups.
Figure 5.9: DARTS for LDH-A testing thermolysin digestion quenching conditions. Western blots of LDH-A from A549 cell lysate aliquots treated with 0 µM or 15 µM thermolysin, all in the presence of 1% EDTA-free protease inhibitor cocktail, immediately prior to the addition of either 0.5 mM or 0.5 M EDTA solutions and the boiling of samples for 10 minutes. The single asterisk (*) denotes the addition of EDTA preceding the addition of cell lysate and thermolysin, and the double asterisks (**) denote the addition of thermolysin to cell lysate immediately before the addition of 0.5 EDTA. Each digestion contained 25 µL lysate (~35 µg of protein). Twelve microliters of each digest (~10 µg of protein) were loaded into each well. Exposure time for all blots was 7 seconds.

After these experimental parameters were optimized, the protection of LDH-A from thermolysin digest in cell lysate and whole cells in the presence of LDH-A inhibitors was assessed. A549 cell lysate aliquots were treated for 30 minutes at room temperature with 250 or 500 µM concentrations of AZ 33 and Glu-4j, and moderate protection of LDH-A from thermolysin digestion was seen in the presence of AZ 33 but not Glu-4j (Figure 5.10 a). Densitometry quantification of these data demonstrated that LDH-A was 20-60% protected from proteolysis even at the highest concentrations of thermolysin used (Figure 5.10 b). Upon repeating this cell lysate assay using additional LDH-A inhibitors, treatment with AZ 33, but not with Genentech 35, AZ 35, 4j, or Glu-4j, protected LDH-A from thermolysin digestion (Figure 5.11 a-b). In this experiment, thermolysin activity seemed to decrease at the higher concentrations in all treatment groups, likely due to autodigestion of thermolysin.
Figure 5.10: DARTS for LDH-A in A549 cell lysate with thermolysin.  

**a)** Western blots of LDH-A from A549 cell lysate aliquots treated for 30 minutes at room temperature with DMSO vehicle or 500 µM compound before being digested with 0 – 15 µM thermolysin in the presence of 1% EDTA-free protease inhibitor cocktail. Each digestion contained 25 µL lysate (~35 µg of protein) and digests were quenched by adding 5 µL 0.5 M EDTA, pH 8.0, and immediately boiling for 10 minutes. Twelve microliters of each digest (~ 10 µg of protein) were loaded into each well. Exposure time for all blots was 5 seconds. 

**b)** Graphical depiction of LDH-A relative band intensity (obtained using densitometry with ImageJ software) versus temperature in all treatment groups.
Figure 5.11: DARTS for LDH-A in A549 cell lysate with thermolysin. a) Western blots of LDH-A from A549 cell lysate aliquots treated for 30 minutes at room temperature with DMSO vehicle or 500 µM compound before being digested with 0 – 15 µM thermolysin in the presence of 1% EDTA-free protease inhibitor cocktail. Each digestion contained 25 µL lysate (~35 µg of protein) and digests were quenched by adding 5 µL 0.5 M EDTA, pH 8.0, and immediately boiling for 10 minutes. Twelve microliters of each digest (~ 10 µg of protein) were loaded into each well. Exposure time for all blots was 15 seconds. b) Graphical depiction of LDH-A relative band intensity (obtained using densitometry with ImageJ software) versus temperature in all treatment groups.
Finally, DARTS was performed in whole cells to assess the ability of LDH-A inhibitors to engage LDH-A in an intact cellular environment. A549 cells were treated with vehicle control or 500 µM concentrations of several classes of LDH-A inhibitors for 1 hour at 37 °C. Cells were washed twice in PBS, sonicated, and incubated with various concentrations of thermolysin for 30 minutes at room temperature. Western blot analysis indicated that AZ 35 (which is cleaved to its active species, AZ 35 MME, in cells and cell lysate [21]) yielded moderate protection of LDH-A from thermolysin digestion, while AZ 33 and Genentech 35 yielded modest protection (Figure 5.12). Multiple whole cell DARTS experiments were conducted with 500 µM concentrations of both 4j and Glu-4j, but these experiments were inconclusive, since loss of protein was observed in all protease digestion aliquots, including the 0 µM thermolysin samples.

