Inhibitory Effects of Galloflavin Against Lactate Dehydrogenase

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Abstract

Lactate dehydrogenase (LDH) is an important enzyme found in nearly every living cell that catalyzes the conversion of pyruvate to lactate, and vice versa, using NADH and NAD+ as cofactors. This oxidoreductase is important in the anaerobic metabolic pathway and exists as five different isozymes in the body, namely LDH-1 through LDH-5, as either homotetrameric or heterotetrametric enzymes (Farhana et. al.). These isozymes differ in containing different numbers of either heart (H) or muscle (M) subunits. Due to the importance of LDH in metabolism and generation of cellular energy, LDH has become a topic of interest in methods to fight the growth of cancerous tumors. Cancer cells depend heavily on their glycolytic pathway to generate ATP (Manerba et. al.) and If LDH activity were to be knocked down, malignant cells could be starved of energy necessary to survive (Miao et. al.). Galloflavin (GF), a novel LDH inhibitor, has recently been proposed as a treatment for this purpose and has shown to induce apoptosis in human breast cancer cells (Farabegoli et. al.) and mitigated malignant behavior in colorectal cancer cells (Guo et. al). Further, galloflavin has been found to, at different K_is, inhibit both LDH-A and LDH-B isoforms (Manerba et. al.), allowing inhibition of enzymes found in different tissues. Current research has provided sufficient initial information to pursue galloflavin further, but more work is required to understand the molecule fully for use as an anti-cancer treatment.

Galloflavin has been discovered as a lactate dehydrogenase inhibitor and has potential as a treatment in various cancerous tumors (Elsisi et. al.). Before further applications can be explored, a further and complete understanding of galloflavin must be done, including method of inhibition, K_i of galloflavin against different isoforms, and potential off target effects against non-cancerous cells. In this study, inhibitory effects of galloflavin were determined against LDH-1 and LDH-3 isoforms in both the forward and reverse directions. Initial velocity experiments across all substrates in varying concentrations and multiple inhibitor concentrations were completed. With this information, initial velocities were plotted on double-reciprocal plots and compared to no inhibitor velocities. Galloflavin was found to act as a mixed inhibitor, leaning toward competitive inhibition, against LDH-1 in the forward direction, preferentially binding to the free-enzyme. Initial velocity measurements yielded unconclusive data of inhibitory effects against LDH-3 in the forward direction, as well as LDH-1 in the reverse direction. From past literature, galloflavin was thought to act as a pure competitive inhibitor (Manerba et. al., Han et. al.)

Materials and Methods

Preparation of Reagents

Premade 0.1M phosphate and 0.1M CAPS buffer were utilized for creating reagents for studying forward and reverse LDH reactions respectively. When studying the forward reaction, 10X, 2mL stock reagents were prepared for 6000uM, 10000uM, and 25000uM NADH and pyruvate solutions were prepared using 0.1M phosphate buffer. When studying the reverse reaction, 10X, 2mL stock reagents were prepared for 150mM, 400mM, and 900mM lactate solutions, and 1000uM, 2500mM, and 5000mM NAD+ solutions using 0.1M CAPS buffer. For all assays, 10X, 2mL stock 200uM, 550uM, and 1000uM galloflavin solutions were prepared using appropriate buffer for forward or reverse reaction. Additionally, 62.5mM lactate and 3.75mM solutions were prepared in 0.1M CAPS buffer for fixed substrate reactions in reverse direction, and 7.5mM pyruvate and 5mM NADH solutions were prepared in 0.1M phosphate buffer for fixed, excess substrate components in forward reaction.

Initial Velocity Assays

Initial velocity measurements were obtained by spectrophotometer, measuring OD340 every five seconds for 30 seconds total. Spectrophotometer was auto blanked with 2mL distilled water prior to each kinetic assay. 1.6mL 10X reagents of excess substrate 0.2mL of 10X test substrate solutions, as well as 0.2mL 10X galloflavin solution were premixed in cuvette, and 10uL of LDH solution was added directly before assay began. For reactions without galloflavin, 0.2mL of phosphate or CAPS buffer were substituted instead depending on reaction direction tested. Once LDH was added, reaction was quickly mixed by inversion and placed into spectrophotometer and recording of OD340 commenced. For each substrate tested with inhibitor, all three galloflavin concentrations were tested for all 18 total kinetics assays performed. No inhibitor reactions were also monitored for 6 total kinetics assays.

