9-1-2013

Inhibition of Bovine Adenosine Deaminase by Anti-Hyperglycemic Agents Metformin and Phenformin

Kaitlin Doucette
Winona State University

Neha Amatya
Winona State University

Elizabeth Daniels
Winona State University

Myoung Lee
Winona State University

Inhibition of Bovine Adenosine Deaminase by Anti-Hyperglycemic Agents Metformin and Phenformin

Kaitlin Doucette, Neha Amatya, Elizabeth Daniels, Dr. Myoung E. Lee
Department of Chemistry, Winona State University, Winona, Minnesota

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. In addition to their anti-hyperglycemic effects, both metformin and phenformin are known to inhibit proliferation of cancer cells. Adenosine deaminase is involved in purine nucleoside metabolism and its activity was elevated in certain cancers such as bladder and renal carcinoma, a possible cause for the proliferation of these cancer cells. 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.

BACKGROUND

Type 2 Diabetes Mellitus (T2D) is the most common form of diabetes, totaling 90% to 95% of all cases of diabetes. It is characterized by high blood glucose levels that result from a decrease in glucose uptake in muscle and adipose tissue, as well as an increase in hepatic gluconeogenesis[1]. One of the most common types of oral medication is metformin, a biguanide anti-hyperglycemic agent. Phenformin was another biguanide anti-hyperglycemic agent that was removed from most markets in the 1970s due to high risk of lactic acidosis[2].

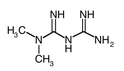


Figure 1: Metformin
(3-carbamimidoyl-1,1-dimethylbiguanide)

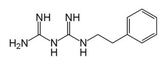


Figure 2: Phenformin
(phenethylbiguanide)

There are three proposed mechanisms for the effects of metformin lowering blood glucose levels in patients with T2D. The first two proposed mechanisms state that metformin causes inhibition of AMP deaminase (AMPD) and mild, nonspecific inhibition of mitochondrial electron complex I[3] . The inhibition of AMPD and complex I would have the overall effect of increasing AMP concentration, which causes the third proposed mechanism: activation of AMP kinase (AMPK)[4]. The activation of AMPK causes the stimulation of fatty acid oxidation, inhibition of cholesterol and triglyceride synthesis, and stimulation of muscle glucose uptake and modulation of insulin secretion[5].
Adenosine deaminase (ADA) is the enzyme that performs a function similar to that of AMPD in that it converts adenosine into inosine: in the case of AMPD, adenosine monophosphate into inosine monophosphate (Figure 3).

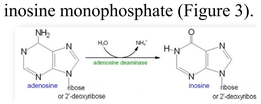


Figure 3: Irreversible deamination of adenosine.

In this research, ADA is used as a comparative enzyme to extrapolate the effects of metformin on AMP deaminase. Additionally, adenosine has been found to accumulate in solid tumors, and has been known to stimulate growth of tumors[6]. The possibility of inhibition of ADA by metformin has been explored in previous research on protein docking simulating software (Figure 3).

Figure 4: ADA with both metformin (left image) and adenine (right image) bound at or very near to the active site of the enzyme. Images obtained from previous Autodock molecular docking simulation.

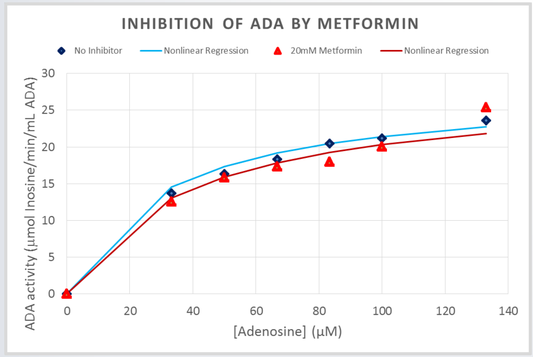
PURPOSE

The purpose of this research is to explore the possibility of inhibition of adenosine deaminase (ADA) by metformin and phenformin.

