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The Effect of pH on Gossypol Inhibition of Rabbit Muscle Lactate Dehydrogenase

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ABSTRACT

Lactate Dehydrogenase (LDH) catalyzes the anaerobic reaction between pyruvate and lactate and is necessary for regeneration of cellular NAD^+. Inhibition of this pathway has been shown to have beneficial effects in some cases. Recent studies indicate that inhibiting LDH will cause oxidative stress in malignant cells, hence limiting tumor propagation. Gossypol and its derivatives are compounds isolated from cottonseed oil and are potent inhibitors of LDH. However, the activity of gossypol within an acidic environment, such as found in cancer cells, is not known. In this research, the inhibition of rabbit muscle LDH was tested at pH levels 6, 7, and 8. The results showed that gossypol strongly inhibits LDH at pH 6, while only moderately inhibiting LDH at pH 7 and 8. The K_i value at pH 6 was found to be 0.001 mM compared with 0.082 mM at pH 7 and 0.107 mM at pH 8. These results indicate that LDH in acidic environments requires a lesser concentration of gossypol to achieve inhibition than at biological pH. This suggests that gossypol-like inhibitors could be used to selectively inhibit LDH under acidic conditions such as those found in cancer cells.

Keywords: *enzyme inhibition, enzyme kinetics, gossypol, lactate dehydrogenase, Warburg Metabolism.*

INTRODUCTION

Lactate dehydrogenase (LDH) is an enzyme that facilitates the reversible conversion of pyruvate, the three-carbon product of glycolysis, into lactate (Swiderek et al., 2015). LDH is present in nearly all organisms; its primary structure is widely conserved across species, making it a frequent subject of biochemical investigation (Dempster et al., 2014). The fate of pyruvate at the end of glycolysis is dependent on the aerobic conditions of the cell: in the presence of oxygen, pyruvate enters the citric acid cycle for energy harvest. In anaerobic conditions, pyruvate is reduced to lactate (Koukourakis et al., 2005). The latter reaction involves the transfer of a hydride from nicotinamide adenine dinucleotide (NADH) to pyruvate. The oxidized form of this co-enzyme, NAD^+, is a necessary compound for continuation of the glycolytic pathway (Shi and Pinto, 2014).

LDH is a tetramer consisting of two subunit types, muscle and heart (abbreviated M and H respectively). The structural arrangement of subunits within the tetramer results in one of five natural isozymes; the specific tetramer present depends on the tissue's metabolic needs. For example, LDH-A (M4 or LDH-5) is found primarily in skeletal muscle and operates most efficiently in anaerobic conditions. In contrast, LDH-B (H4, LDH-1) is found in heart tissue where the aerobic citric acid cycle predominates (Le et al., 2010). Due to slight differences in protein structure between isozymes, LDH-A has a higher affinity for pyruvate than other variants and favors the forward reaction of pyruvate to lactate (Valvona et al., 2015). Because of the importance of LDH to cellular metabolism, especially under anaerobic conditions, inhibition of the enzyme has negative biological consequences such as the creation of cellular stress. This is partially due to reduced levels of NAD^+, preventing further glycolysis (Le et al., 2010).

Scientists are exploring how to use this inhibitory activity in medical research (Porporato et al., 2011). In one study, inhibition of LDH in *plasmodium falciparum* was investigated in malaria treatment (Shoemark et al., 2007). Further, inhibition of LDH is showing promise in cancer research (Pelicano et al., 2006). Even in the presence of oxygen, malignant cells tend to utilize the anaerobic pathway, a phenomenon known as Warburg Metabolism (Vander Heiden et al., 2009). Thus, frequent hypoxic conditions within cancerous tissues cause LDH to play an enhanced role in cellular metabolism (Augoff et al., 2015). To maximize anaerobic efficiency, cancer cells produce high levels of the muscle type LDH-A tetramer. Therefore, tumor dependence on LDH-A makes this particular tetramer a potential target in cancer treatment (Porporato et al., 2011).