Interpretation of OD340 Measurements

Following data collection, initial velocity of each assay was determined by calculating OD340/sec by dividing delta values between OD340 measurements by five while ΔOD340 was linear. This was repeated for all kinetics assays. Initial velocities were plotted on a double reciprocal plot of 1/v and 1/[substrate], a linear trendline was extrapolated, and equation of this trendline was obtained for further analysis. K_m of LDH in each condition was found by determining x-intercept of the trendline, and V_{max} was found by determining y-intercept of the trendlines. K_i and α values of inhibitor were also determined using trendline of each case.

Results

Galloflavin Affects LDH1 in Forward Reaction

Initial velocity tests were performed with varying concentrations of NADH or pyruvate in the presence of varying concentrations of galloflavin by measuring OD340 over time. Increasing concentrations of either NADH or pyruvate, while the other substrate was held constant, resulted in a higher OD/sec and initial enzyme velocity. When galloflavin was introduced into reactions containing excess NADH and fixed pyruvate, some changes in initial velocity, K_m, and V_{max} were observed (Figure 1), but did not exhibit traditional inhibitor changes in these values. When galloflavin was introduced into reactions containing excess pyruvate and fixed NADH concentrations, a decrease in initial velocity was observed as galloflavin concentration was increased, K_m increased, and V_{max} decreased (Figure 2).

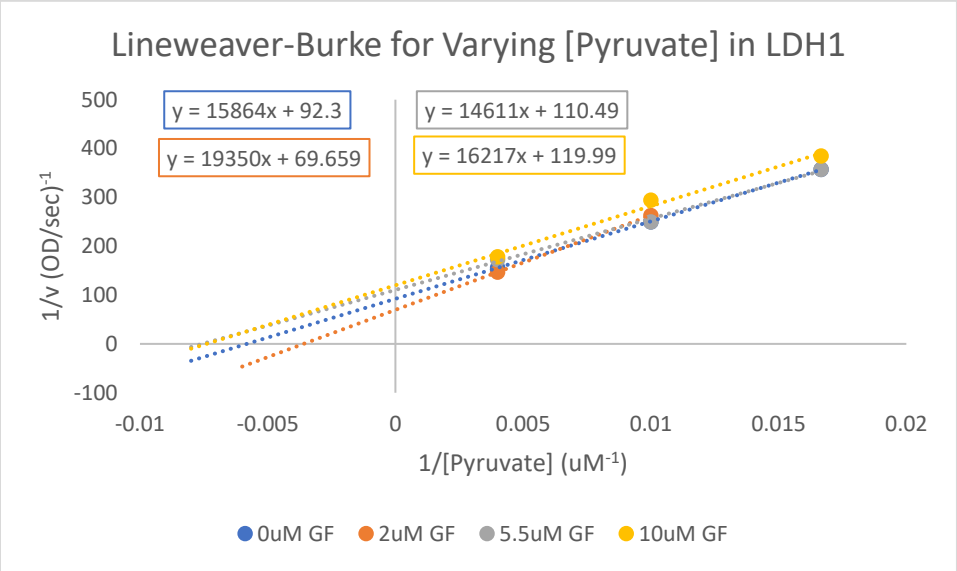

Figure 1: Lineweaver-Burke Plots for Constant [NADH} and Changing [Pyruvate] In LDH1 In Presence of Galloflavin (GF)