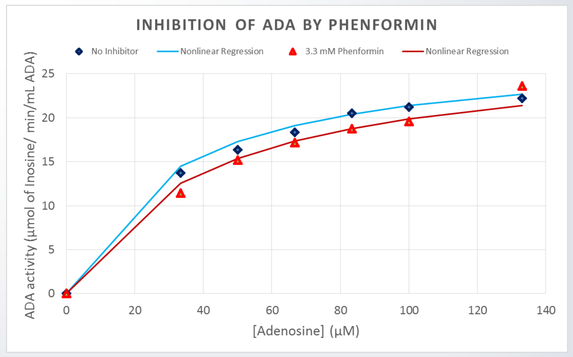
MATERIALS AND METHODS

In this 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.

RESULTS AND DISCUSSION



Graph 1: Nonlinear Regression Plot for 20 mM Metformin.



Graph 2: Nonlinear Regression Plot for 3.3 mM Phenformin

Non-Linear 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

Table 1: Nonlinear Regression Analysis Data

RESULTS AND DISCUSSION

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 1. 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.

CONCLUSIONS

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.

FUTURE WORK

Future work on this research will involve testing AMPD and determining mode of inhibition of metformin and phenformin on it.

ACKNOWLEDGEMENTS

Dr. Myoung E. Lee for instructing and assisting in the experiment, and Winona State University for the student research grant as well as the student travel grant.

REFERENCES

(1) Schramm, T. K.; Gislason, G. H.; Vaag, A.; Rasmussen, J. N.; Folke, F.; Hansen, M. L.; Fosbøl, E. L.; Køber, L.; Norgaard, M. L.; Madsen, M.; et al. Mortality and Cardiovascular Risk Associated with Different Insulin Secretagogues Compared with Metformin in Type 2 Diabetes, with or without a Previous Myocardial Infarction: A Nationwide Study. *Eur Heart J* **2011**, *32*, 1900–1908.
(2) Woollhead, A. M.; Scott, J. W.; Hardie, D. G.; Baines, D. L. Phenformin and 5-Aminoimidazole-4-Carboxamide-1-B-D-Ribofuranoside (AICAR) Activation of AMP-Activated Protein Kinase Inhibits Transepithelial Na+ Transport across H441 Lung Cells. *The Journal of Physiology* **2005**, *566*, 781–792.
(3) Zakikhani, M.; Dowling, R.; Fantus, I. G.; Sonenberg, N.; Pollak, M. Metformin Is an AMP Kinase–Dependent Growth Inhibitor for Breast Cancer Cells. *Cancer Res* **2006**, *66*, 10269–10273.
(4) Ouyang, J.; Parakhia, R. A.; Ochs, R. S. Metformin Activates AMP Kinase through Inhibition of AMP Deaminase. *J. Biol. Chem.* **2011**, *286*, 1–11.
(5) Mahmood, K.; Naeem, M.; Rahimnajjad, N. A. Metformin: The Hidden Chronicles of a Magic Drug. *European Journal of Internal Medicine* **2013**, *24*, 20–26.
(6) Spychala, J. Tumor-Promoting Functions of Adenosine. *Pharmacology & Therapeutics* **2000**, *87*, 161–173.

RESEARCH / CREATIVE PROJECT ABSTRACT / EXECUTIVE SUMMARY
FINAL REPORT FORM

Title of Project
Inhibition of bovine adenosine deaminase by anti-hyperglycemic agents metformin and phenformin

---

Student Name Kaitlin A. Doucette

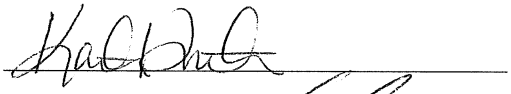
Faculty Sponsor Dr. Myoung E. Lee

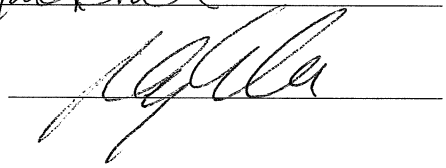
Department Chemistry

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. In addition to their anti-hyperglycemic effects, both metformin and phenformin are known to inhibit proliferation of cancer cells. Adenosine deaminase is involved in purine nucleoside metabolism and its activity was elevated in certain cancers such as bladder and renal carcinoma, a possible cause for the proliferation of these cancer cells. 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, KI, of 88 mM. Phenformin was slightly more effective than metformin as a competitive inhibitor with KI of 10 mM. The KM 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.

