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ABSTRACT

Metformin (1,1-dimethylbiguanide) is an anti-hyperglycemic agent that is commonly used in the oral treatment of Type 2 Diabetes Mellitus. Phenformin (phenethylbiguanide) is its structural analog. As an anti-hyperglycemic agent, metformin is known to affect glucose transporters. *Lactobacillus acidophilus* is a bacteria that ferments lactic acid under anaerobic conditions and is known to contain numerous glucose transporters. Inconclusive results were seen during NMR analysis due to the lack of lactic acid and the increase of glucose over time. With completely anaerobic conditions and longer measurement periods, it is hopeful that further experimentation will be successful. Adenosine deaminase is involved in purine nucleoside metabolism. Based on molecular docking simulations, we have hypothesized that adenosine deaminase could be inhibited by metformin and phenformin, and that phenformin is a better inhibitor than metformin. The inhibitory effect of metformin and phenformin on adenosine deaminase from bovine spleen was studied spectrophotometrically. Non-linear regression analysis was used to obtain the inhibition constants. Metformin was a competitive inhibitor of deamination of adenosine with the inhibition constant, K_I, of 88 mM. Phenformin was slightly more effective than metformin as a competitive inhibitor with K_I of 10 mM. The K_M of adenosine was 31 μM. The inhibition constant values indicate that neither metformin nor phenformin is a potent inhibitor of adenosine deaminase. It is unlikely that at the physiological concentration, adenosine deaminase activity would be greatly affected by either drug.

INTRODUCTION

Diabetes Mellitus is an increasingly prevalent disease that affects an estimated 25.8 million people in the United States alone.[1] The majority of these people are diagnosed as having Type 2 Diabetes Mellitus (T2DM). T2DM is characterized by insulin resistance resulting in abnormally high blood glucose levels due to the inability of glucose uptake by cells.[2] Along with careful regulation and monitoring of blood glucose levels, T2DM patients can also be prescribed other treatment options, such as metformin. Metformin is a biguanide, antihyperglycemic agent that is known to lower blood glucose concentration, increase glucose uptake in cells, and improve insulin sensitivity.[3] Phenformin was another treatment option similar to metformin, until it was taken off the market due to problems associated with lactic acidosis.[4]

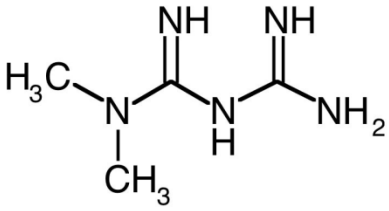


Figure 1: Structure of Metformin

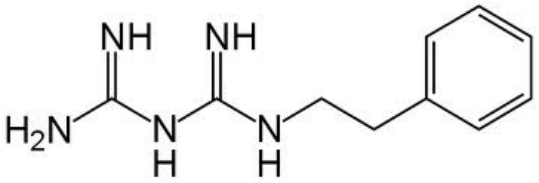


Figure 2: Structure of Phenformin

There are several proposed mechanisms associated with the specific functioning of metformin within Type 2 Diabetics. The most commonly accepted mechanisms involve the inhibition of AMP deaminase (AMPD), an enzyme the converts AMP into IMP, and mitochondrial complex 1, an enzyme in the electron transport chain. Together, the inhibition of both of these enzymes essentially increase the concentration of AMP, stimulating AMP-activated protein kinase (AMPK).[3] Activation of AMPK causes the inhibition of synthetic pathways including gluconeogenesis and fatty acid synthesis; and, the stimulation of catabolic pathways including glucose degradation and β-oxidation.[3] Adenosine deaminase (ADA), an enzyme that converts adenosine into inosine, has a similar function as AMPD.[5] Comparatively speaking, researching the inhibition of ADA by metformin will help extrapolate inhibition of AMPD. Molecular docking simulations, seen below, show that metformin will bind tightly and similarly to adenine at the active site on ADA.