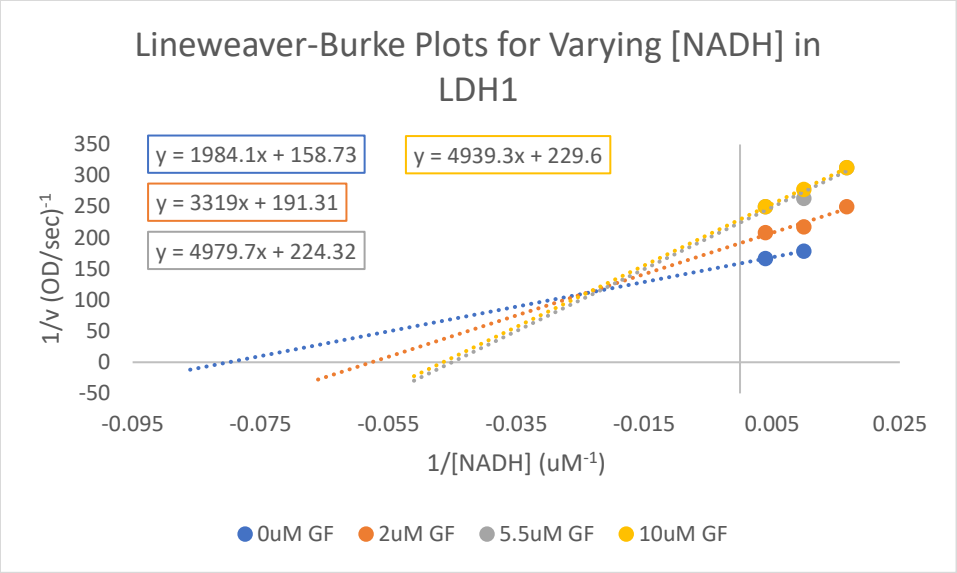

Figure 2:Lineweaver-Burke Plots for Constant [Pyruvate] and Changing [NADH] In LDH1 In Presence of Galloflavin (GF)

Galloflavin Shows Unclear Inhibitory Effects on LDH3 In Forward Reaction

Initial velocity tests of LDH3 for the forward reaction were completed in the same manner. Varying concentrations of pyruvate or NADH in reaction were again tested with varying concentrations of galloflavin. When pyruvate was varied and NADH was held constant, increasing concentrations of galloflavin changed K_m and V_{max} each, but did not follow any noticeable trends (Figure 5). Initial velocity measurements were taken again, this time varying NAD+ and holding pyruvate constant through testing. As [galloflavin] increased, K_m also increased, but V_{max} decreased (Figure 6).

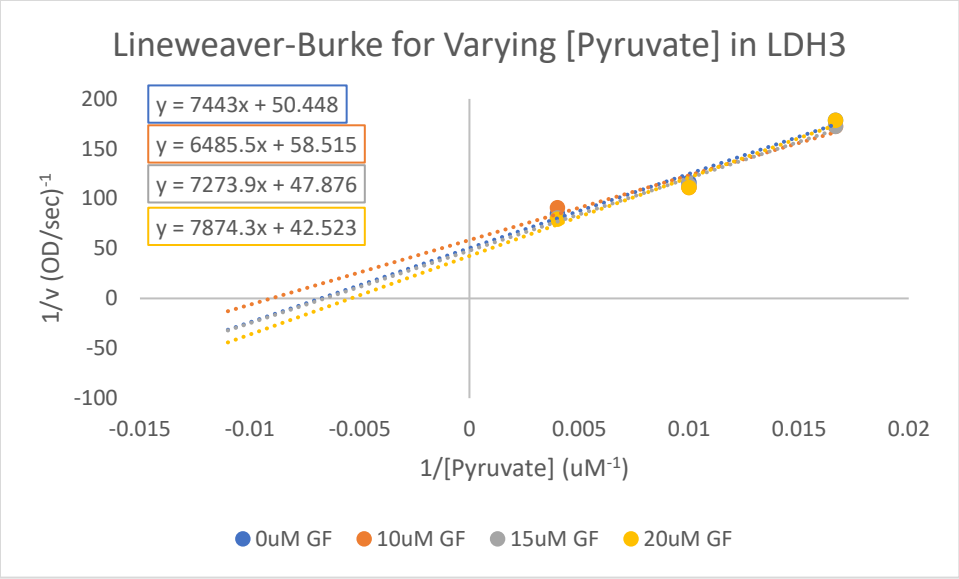

Figure 3: Lineweaver-Burke Plots for Constant [NADH} and Changing [Pyruvate] In LDH3 In Presence of Galloflavin (GF)