In addition to increased reliance on LDH, cancerous tumors show a wider range of pH variance between intracellular and extracellular microenvironments when compared to normal tissue (Estrella et al., 2013). The intracellular pH of cancer cells is reported as basic in some instances and acidic in others (Aredia and Scovassi, 2014).

Gossypol, a derivative of cottonseed oil and a clinically known male contraceptive, is a natural inhibitor of LDH (Keshmiri-Neghab and Goliael, 2014). Research shows that gossypol and its derivatives increase oxidative stress in tumors, killing cancer cells and inhibiting tumor propagation (Le et al., 2010). While gossypol and its analogs have been

shown to be potent inhibitors of LDH, their inhibition at various pH levels is not well known (Porporato et al., 2011). Past studies have demonstrated, however, that gossypol influences enzymes such as pepsinogen in unexpected ways when pH is taken outside of normal ranges (Tanksley et al., 1970). Due to the variant pH levels seen in cancerous tissue, it becomes possible that conformational changes due to varying pH may influence the ability of gossypol to inhibit LDH.

The aim of this study was to evaluate the effectiveness of gossypol in inhibiting LDH in cellular environments outside of normal biological pH levels, hoping to find that gossypol inhibits LDH more effectively in certain pH ranges; this knowledge would further our understanding of gossypol's effectiveness in conditions similar to malignant tumors.

MATERIALS AND METHODS

The experimental design involved a kinetic analysis of LDH in the presence of NADH and pyruvate. These kinetic runs were repeated in the presence of a known LDH inhibitor, gossypol. Each analysis was performed at different pH levels to evaluate the effectiveness of varying gossypol concentration on LDH activity. All experiments were performed in triplicate to assess variation (Zivadinovic and Nikcevic, 2010).

LDH isolated from *Oryctolagus cuniculus* (Rabbit) muscle was used in this study as a readily available representative of LDH-A; its primary structure is widely conserved across species (Dempster et al., 2014). Reagents purchased from Sigma-Aldrich, included LDH isolated from rabbit muscle LDH (item 1012776001), NADH (N8129-100MG), sodium pyruvate (P2256-5G), and gossypol (G876-100MG). McPherson College Natural Science Department provided other chemicals, including concentrated hydrochloric acid (HCl) and tris base reagents. A Genesys 20 UV-Vis spectrometer with quartz cuvettes, an Accumet AB15 digital pH meter, P200 and P1000 micropipetteman, and volumetric glassware were also provided by McPherson College.

LDH is most active between pH 5 and pH 8 (Javed et al. 1997). Thus, 80 mM tris buffer was prepared at three pH levels (6.00, 7.00, and 8.00) by the careful addition of concentrated HCl to Tris Base, as measured by digital pH meter with a slope in excess of 90% at 23 °C (Rabiu et al. 2013).

Rabbit Muscle LDH was dissolved and diluted with tris HCl buffer at pH 7.0 in order to obtain the change in absorbance of 0.2 to 0.4 absorbance/minute at 340 nm during assay, following standard protocol (Worthington Biomedical Protocol, 1993). The stock enzyme was diluted at 1 mg protein per ml and subsequently diluted 200 fold, obtaining a 0.2-0.4 change in absorbance during assay. When not in use, the enzyme solution was kept cold at 4 °C (Javed et al. 1997).

Stock substrate solutions of 1.25 mM NADH and 12.5 mM pyruvate were prepared quantitatively with the use of a four-place analytical balance and serial dilution techniques. From this stock, appropriate volumes of each solution were added to the cuvette for each kinetic run (See Table 1).

Gossypol is poorly soluble in aqueous solutions; therefore, dimethylformamide (DMF) was used to dissolve the product. An appropriate mass of solid gossypol was dissolved into DMF using a volumetric flask to create a stock solution of 150 µM gossypol. From the stock solution, aliquots were further diluted so that the final concentrations within the cuvette varied at three levels of 5.8, 11.5, and 57.7 µM gossypol (Gupta et al., 1988). For the control, DMF alone is used, without dissolved gossypol. All the above solutions were used within two of preparation.