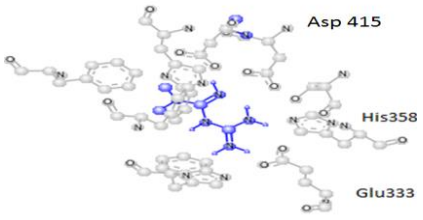


Figure 3: Metformin bound to ADA

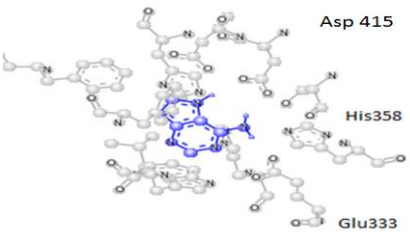


Figure 4: Adenine bound to ADA

In addition to studying the effects of metformin on certain enzymes, glucose transporters were also studied using *Lactobacillus acidophilus*, a bacterium primarily functioning to ferment sugar into lactic acid.[6] Since this bacterium is known to metabolize glucose into lactic acid, its membrane contains numerous glucose transporters.[6] In general, metformin increases uptake of glucose in cells; however, it is unclear exactly how metformin interacts mechanistically with glucose transporters.

EXPERIMENTAL PROCEDURE

To study glucose transporters, *Lactobacillus acidophilus* 4365 and its recommended growth media 416 was purchased from ATCC. The Lactobacilli MRS broth (BD 288130) was made using 55 grams/ 1 L. A total of 2 Liters of broth was made and autoclaved at 121 degrees Celsius to ensure sterility. A small sample of 24 hour incubation cells were prepared at 80 rpm at 35.5 degrees Celsius. The broth was a dark brown color, when cells started growing the broth-cell solution turned a lighter brown color and had a cloudy appearance. Next, the rate of bacterial growth was measured over time using a broth sample as the standard auto-zero. The cells from the 24 hour incubation small sample were measured to have an absorbance of 1.61 at 600 nm wavelength using UV-vis spectroscopy. Bacterial growth measurements were taken from samples prepared with 25 mL MRS broth to 1 mL of small sample incubation cells every 30 minutes until growth increase plateaued based on the absorbance values at 600 nm. Growth was slow but leveled off around 14 hours; the primary growth slope took approximately 10 hours as seen in the Graph below. A large culture of *Lactobacillus acidophilus* was then made using this timeframe at the same incubation settings.

The cells were centrifuged at 1500 rpm for 10 minutes and the supernatant was discarded. 10 mL of Buffer A was added per gram of cell; there was 1.457 grams of cells so 14.57 mL of buffer was added. The cells were combined into the same test tube and the following solutions were made according to Table 1 and Table 2. The solutions were vortexed and incubated for the allotted time (Note that 0 minutes indicates the cells should be killed immediately).

Table 1: Control Solutions

Tube	Cell Suspension (mL)	DI Water (mL)	200 mg/mL Labeled Glucose (mL)	Incubation Time (minutes)
1	1	0.025	0.025	0
2	1	0.025	0.025	2
3	1	0.025	0.025	4
4	1	0.025	0.025	6
5	1	0.025	0.025	8
6	1	0.025	0.025	10