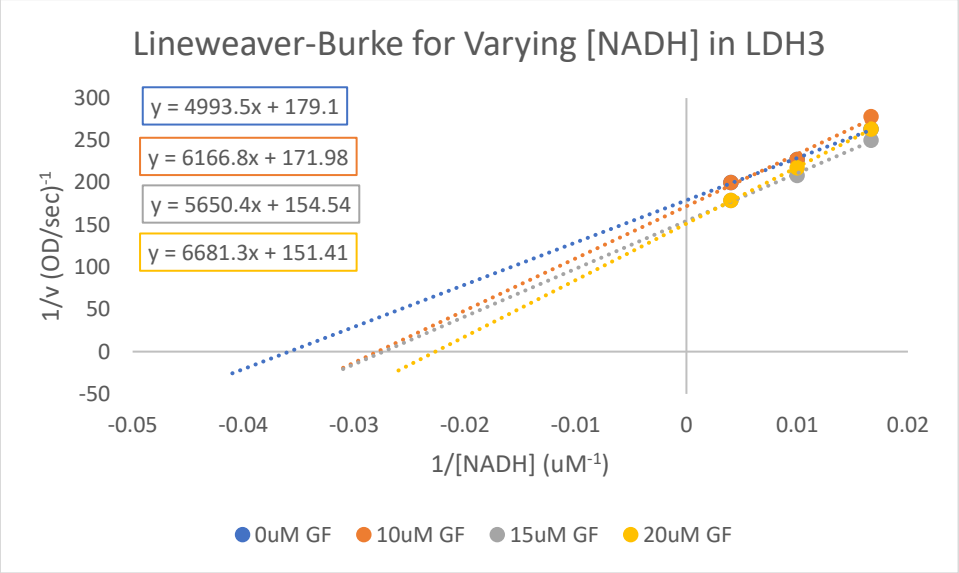

Figure 4: Lineweaver-Burke Plots for Constant [Pyruvate] and Changing [NADH] In LDH3 In Presence of Galloflavin (GF)

Galloflavin Shows Unclear Inhibitory Effects on LDH1 in Reverse Reaction

Initial velocity tests were performed in a similar manner, this time examining LDH in the reverse direction, by measuring OD340 over time. Varying concentrations of NAD+ or lactate in reaction were tested with varying concentrations of galloflavin. Again, as one substrate was in excess while the other was varied, initial velocity increased as test substrate concentration was increased. When NAD+ was held in excess and lactate concentrations were varied, minimal

changes in initial velocity, K_m, and V_{max} were detected, but did not follow traditional inhibitor expectations (Figure 5). Additionally, as lactate was held in excess and NAD+ concentrations were varied, minimal changes in initial velocity, K_m, and V_{max} were observed, but again did not yield traditional inhibitor expected changes (Figure 6).

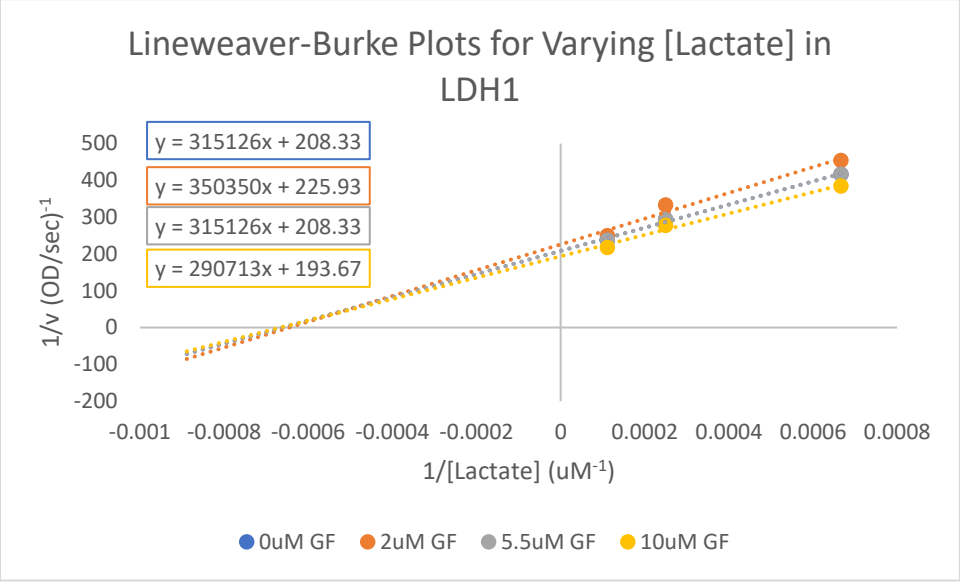

Figure 5: Lineweaver-Burke Plots for Constant [NAD+] and Changing [Lactate] In LDH1 In Presence of Galloflavin (GF)