The end product of this project in electronic format has been submitted to the Provost/Vice President for Academic Affairs via the Office of Grants & Sponsored Projects Officer (Maxwell 161, npeterson@winona.edu).

Student Signature  Date 5/5/2014

Faculty Sponsor Signature  Date May 5, 2014

Inhibition of Bovine Adenosine Deaminase by Anti-Hyperglycemic Agents Metformin and Phenformin

Kaitlin Doucette, Neha Amatya, Elizabeth Daniels, Dr. Myoung E. Lee

Department of Chemistry, Winona State University

4/14/2014

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. In addition to their anti-hyperglycemic effects, both metformin and phenformin are known to inhibit proliferation of cancer cells. Adenosine deaminase is involved in purine nucleoside metabolism and its activity was elevated in certain cancers such as bladder and renal carcinoma, a possible cause for the proliferation of these cancer cells. 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:

Type 2 Diabetes Mellitus (T2D) is the most common form of diabetes, totaling 90% to 95% of all cases of diabetes. It is characterized by high blood glucose levels that result from a decrease in glucose uptake in muscle and adipose tissue, as well as an increase in hepatic gluconeogenesis[1]. One of the most common types of oral medication is metformin, a biguanide anti-hyperglycemic agent. Phenformin was another biguanide anti-hyperglycemic agent that was removed from most markets in the 1970s due to high risk of lactic acidosis[2].

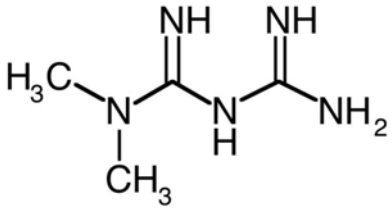


Figure 1: Metformin
(3-carbamimidoyl-1,1-dimethylbiguanide)

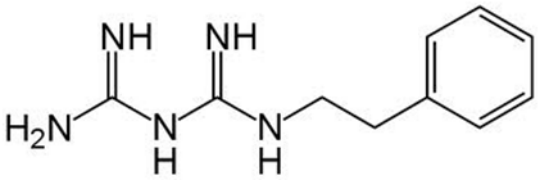


Figure 2: Phenformin
(phenethylbiguanide)

There are three proposed mechanisms for the effects of metformin lowering blood glucose levels in patients with T2D. The first two proposed mechanisms state that metformin causes inhibition of AMP deaminase (AMPD) and mild, nonspecific inhibition of mitochondrial electron complex I[3] . The inhibition of AMPD and complex I would have the overall effect of increasing AMP concentration, which causes the third proposed mechanism: activation of AMP kinase (AMPK)[4]. The activation of AMPK causes the stimulation of fatty acid oxidation, inhibition of cholesterol and triglyceride synthesis, and stimulation of muscle glucose uptake and modulation of insulin secretion[5].
Adenosine deaminase (ADA) is the enzyme that performs a function similar to that of AMPD in that it converts adenosine into inosine: in the case of AMPD, adenosine monophosphate into inosine monophosphate (Figure 3).

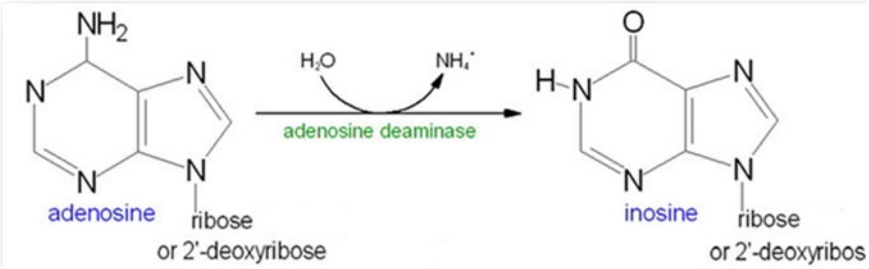


Figure 3: Irreversible deamination of adenosine.