Table 2: Metformin Solutions

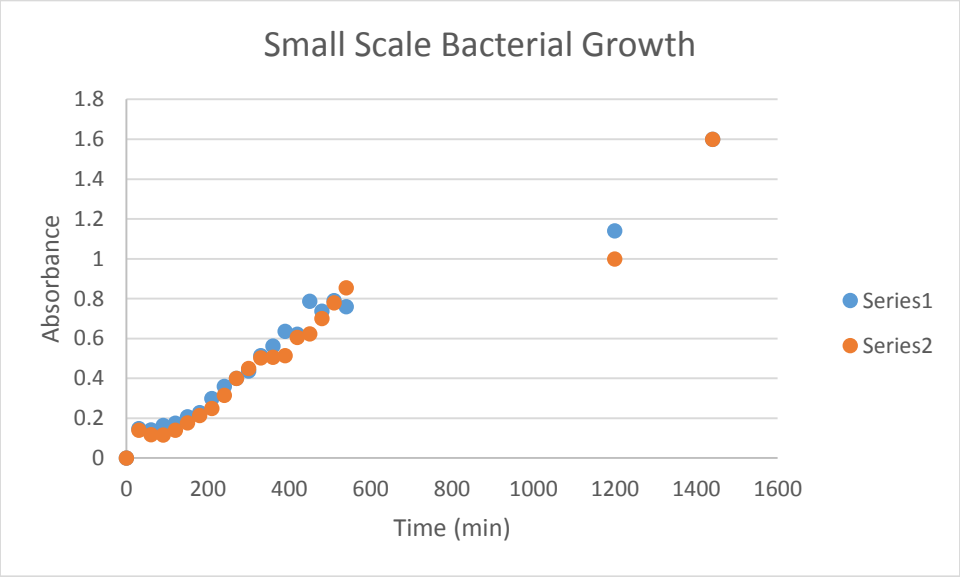
Tube	Cell Suspension (mL)	500 mM Metformin (mL)	200 mg/mL Labeled Glucose (mL)	Incubation Time (minutes)
1	1	0.025	0.025	0
2	1	0.025	0.025	2
3	1	0.025	0.025	4
4	1	0.025	0.025	6
5	1	0.025	0.025	8
6	1	0.025	0.025	10

After the incubation time is complete, the cells were killed with 0.125 mL of 70% perchloric acid. The cells were then broken up by freezing each tube in liquid nitrogen and thawing in water bath three times. Then, the solutions were centrifuged at 2,000 rmp for 2 minutes. 0.145 mL of 10M KOH was added to the solution for neutralization. Then, 0.122 mL D_2O was added and the tubes were centrifuged at 5,000 rpm for two minutes in order to remove any precipitates. The supernatant was then removed using a pipet, making sure not to disturb the pellet, and transferred into a clean NMR tube. The ^{13}C NMR was done using D_2O for locking and 100 proton-decoupled scans per spectrum were collected.

In the next experiment, the inhibition of ADA obtained from bovine spleen by anti-hyperglycemic agents metformin and phenformin was studied. The activity of ADA was measured through continuous spectrophotometric rate determination via UV-vis spectrophotometer at a 265 nm wavelength. Quartz cuvettes containing a 50 mM phosphate buffer at pH 7.4 and adenosine concentrations ranging from 15 µM to 133 µM provided a control group. Metformin was studied at concentrations of 10 mM, 20 mM, and 40 mM while phenformin was studied at concentrations of 2 mM, 3.3 mM, 4 mM, and 30 mM. All chemicals used in these reactions were purchased from Sigma-Aldrich. The data obtained was used to calculate ADA activity and was used to create a nonlinear regression graph. Through non-linear regression analysis of these graphs, calculations of V_{max}, K_m, and K_I were carried out in order to help characterize the type of inhibition metformin and phenformin present on adenosine deaminase.

It is important to note that this report was done to describe the experiments pertaining to glucose transporters in *Lactobacillus acidophilus.* The adenosine deaminase experiment was included to help describe further experiments done on a similar topic that help in understanding and analyzing this particular research experiment.