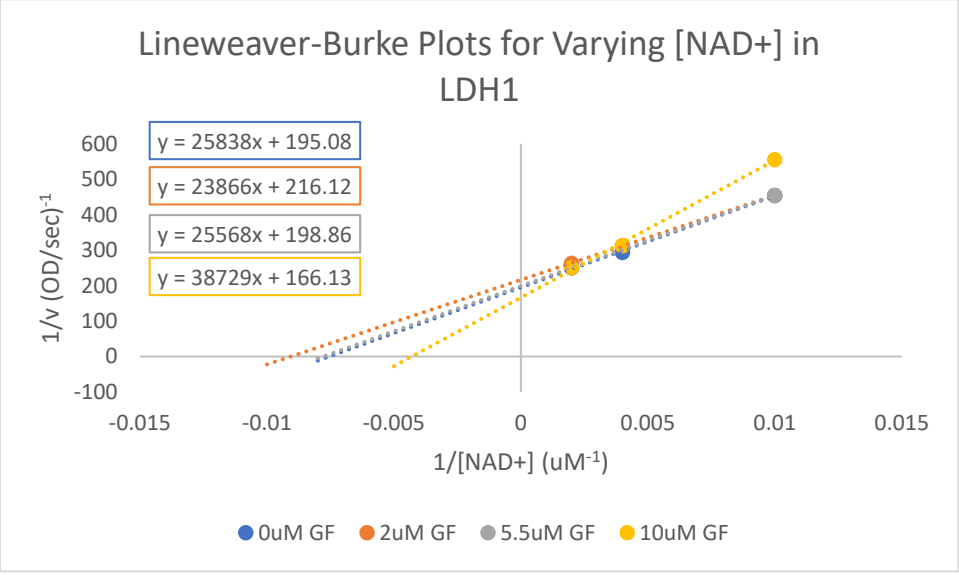

Figure 6: Lineweaver-Burke Plots for Constant [Lactate] and Changing [NAD+] In LDH1 In Presence of Galloflavin (GF)

α, α', K_i, and K_i' Measurements of Lineweaver-Burke Plots

From linear trendlines obtained from Lineweaver-Burke plots of all test cases varying each substrate in forward and reverse reactions, α, α', K_i, and K_i' values were obtained (Tables 1, 2, and 3).

Table 1: α, α', Ki, and Ki' values of Lineweaver-Burke Trendlines for LDH1 in Forward Reaction

	LDH1 Forward Reaction Varying [Pyruvate]				**LDH1 Forward Reaction Varying [NADH]**			
[GF] (uM)	0	2	5.5	10	0	2	5.5	10
α	1	1.22	0.92	0.92	1	1.67	2.51	2.5
α'	1	0.75	1.2	1.2	1	1.2	1.41	1.45
K_i		9.09	-68.75	-125		2.99	3.64	6.67
K_i'		-8	27.5	50		10	6.67	4.44

Table 2: α, α', Ki, and Ki' values of Lineweaver-Burke Trendlines for LDH3 in Forward Reaction

	LDH3 Forward Reaction Varying [Pyruvate]				**LDH3 Forward Reaction Varying [NADH]**			
[GF] (uM)	0	10	15	20	0	10	15	20
α	1	0.87	0.98	1.06	1	1.23	1.13	1.34
α'	1	1.15	0.95	0.84	1	0.86	0.86	0.85
K_i		-76.9	-750	333.3		43.47	115.38	58.82
K_i'		66.6	-300	-125		-250	-107.1	-133.3

Table 3: α, α', Ki, and Ki' values of Lineweaver-Burke Trendlines for LDH1 in Reverse Reaction