![Figure 5.12: DARTS for LDH-A with thermolysin. Western blots of LDH-A from A549 whole cells treated for 1 hour at 37 °C with DMSO vehicle or 500 µM compound, washed twice in sterile PBS, pH 7.4, lysed, and then digested with 0 – 15 µM thermolysin in the presence of 1% EDTA-free protease inhibitor cocktail. Each digestion contained 25 µL lysate, and digests were quenched by adding 5 µL 0.5 M EDTA, pH 8.0, and immediately boiling for 10 minutes. Twelve microliters of each digest were loaded into each well. Exposure time for all blots was 5 seconds.](image)
5.6: Summary and conclusions

In summary, CETSA and DARTS techniques were utilized to investigate target engagement of LDH-A inhibitors in cell lysate and whole cells. CETSA studies examining LDH-A were unsuccessful due to an inability of LDH-A to melt at temperatures up to 96 °C in the experimental conditions that achieved melting of every other protein assessed. CETSA indicated a $T_m$ of 73 °C for GLUT-1, which is comparable to the previously reported $T_m$ value [17]. However, the $T_m$ of GLUT-1 in CETSA was only modestly elevated in the presence of GLUT substrate Glu-4j, and further attempts to characterize $T_m$ shifts of GLUT-1 in the presence of other GLUT substrates or inhibitors were unsuccessful.

DARTS experiments were performed using a variety of proteases with broad substrate specificity. Thermolysin digestion conditions were optimized to identify concentrations of protease that yielded dose-dependent digestion of LDH-A, as well as quenching conditions that terminated most enzymatic digestion. DARTS experiments with thermolysin in A549 cell lysate repeatedly demonstrated protection of LDH-A in the presence of 250 and 500 µM concentrations of AZ 33, but not in the presence of weaker-affinity LDH-A inhibitors such as 4j, Glu-4j, AZ 35, or Genentech 35. In the whole cell DARTS experiments, only the cell-permeable LDH-A inhibitor AZ 35 yielded modest protection of LDH-A from thermolysin digestion.

5.7: Materials and methods

CETSA protocol – These experiments were performed according to the protocol outlined by Nordlund and coworkers [2]. Briefly, A549 cells were cultured in RPMI 1640 medium + 10% FBS + 1% Penstrep, then washed and counted in sterile PBS. Then, 1% v/v protease inhibitor cocktail III (EMD Millipore # 539134, Billerica, MA) was added to each sample prior
to lysing cells by sonication. For MetAP2 and GLUT-1 experiments, cell lysate was incubated for 10 minutes at room temperature with compound or vehicle control. For all experiments, equal volumes of lysates from each treatment group were then divided into thin-walled PCR tube aliquots, and each aliquot was incubated for a given incubation time (3-10 minutes depending on the experiment) on a PCR block at a given temperature between 55-96 °C. All samples were then cooled to room temperature, and stored at -20 °C pending analysis.

**DARTS in cell lysate protocol** – A549 cells were cultured in RPMI 1640 medium + 10% FBS + 1% Penstrep. Cells were harvested, resuspended in PBS, and adjusted to concentration of 300,000 cells/mL, to which 1% v/v protease inhibitor cocktail III was added. (This protease inhibitor solution is EDTA-free, which is important because thermolysin is inhibited by EDTA.) The cell solution in PBS was lysed by sonication, and lysate aliquots were incubated for 10-30 minutes at room temperature in microfuge tubes containing various concentrations of compounds or vehicle control. Compounds were prepared as DMSO stocks, and the final concentration of DMSO in each tube never exceeded 1%.

Following incubation with compound, cell lysates were subjected to protease digestion (**Table 5.1**). Protease solutions were prepared freshly from solid stock at the time of each experiment. Following the designated incubation time, protease reactions were quenched, 6x SDS-PAGE loading dye + 5% v/v β-mercaptoethanol was added, and samples were boiled. Samples were stored at -20 °C until Western blotting could be performed.
Table 5.1: Proteases used in DARTS experiments