RESULTS AND DISCUSSION



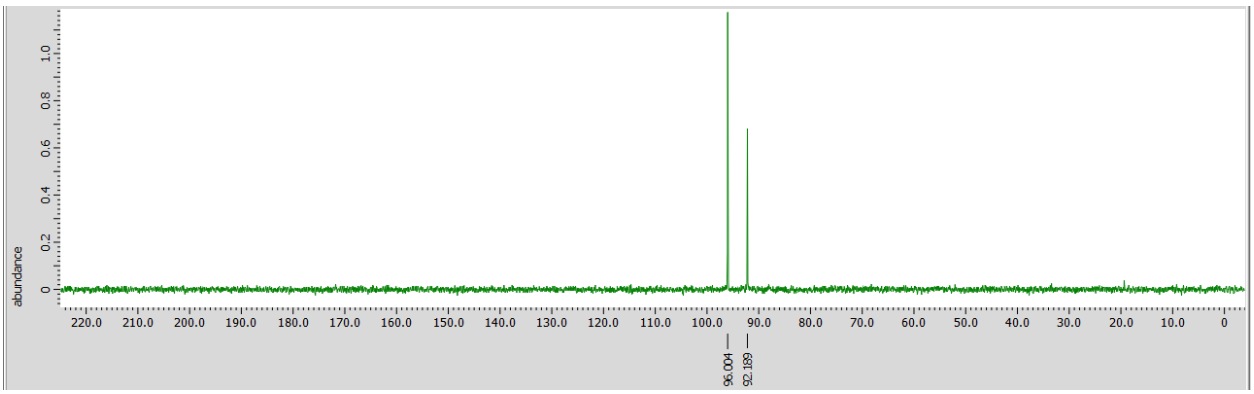
Graph 1: Growth Rate of *Lactobacillus acidophilus* as a Function of Absorbance

Table 3: NMR Data for Control Solutions

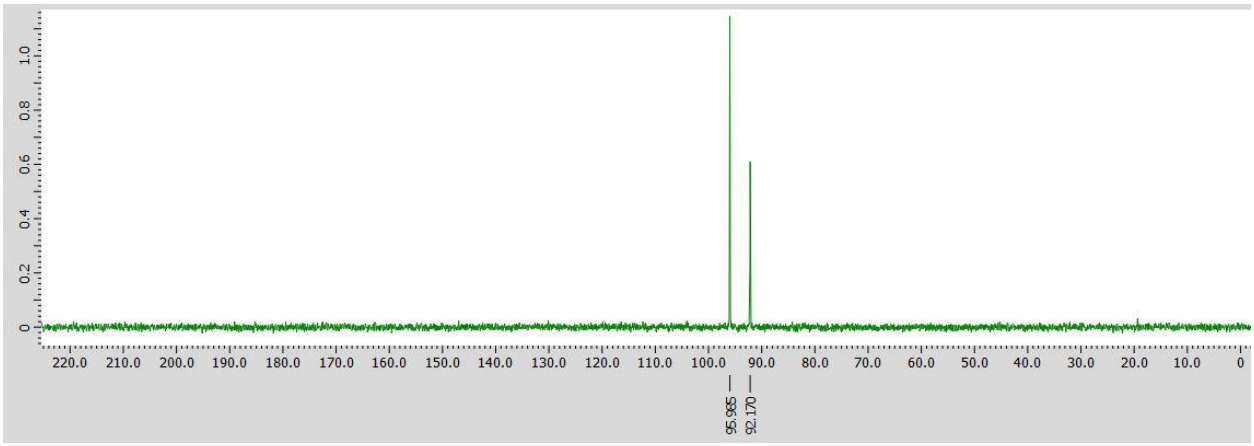
	β-Glucose		α-Glucose	
Time	Peak (ppm)	Peak Height [abn]	Peak (ppm)	Peak Height [abn]
0	96.0	0.75183	92.2	0.41317
2	96.0	1.35312	92.2	0.6978
4	95.9	1.10188	92.17	0.6444
6	95.9	1.21526	92.17	0.69443
8	95.9	1.07791	92.18	0.57009
10	95.9	1.14678	92.16	0.60712