	LDH1 Reverse Reaction Varying [Lactate]				**LDH1 Reverse Reaction Varying [NAD+]**			
[GF] (uM)	0	2	5.5	10	0	2	5.5	10
α	1	1.11	1	0.92	1	0.92	0.98	1.5
α'	1	1.08	1.08	0.93	1	1.11	1.02	0.85
K_i		18.18	50	-125		-25	-275	20
K_i'		25	68.75	-142.9		18.18	275	-66.67

Discussion

Galloflavin Has Some Inhibitory Effects on LDH1 In Forward Reaction

Galloflavin was introduced at 2uM, 5.5uM, and 10uM into varying concentrations of pyruvate and NADH, and reaction was monitored by spectrophotometer. When examining galloflavins effects when pyruvate is changed, there were some small effects. K_m was increased in two cases and V_{max} decreased in one case. In the other cases, K_m decreased, and V_{max} increased, or did not substantially differ from the no inhibitor control. In this analysis, galloflavin did not exhibit expected results of a pure inhibitor, and a general trend was not observed across concentrations of galloflavin. Galloflavin could acts as a mixed non-competitive inhibitor. Due to this, galloflavin is not a consistent inhibitor of LDH1 when examining pyruvate.

When galloflavin was introduced into varying concentrations of NADH, more typical inhibitor results were observed. As [Galloflavin] increased, K_m increased, and V_{max} decreased. The slope of trendlines increased as [Galloflavin] increased, but trendlines did not converge onto a single V_{max}. Instead, plotted trendlines converged onto a point at a negative 1/[NADH]. This also does not follow expected pure inhibitor results. Instead, galloflavin likely acts as a mixed inhibitor leaning toward competitive inhibition against NADH. This trend increased as concentration of galloflavin in reaction increased, but began to level off as higher amounts were present. In addition, α and α' values were both positive for all tests, and $\alpha > \alpha'$, further pointing to mixed competitive inhibition. Galloflavin is likely a viable inhibitor of LDH1 in the forward reaction when examining its effect on NADH.

Galloflavin Exhibits Less Typical Inhibitor Effects on LDH3 in Forward Reaction

Identical experiments were conducted against LDH3 against varying [Pyruvate]. This time, less discernible effects were observed when compared to LDH1. There were minor differences in K_m and V_{max}, but these changes did not follow a discernible trend. K_m decreased in two cases, and increased in one. V_{max} also decreased in two cases and increased in one case. Due to this, it is impossible to determine whether galloflavin is an inhibitor of LDH3 when examining varying pyruvate concentrations.

Similar results to LDH1 were observed when examining galloflavins effects against [NADH] in LDH3. In all inhibitor concentrations, K_m increased, suggesting some competitive binding with NADH. This ratio of increase in K_m was similar with that observed in LDH1, but the change leveled off more quickly with increasing [Galloflavin]. Despite this, V_{max} tended to increase as [Galloflavin] increased. This is, again, not consistent with results expected with a pure inhibitor. Taking this into account, galloflavin could act as a mixed competitive inhibitor of LDH3 when examining varying [NADH], but could not be reliable concluded based on data collected.

Galloflavin Shows Minimal Inhibitory Effects Against LDH1 in the Reverse Direction

Experiments were completed again, this time examining the effect of galloflavin against LDH1 in varying [Lactate] and [NAD+]. Changes in V_{max} were seen with different concentrations of galloflavin compared to inhibitor control, but a visible trend in these changes could not be extrapolated. However, the trendlines of plotted points seems to converge along the x-axis. When

comparing this intersection, the calculated K_m of these trendlines converge on very similar values. This points to the idea that galloflavin could act as a mixed noncompetitive inhibitor of LDH1 in the reverse direction when examining its effect on lactate, but results remain inconclusive at present.

Galloflavin's effect against LDH1 with varying [NAD+] was not expected. In the forward direction, increasing [Galloflavin] data suggested that it acts as a mixed competitive inhibitor against NADH. These same results were not observed in the reverse direction. Increases and decreases in K_m and V_{max} were observed across assays, but neither followed a consistent trend. Galloflavin could have some inhibitory effects against LDH1 in the reverse reaction in regard to NAD+, but no definitive conclusions could be made.