Table 1. A description of the experimental design and reagents within the cuvette for each set of runs (listed in order of addition). The substrate pyruvate was varied and other concentrations were held constant. In the inhibitor group, each set of runs was completed separately against three inhibitor concentrations. This design was repeated in triplicate at 6.00, 7.00 and 8.00 pH levels.

Trial	pH	Cuvette Reagents
Control: DMF	6.00 7.00 8.00	100µL pyruvate: 0.192, 0.385, 0.769, or 0.962 mM 200µL of 0.25mM NADH 50 µL DMF 900 µL pH Buffer 50 µL Enzyme added last
Inhibitor: 5.8 11.5 57.7µL in DMF	6.00 7.00 8.00	100µL pyruvate: 0.192, 0.385, 0.769, or 0.962 mM 200µL of 0.25mM NADH 50 µL DMF with inhibitor 900 µL pH Buffer 50 µL Enzyme added last
		Final volume in cuvette: 1.3 ml

Kinetics studies were carried out at 340 nm in the UV spectrometer at approximately 23 °C (Talaiezadeh et al. 2015). 50 µl DMF, 200 µl NADH, 20-100 µl pyruvate, 900 µl tris buffer, and 50 µl enzyme (introduced last) were added to the cuvette and thoroughly mixed by pipetting technique. NADH was held constant at 0.250 mM within the cuvette, while the pyruvate concentration was varied. From the pyruvate stock solutions, appropriate volumes were dissolved into the cuvette to adjust the concentration of each run to 0.192, 0.385, 0.769, and 0.962 mM pyruvate (Javed et al. 1997; Talaiezadeh et al. 2015; Zivadinovic and Nikcevic, 2010). Please see Table 1 for a clarification of cuvette contents.

The addition of the enzyme initiated the reaction; samples were analyzed every 5.0 seconds for 1.0 minute, monitoring decrease in absorbance as assessed by the instrument. Steady state kinetics were shown by straight lines of change in absorbance per minute at these concentrations, and the results were analyzed on Lineweaver-Burk plots (Wang, Y et al. 2016). Data points were processed using Microsoft Excel® 2013.

RESULTS

The results of this study are reported as findings from the experimental conditions of this particular enzyme and its substrate concentrations. Following the current convention for reporting enzyme kinetics, all data was plotted on Lineweaver-Burk plots for determination of the Michaelis-Menton constant (K_m) and the maximum velocity (V_{max}) of the enzyme at this study's experimental conditions (Figures 1–3). The following equation was used:

$$\frac{1}{V} = \frac{Km}{Vmax}\frac{1}{[S]} + \frac{1}{Vmax}$$

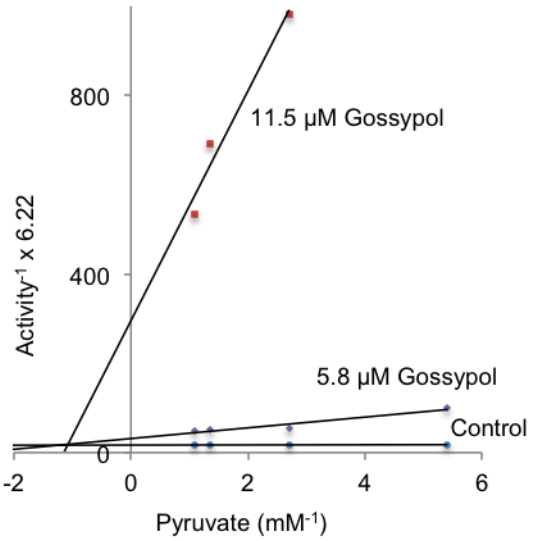

Figure 1. pH 6.00 Lineweaver-Burk, double reciprocal plot. Each line represents runs completed at a single gossypol concentration, or a control with DMF only. The individual data points of each line represent the average rate (n=3) of a 1.0 minute kinetics run. The y axis activity is multiplied by 6.22, the molar absorbtivity of NADH (Worthington Bio). The steep increase in slope with increasing inhibitor concentration indicates that very little of the inhibitor is required to acheive substantial inhibition. Note the interesection of the lines behind the y-axis, displaying mixed inhibition behavior.