Table 4: NMR Data for Metformin Solutions

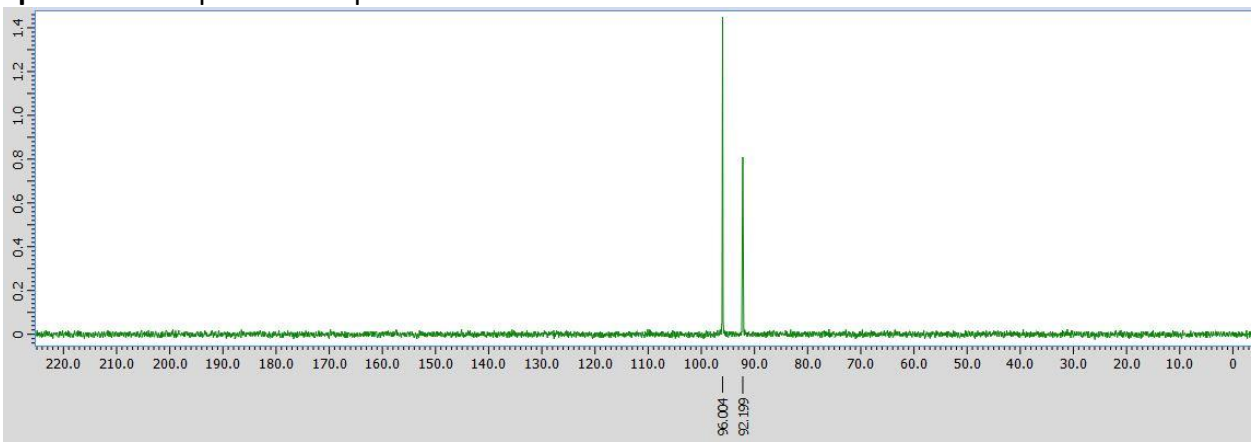
	β-Glucose		α-Glucose	
Time	Peak (ppm)	Peak Height [abn]	Peak (ppm)	Peak Height [abn]
0	96.0	1.44792	92.1	0.81084
2	95.9	1.23372	92.1	0.63353
4	95.8	0.74083	92.1	0.30322
6	95.7	0.71139	92.0	0.25662
8	95.9	1.13356	92.17	0.61201
10	96.0	1.17733	92.18	0.66894