Galloflavin's Mechanisms of Inhibition

A variety of effects were observed when increasing [Galloflavin] was introduced into forward reactions of LDH1 and LDH3, and reverse reaction on LDH1. In both LDH1 and LDH3, the effect of galloflavin on enzyme kinetics when varying pyruvate was minimal at best, and drawing conclusions on its effect is impossible at this time; however, preliminary data suggest it could act as a mixed inhibitor. When examining galloflavin's effect on NADH in LDH1 and LDH3, results were more easily discernible. K_m noticeably increased as galloflavin concentration was increased. V_{max} change was less concrete, but sill present. From this information, galloflavin could act as a mixed competitive inhibitor of LDH1 and LDH3 when examining [NADH]. Noticeably, LDH1 seemed to react more substantially to galloflavin, and could therefore be more susceptible to its inhibitory effects when compared to LDH3. Inhibitory effects of galloflavin against LDH1 in its reverse direction were minimal. Small changes were observed in LDH1 K_m and V_{max}, but these changes were not consistent. Galloflavin might not be as effective of an inhibitor of LDH1 in the reverse reaction, compared to its forward direction. This was especially noticeable when examining varying [NAD+]. Instead of mixed competitive inhibition that was seen in varying [NADH], trends pointed toward some other kind of mixed inhibition.

To determine the inhibitory effects of galloflavin more accurately against LDH1 and LDH3, further testing is required. Running reactions in triplicate would allow for better trendlines to be plotted on double reciprocal plots and including more reactions with additional [Substrate] and [Galloflavin] reactions would provide more datapoints to be compared. At present, galloflavin seems to act most as a mixed competitive inhibitor against LDH1, with an emphasis on its effect on NADH in the forward direction.

References

1) Farhana A, Lappin SL. Biochemistry, Lactate Dehydrogenase. [Updated 2023 May 1]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2023 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK557536/
2) Manerba, M., Vettraino, M., Fiume, L., Di Stefano, G., Sartini, A., Giacomini, E., Buonfiglio, R., Roberti, M. and Recanatini, M. (2012), Galloflavin (CAS 568-80-9): A Novel Inhibitor of Lactate Dehydrogenase. ChemMedChem, 7: 311-317. https://doi.org/10.1002/cmdc.201100471
3) Miao, P., Sheng, S., Sun, X., Liu, J. and Huang, G. (2013), Lactate dehydrogenase a in cancer: A promising target for diagnosis and therapy. IUBMB Life, 65: 904-910. https://doi.org/10.1002/iub.1216
4) Farabegoli F, Vettraino M, Manerba M, Fiume L, Roberti M, Di Stefano G. Galloflavin, a new lactate dehydrogenase inhibitor, induces the death of human breast cancer cells with different glycolytic attitude by affecting distinct signaling pathways. Eur J Pharm Sci. 2012 Nov 20;47(4):729-38. doi: 10.1016/j.ejps.2012.08.012. Epub 2012 Aug 30. PMID: 22954722.
5) Guo L, Yang Y, Sheng Y, Wang J, Li W, Zhou X, Ruan S, Han C. Galloflavin Relieves the Malignant Behavior of Colorectal Cancer Cells in the Inflammatory Tumor Microenvironment. Front Pharmacol. 2021 Dec 10;12:752118. doi: 10.3389/fphar.2021.752118. PMID: 34955826; PMCID: PMC8702829.
6) Han, X., Sheng, X., Jones, H.M. *et al.* Evaluation of the anti-tumor effects of lactate dehydrogenase inhibitor galloflavin in endometrial cancer cells. *J Hematol Oncol* **8**, 2 (2015). https://doi.org/10.1186/s13045-014-0097-x
7) Elsisi, A., Sokar, S., El-Mahrouk, S., & Abu-Risha, S. (2021). Potentiation of paclitaxel antitumor activity by galloflavin or oxamate as lactate dehydrogenase inhibitors. *Journal of Advanced Medical and Pharmaceutical Research*, *2*(2), 64-76. doi: 10.21608/jampr.2021.98224.1020