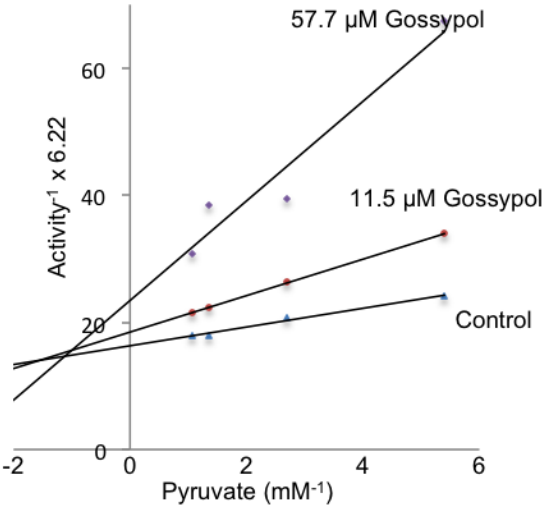

Figure 2. pH 7.00 Lineweaver-Burk plot, obtained as in Figure 1. Less inhibition is noted than at pH 6.00. Mixed inhibition behavior is similar to Figure 1.

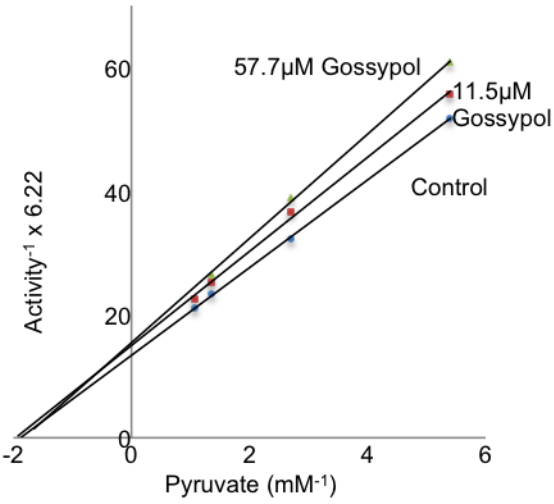

Figure 3. pH 8.00 Lineweaver-Burk plot, obtained as in the previous figures. Note that the lines intersect differently than in previous figures, indicating noncompetitive inhibition behavior.

Table 2. Comparison of observed K_m values between control and 11.5 µM gossypol groups. K_m values are mean ± standard deviations and in units of mM. These values were obtained from the Lineweaver-Burk plots using the equation above to find K_m. *Note that inhibitor concentration of 57.7 µM resulted in complete inhibition with no enzyme activity.

pH	Control	11.5 µM	57.7 µM
6.0	0.010 ± 0.002	1.9 ± 2	0 ± 0*
7.0	0.10 ± 0.06	0.17 ± 0.06	0.35 ± 0.11
8.0	0.55 ± 0.02	0.5 ± 0.1	0.6 ± 0.2

Table 3. Comparison of mean Vmax between control and gossypol groups ± standard deviation. These values were also obtained from the Lineweaver-Burk plots using the equation above to solve for V_{max}. Velocities are in units of mM per minute. *Note that inhibitor concentration of 57.7 µM resulted in complete inhibition with no enzyme activity at pH 6.0.

pH	Control	11.5 µM	57.7 µM
6.0	0.059 ± 0.001	0.005 ± 0.005	0 ± 0*
7.0	0.06 ± 0.01	0.054 ± 0.002	0.043 ± 0.008
8.0	0.075 ± 0.001	0.067 ± 0.008	0.67 ± 0.01

Because mixed inhibition presents challenges to calculations, the inhibitor constant (K_i) for each group was found by assuming noncompetitive inhibition of gossypol, as reported by the literature (Gupta et al., 1988; Olgiati and Toscano, 1983; Rabiu et al. 2013). K_i is calculated by the inhibitor concentration of 11.5 µM gossypol divided by a comparison of V_{max} and apparent V_{max}. (Nelson and Cox, 2008).

$$Ki = \frac{[I]}{\left[\frac{Vmax}{\alpha Vmax} - 1\right]}$$

Smaller K_i values indicate increased enzyme susceptibility to inhibition. At pH 8.00, the enzyme was shown to be more robust in its ability to overcome gossypol, shown by a higher K_i.