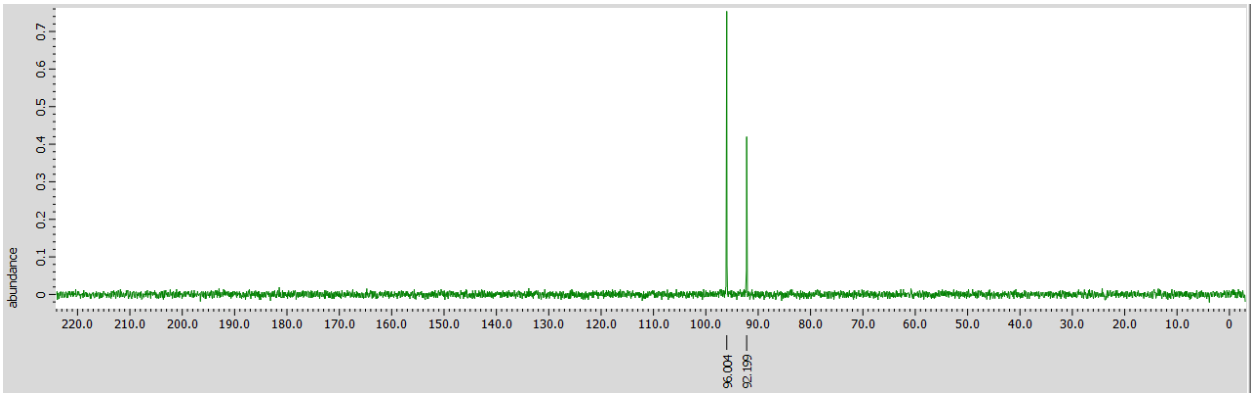
Spectra 1: Sample 1 NMR spectrum



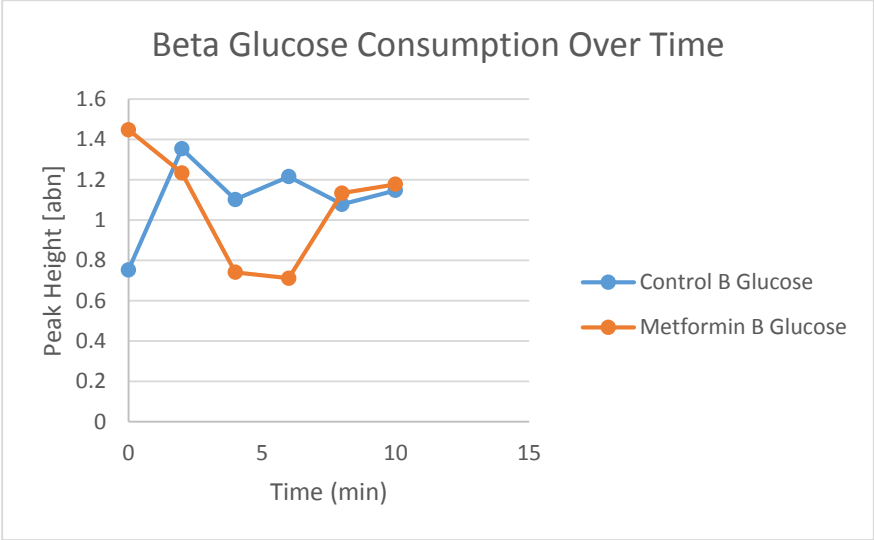
Spectra 2: Sample 6 NMR spectrum



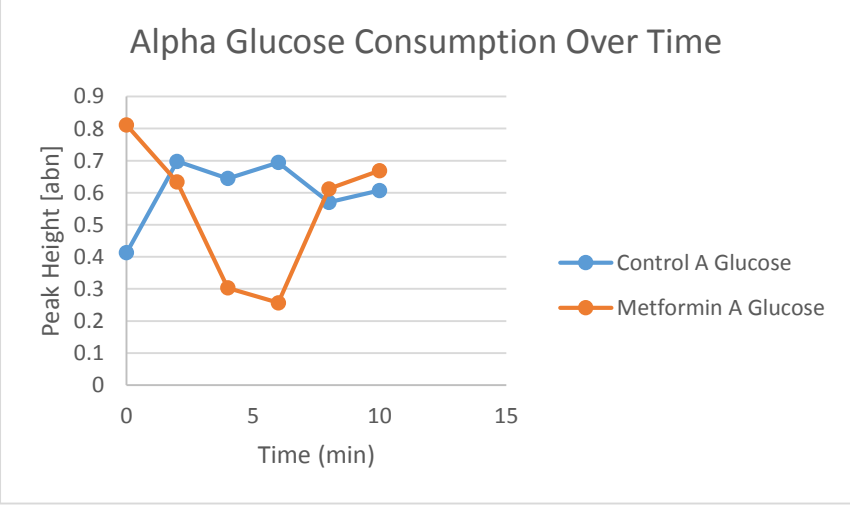
Spectra 3: Sample 11 NMR spectrum



Spectra 4: Sample 16 NMR spectrum

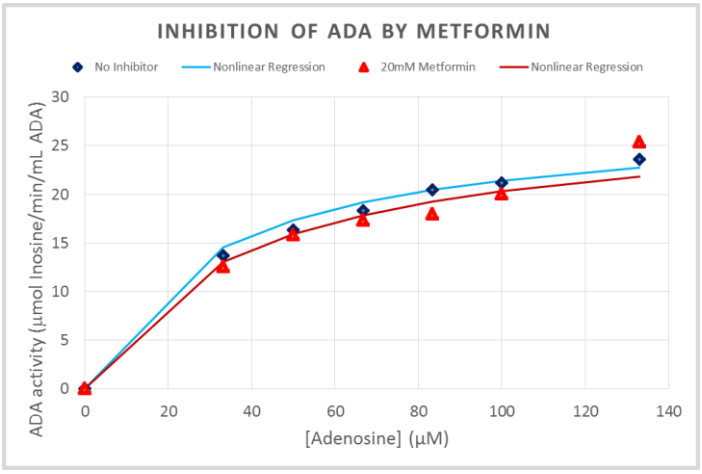


Graph 2: Peak Height vs. Incubation Time for Beta Glucose

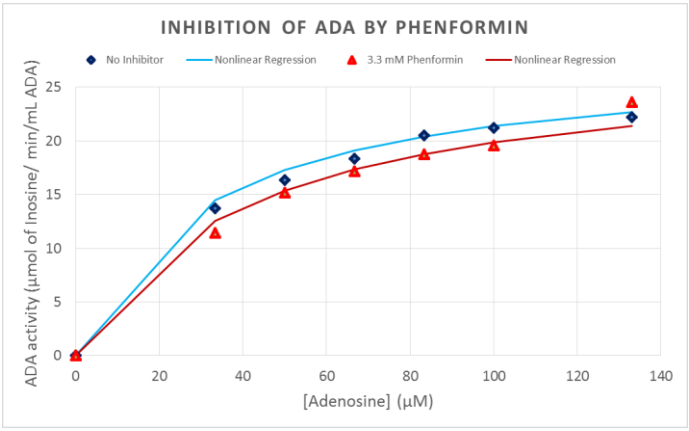


Graph 3: Peak Height vs. Incubation Time for Alpha Glucose

The data seen above shows inconclusive results. All spectra analyzed showed peaks around 96 ppm and 92 ppm. Using the Spectral Database for Organic Compounds SDBS, it was determined that the peak at 96 ppm represents β-glucose while the peak at 92 ppm represents α-glucose.[7] It was expected that the spectra would show a lactic acid peak in Sample 6 and Sample 16 due to the cells undergoing lactic acid fermentation. However, this is not seen in those respective spectra's. This could be due to the fact that the cells were not under completely anaerobic conditions, which allowed them to carry out aerobic respiration. If the cells were going through aerobic respiration, the labeled glucose would disappear as carbon dioxide released from the citric acid cycle, and thus the α-glucose and β-glucose peaks would show a decreased peak height (Since carbon dioxide is released as a gas, it cannot be picked up by NMR analysis so a peak will not be present). Unfortunately, this is not seen in Graphs 2 and 3 due to the fact that the amount of glucose increases. In future studies, the cells can be flushed with nitrogen gas to ensure completely anaerobic conditions. Also, the measurements can be made over a longer period of time, allowing the cells more time to undergo metabolism of glucose.



Graph 4: Nonlinear Regression Plot for 20 mM Metformin



Graph 5: Nonlinear Regression Plot for 3.3 mM Phenformin

Table 5: Nonlinear Regression Analysis