<table>
<thead>
<tr>
<th>Protease</th>
<th>Manufacturer and product number</th>
<th>Concentrations or ratios used</th>
<th>Quenching conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thermolysin from <em>Geobacillus stearothermophilus</em></td>
<td>Sigma Aldrich #P1512</td>
<td>1.25-30 µM</td>
<td>0.5 M EDTA, pH 8 followed by immediate boiling for 10 minutes</td>
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<tr>
<td>Subtilisin A (protease from <em>Bacillus licheniformis</em>)</td>
<td>Sigma Aldrich #P5380</td>
<td>1-20 µM</td>
<td>boiling for 10 minutes</td>
</tr>
<tr>
<td>Pronase (protease mixture from <em>Streptomyces griseus</em>)</td>
<td>Sigma Aldrich #P5147</td>
<td>1:3000 – 1:300</td>
<td>boiling for 10 minutes</td>
</tr>
</tbody>
</table>

**DARTS in whole cells protocol** – The day prior to an experiment, A549 cells were seeded at a concentration of 300,000 cells/well in a 6-well plate in RPMI 1640 medium + 10% FBS + 1% Penstrep. Cells were allowed to adhere and grow overnight at 37 °C. The next day, medium was aspirated from each well, and 400 µL of medium containing vehicle control or compound was added to each well. Following a 1 hour incubation at 37 °C, cells in each well were collected by scraping, and cell solutions were washed twice and resuspended in PBS + 1% v/v protease inhibitor cocktail III. Each aliquot of cell solution was individually sonicated for lysis. Lysates were incubated with protease for the designated time, and reactions were quenched as describe above.

**SDS-PAGE and Western blotting protocol** – For CETSA experiments, the bicinchoninic acid (BCA) assay was performed using a commercially-available kit (Thermo Scientific Pierce, # PI-23225, Walthman, MA) to determine the protein concentration of each sample. Samples for SDS-PAGE electrophoresis were prepared by combining 20 µg of protein (diluted in PBS for a final volume of 12 µL) with 3 µL 6x SDS-PAGE loading dye + 5% v/v β-mercaptoethanol. These 15 µL aliquots were then boiled for 5 minutes, and 12 µL of each
aliquot were loaded into each well of a 4–20% Mini-PROTEAN® TGX™ Precast Gel (BIO-RAD # 456-1096EDU, Hercules, CA). Electrophoresis was performed for 1 hr at 120 V. Following electrophoresis, gel proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Thermo Scientific Pierce # 88518) for 2 hours at 45 V.

For DARTS experiments, protease digests were quenched by adding 6x loading dye + 5% v/v β-mercaptoethanol and immediately boiling samples, so BCA assays were not performed. Instead, to ensure equal loading of protein in each well from the same treatment group, equal volumes (12 µL) of each quenched sample were loaded directly into each well, and the same electrophoresis protocol was used.

Membranes were blocked with 5% bovine serum albumin (BSA) prepared in Tris-Buffered Saline + 1% Tween 20 (TBST) solution for 1 hour at room temperature on a rocker. Following three washes with TBST, membranes were then incubated overnight on a rocker at 4 °C in primary antibody solution (Table 5.2). Membranes were then washed thrice in TBST and incubated for 1-2 hours at room temperature, rocking, in secondary antibody: goat anti-rabbit IgG linked with horse radish peroxidase (HRP) for visualization (Thermo Scientific Pierce #32460), prepared in a 1:5000 dilution in 5% BSA in TBST. Membranes were washed thrice in TBST, then incubated with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific Pierce # 34078) for 5-10 minutes at room temperature on a rocker. Imaging was performed using X-ray film developed in a chemiluminescent imaging system. Densitometry analysis was performed using ImageJ software.
Table 5.2: Primary antibodies used in CETSA and DARTS Western blotting assessment:

<table>
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<tr>
<th>Antibody</th>
<th>Type and species</th>
<th>Manufacturer and product number</th>
<th>Working dilution</th>
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</thead>
<tbody>
<tr>
<td>Anti-LDH-A</td>
<td>Monoclonal rabbit</td>
<td>Cell Signaling (Danvers, MA) #3582</td>
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<tr>
<td>Anti-GLUT-1</td>
<td>Monoclonal rabbit</td>
<td>Abcam (Cambridge, MA) ab115730</td>
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<tr>
<td>Anti-MetAP2</td>
<td>Monoclonal rabbit</td>
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5.8: References