Table 4. Determination of K_i values. Units are in mM gossypol. A smaller K_i value indicates that a lesser concentration of gossypol is needed to effectively inhibit LDH. The K_i value was calculated from the V_{max} values at 11.5 µM inhibitor with the assumption that gossypol is noncompetitive for pyruvate.

pH	6.0	7.0	8.0
K_i	0.001	0.082	0.107

Concentrations above 11.5 µM gossypol almost completely inhibited the enzyme with little activity noted. At pH 6.00 and 57.7 µM gossypol, no LDH activity was seen. This is in contrast to the pH 7.00 and pH 8.00 groups, which continued to show reasonable activity at 57.7 µM gossypol.

DISCUSSION

The inhibition of lactate dehydrogenase has been the subject of much study because of its medicinal applications to cancer, contraception, and malaria (Porporato et al., 2011). In most publications, the focus of the inhibition kinetics is evaluated by varying the inhibitor concentration in the presence of consistent reaction conditions and a single pH (Gupta et al., 1988; Rabiu et al., 2013; Wang et al., 2016). In the present study, the reaction conditions were varied to compare the effectiveness of gossypol inhibition at differing pH levels.

pH is known to have an effect on enzyme kinetics; at hydrogen concentrations outside of normal conditions, the conformational structures can change, influencing both the enzyme's active site and the overall shape of the enzyme. Changes in the conformational shape of rabbit muscle LDH due to pH have been shown for decades (Fritz, 1967; Javed et al. 1997). As structural changes occur, the binding of substrates and cofactors can also be influenced. Further, changes in the shape of the enzyme can also affect the ability of inhibitors to bind to the protein. Thus, in an abnormal pH environment, a combination of both enzyme kinetic instability and changes in inhibitor behavior are likely to occur (De Arriaga et al., 1982).

The results of this study show that lowered pH has a significant effect on gossypol and its ability to inhibit LDH. Particularly, the near complete inhibition seen at pH 6.00 with 57.7 µM inhibitor concentration stands in contrast to the same inhibitor concentration at the other two pH groups. The rapid decline of enzyme activity at pH 6.00, as shown in Figure 1 and Table 4, demonstrates that very little gossypol is required to inhibit rabbit muscle LDH at this pH. Comparing near-biological pH (7.00) and acidic pH (6.00) shows a wide difference in K_i values. Based on existing knowledge, it appears that the observed effect is most likely due to a change in the enzyme's conformational shape at differing pH levels.

The initial velocities of the highest concentration of pyruvate (without gossypol) are reported in Table 5. This is a comparison across the pH groups, demonstrating that the enzyme is active within each group. This suggests that pH 6.00 alone does not significantly cause enough conformational change in the enzyme to sufficiently explain the substantial decrease in activity when gossypol is added.

Table 5. Comparison of control group initial enzyme activities (without inhibitor) at each pH ± standard deviation. These velocities are at the highest pyruvate concentration of 0.9615 mM. This shows that the enzyme is active at pH 6.00 without inhibitor, as it is at pH 7.00 and 8.00.

pH	6.0	7.0	8.0
Activity	0.364±0.002	0.348±0.04	0.297±0.005

This study follows the Michaelis-Menton assumption of steady state kinetics. LDH is a bisubstrate enzyme; the current model suggests that NADH binds first, followed by the binding of pyruvate (Swiderek et al., 2015). Gossypol has been shown in some studies to be a noncompetitive inhibitor of LDH, binding to the site for NADH; however, other researchers debate this finding (Gupta et al., 1988; Olgiati and Toscano, 1983; Rabiu et al. 2013). The Lineweaver-Burk plots in the present study show the intersection of lines behind the Y-axis but above the

X-axis, characteristic of mixed inhibition (features of both noncompetitive and competitive inhibition). If gossypol is acting strictly as a noncompetitive inhibitor, as reported by Gupta and colleagues, the observation of mixed inhibition suggests that DMF may also be influencing the enzyme as an inhibitor (Gupta et al., 1983). During trial runs, it was noted that increased DMF concentration in the cuvette caused inconsistencies in the enzyme's activity. DMF was used as a solvent in accordance with assay protocols (Gossypol Product Information, 2013). The inhibitory effects of the solvent itself have not been published and could be the subject of future investigation, perhaps focusing on the effect of increasing DMF concentrations upon the enzyme. However, it is important to note that our pre-experimental trials did not indicate any effect of DMF on enzyme activity.