In this research, ADA is used as a comparative enzyme to extrapolate the effects of metformin on AMP deaminase. Additionally, adenosine has been found to accumulate in solid tumors, and has been known to stimulate growth of tumors[6]. The possibility of inhibition of ADA by metformin has been explored in previous research on protein docking simulating software (Figure 3).

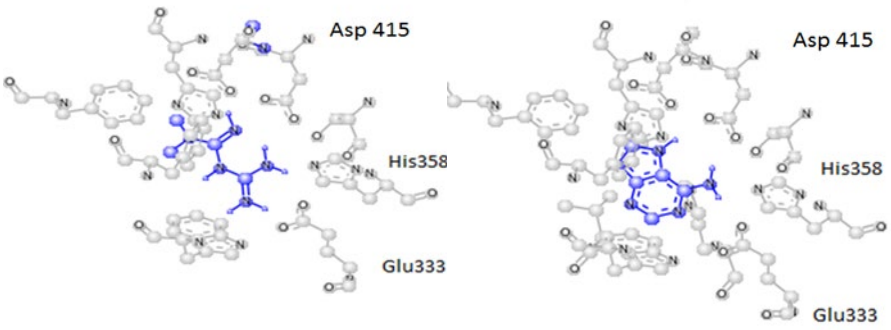


Figure 4: ADA with both metformin (left image) and adenine (right image) bound at or very near to the active site of the enzyme. Images obtained from previous Autodock molecular docking simulation.

Results and Discussion:

In this 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.

Solutions of adenosine, enzyme, and phosphate buffer were prepared with either metformin or phenformin, or neither, as a control group. The solutions were made in quartz cuvettes consisting of a 50 mM phosphate buffer at pH 7.4 and adenosine concentrations ranging from 15 µM to 133 µM. The solutions of enzyme solution containing metformin and phenformin were analyzed spectrophotometrically at 265 nm, and the activity of the adenosine deaminase was calculated and used to create a nonlinear regression graph for each.