	Metformin	**Phenformin**
Concentration (mM)	20	3.3
V_{max} (µmol inosine/min/mL ADA)	28	28
K_m (µM)	31	31
K_I (mM)	88	10
Mode of Inhibition	Competitive	Competitive

It was determined that the K_m value is equal to 31 µM. The K_I for 20 mM metformin is 88 mM while the K_I for 3.3 mM phenformin is 10 mM as seen in Table 3. Since both K_I values are larger than the K_m, it can be assumed that the inhibitors reduce the binding affinity of the enzyme. Phenformin has a smaller K_I value than metformin indicating that it is better at inhibiting ADA activity. The V_{max} values are very similar; therefore, it can be said that the total amount of substrate being converted into product is similar in all three cases. This suggests that the inhibitor reduces the affinity of the enzyme for the substrate but does not decrease the ability of the enzyme to convert substrate into product if the substrate were to bind to it. Thus, the mode of inhibition of metformin and phenformin is competitive. The actual substrate binds tighter to the enzyme than either inhibitor is able to, implying that a subtle competitive inhibition is taking place.

CONCLUSION

In conclusion, it has been determined by nonlinear regression analysis that ADA activity is competitively inhibited by metformin and phenformin. However, the competitive inhibition is subtle due to the fact that the adenosine substrate has a higher binding affinity toward ADA in a comparison to the inhibitors, metformin and phenformin. As for the experiment involving glucose transporters in *Lactobacillus acidophilus*, the NMR results were inconclusive due to the lack of a peak representing lactic acid, initially suggesting that aerobic respiration was taking place. However, the amount of glucose seen in the spectrum did not decrease as should be seen in aerobic respiration by the release of carbon dioxide in the citric acid cycle.

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