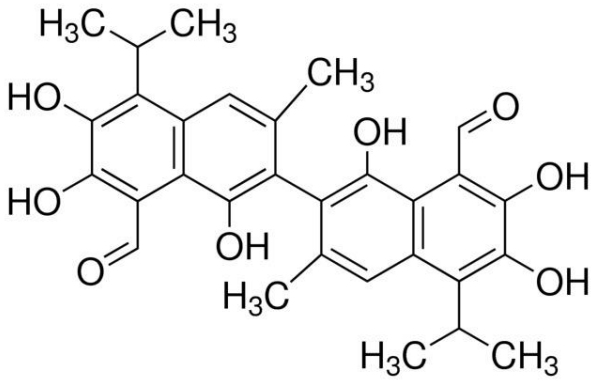

Figure 4. Structure of gossypol. A natural compound isolated from cottonseed oil and a potent inhibitor of LDH, it has a molecular weight of 518.55 g/mol and has two pKa values of note to this experiment: 6.73 and 7.38 (Image from Sigma Aldrich, 2017).

Gossypol is a large organic compound that is poorly soluble in water (Figure 4). It has five pKa values, the final two of which are reported as 6.73 and 7.38, both within the experimental pH groups (Toxnet, 2012). The protonation state of gossypol could be contributing to the changes in inhibition behavior seen at various pH levels. At pH 6, it is likely a fully protonated species and is predominantly in a neutral state. In the pH groups of 7 and 8, gossypol may exhibit a prevalent negatively charged compound. Thus, the protonation of gossypol functional groups could influence the binding of the inhibitor to LDH. Further, it is known that intramolecular hydrogen bonding occurs between the carbonyl and the nearby alcohol groups of the compound (Kenar, 2006). The presence or absence of hydrogen bonds could be altered by pH and may change its binding to LDH, modifying the inhibition behavior.

Direct comparison of the results in this study to previous studies is difficult, due to the wide variety of published experimental methods; the materials utilized in the literature contain a wide variety of LDH types, from goat to pika muscle. Further, the experimental designs differ; some researchers introduced pyruvate last, allowing the enzyme to "incubate" with NADH and the inhibitor for a short time (Gupta et al., 1983; Swiderek et al., 2015). This study did not aim to give gossypol an advantage, so the enzyme was added last. Despite these differences, the LDH-A K_i value reported by Gupta et al. of 0.020 mM Gossypol at pH 7.00 is within reasonable magnitude to the 0.081 mM found in this study. The K_m values were also similar (Gupta et al., 1983). The findings detailed by De Arriaga and coworkers showed that other inhibitors strongly affect LDH function at a pH range from 5.0 to 6.8, suggesting that the inhibition of LDH is very dependent upon pH (De Arriaga et al., 1982).

The manner in which the K_i values are calculated involves several computations of slopes and intercepts to obtain V_{max} and apparent V_{max}. By feeding the original data into these calculations, propagation of uncertainty becomes problematic and uncertainty is not reported with K_i values. However, the results should not be discounted. The data strongly and repeatedly indicates that very little gossypol was required to inhibit LDH at pH 6.00.

In this study, it was demonstrated that gossypol is a potent inhibitor of rabbit muscle LDH at pH 6.00, while less potent at 7.00 and 8.00. Since cancer cells show pH ranges outside of normal, it is helpful to know that a lower dose of gossypol may be able to inhibit acidic cancer cell LDH-A in order to slow tumor propagation at certain pH levels. This is preliminary research, involving the effect of pH on rabbit muscle LDH inhibition. The exact kinetics of malignant cell LDH should be studied separately, but under a similar experimental design.

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