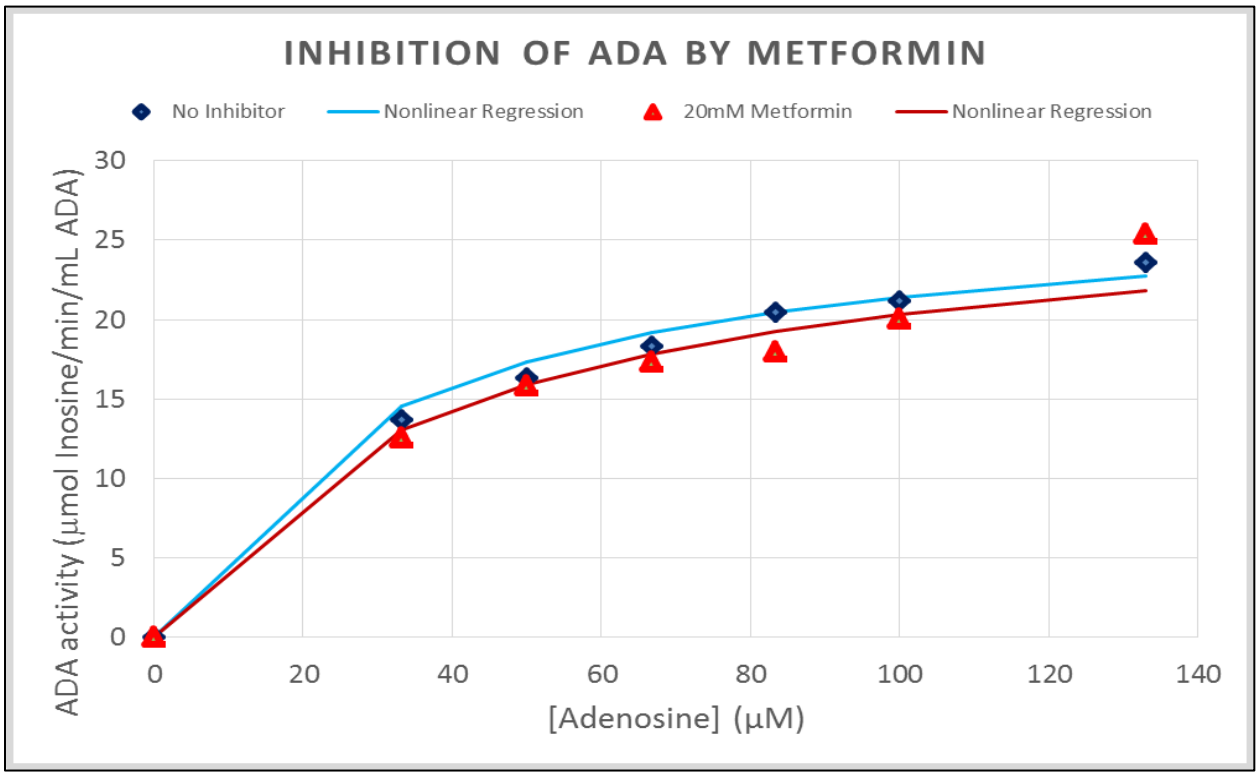


Figure 5: Nonlinear regression plot of 20 mM metformin

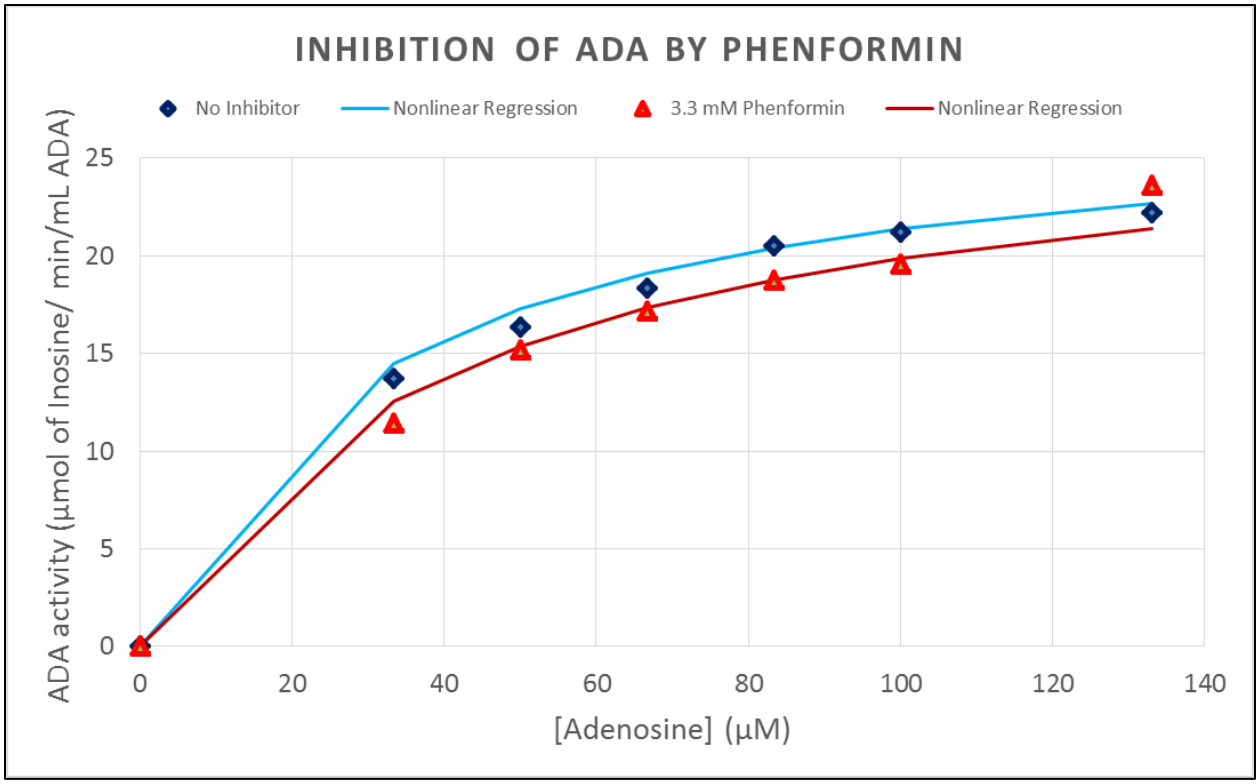


Figure 6: Nonlinear regression plot for 3.3 mM phenformin

The nonlinear regression plots were then used to calculate the V_{max}, K_M and K_I of the enzyme activity. These calculations were then used to determine the type of inhibition taking place by both metformin and phenformin, as seen in Table 1.

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

Table 1: Nonlinear regression analysis

Based on the data calculated and shown in Table 1, since both K_I values are larger than the K_M it is observed that both metformin and phenformin reduce the binding affinity of adenosine deaminase. Since phenformin's K_I is smaller than that of metformin, this indicates that it is a stronger inhibitor to adenosine deaminase than metformin. The V_{max} values are similar and thus the total amount of substrate being converted into product is similar in all three cases: with metformin, with phenformin, and without any inhibitor. Thus it is implied that both inhibitors reduce the affinity of adenosine deaminase for adenosine, but does not decrease the ability of the enzyme to convert substrate into product if the substrate were to bind to it. This suggests that the mode of inhibition of metformin and phenformin is competitive. However, a very subtle inhibition is taking place, as the actual substrate

binds tighter to the enzyme than either inhibitor. High concentrations of inhibitor are required for any noticeable effect on the enzyme.

Conclusions:

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.

Future Work:

Future work on this research will involve testing AMPD and determining mode of inhibition that metformin and phenformin have with regards to the enzyme.

Acknowledgements:

Dr. Myoung E. Lee for instructing and assisting in the experiment, and Winona State University for the student research grant as well as the student travel grant.

References

(1)Schramm, T. K.; Gislason, G. H.; Vaag, A.; Rasmussen, J. N.; Folke, F.; Hansen, M. L.; Fosbøl, E. L.; Køber, L.; Norgaard, M. L.; Madsen, M.; et al. Mortality and Cardiovascular Risk Associated with Different Insulin Secretagogues Compared with Metformin in Type 2 Diabetes, with or without a Previous Myocardial Infarction: A Nationwide Study. *Eur Heart J* **2011**, *32*, 1900–1908.

(2) Woollhead, A. M.; Scott, J. W.; Hardie, D. G.; Baines, D. L. Phenformin and 5-Aminoimidazole-4-Carboxamide-1-B-D-Ribofuranoside (AICAR) Activation of AMP-Activated Protein Kinase Inhibits Transepithelial Na+ Transport across H441 Lung Cells. *The Journal of Physiology* **2005**, *566*, 781–792.

(3) Zakikhani, M.; Dowling, R.; Fantus, I. G.; Sonenberg, N.; Pollak, M. Metformin Is an AMP Kinase–Dependent Growth Inhibitor for Breast Cancer Cells. *Cancer Res* **2006**, *66*, 10269–10273.

(4) Ouyang, J.; Parakhia, R. A.; Ochs, R. S. Metformin Activates AMP Kinase through Inhibition of AMP Deaminase. *J. Biol. Chem.* **2011**, *286*, 1–11.

(5) Mahmood, K.; Naeem, M.; Rahimnajjad, N. A. Metformin: The Hidden Chronicles of a Magic Drug. *European Journal of Internal Medicine* **2013**, *24*, 20–26.

(6) Spychala, J. Tumor-Promoting Functions of Adenosine. *Pharmacology & Therapeutics* **2000**, *87*, 161–173.