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The Effects of Valproate Prodrugs on the Inositol Biosynthetic Pathway in Saccharomyces Cerevisiae Yeast

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Honors Thesis

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Abstract

Bipolar disorder is a psychiatric disorder characterized by episodes of abnormally elevated mood followed by periods of extreme depression. Currently, valproate is one of the drugs used to treat the disorder but it is associated with many negative side effects. The goal of this project is to create a prodrug with structural similarities to valproate and determine if its effect on growth of the yeast *Saccharomyces cerevisiae* is similar to that of valproate. Yeast cells were grown in media with or without the prodrug in the presence or absence of inositol. The results showed that the prodrug inhibited growth.

Background

Bipolar disorder is a severe and chronic illness affecting approximately 1.5% of the American population (Azab et al., 2007; Azab and Miriam L. Greenberg, 2006). The major symptoms of the disorder are episodes of abnormally elevated mood followed by periods of extreme depression. One of the drugs currently used to treat patients with bipolar disorder is valproate. It is thought to accomplish treatment through the depletion of intracellular inositol. Inositol is a chemical compound that forms the basis of many second messenger systems in the body. A second messenger is a molecule that relays signals from receptors on the cell surface to target molecules within the cell, therefore causing a change in the activity of the cell. The original signal usually comes from a hormone.

Valproate is effective in managing the symptoms of bipolar disorder, as it is a mood stabilizer and anti-convulsant. However, there are many negative side effects associated with the drug. These include nausea, vomiting, diarrhea, indigestion, and in some rare cases, liver failure and pancreatitis. The purpose of this research project is to create a valproate prodrug and test its effect on the inositol biosynthetic pathway of the yeast *Saccharomyces cerevisiae.* To make a prodrug, an inactive group is attached to a known pharmacologically active molecule (in this case, valproate) via an easily metabolized ester linkage. The two inactive groups used in this project were an aldehyde and a chlorine atom. Activation of the prodrug is then achieved by the action of enzymes in the body. The reason for using a prodrug is to promote easier absorption and solubility, thereby minimizing the side effect while still treating the target symptoms.

The specific compound that will be made is called α-chlorooctyl valproate, which belongs to the organic family of α-haloacylals or 1-haloalkyl esters. This prodrug will then be tested for its ability to affect yeast growth of the strain SMY7 in the presence or absence of inositol. The success of the prodrug will depend on its ability to act similar to that of valproate by decreasing intracellular inositol. Yeast growth is dependent on inositol and therefore inhibited growth should indicate a decrease in inositol levels. The reason for using yeast as the biological system is that yeast and humans have a similar inositol biosynthetic pathway. Therefore, how the prodrug affects inositol levels in yeast will be indicative of how it possibly would affect inositol levels in humans and thus whether it is a viable option for the treatment of bipolar disorder.

Methodology

SYNTHESIS OF VALPROATE PRODRUG (α-CHLOROOCTYL VALPROATE):

Trial 1

A 50 mL round bottom flask and spin vane were dried in the oven for about 15 minutes. Valproic acid (VPA) and thionyl chloride were refluxed for 45 minutes in dichloromethane to produce valproyl chloride. Reflux involves boiling the solution with minimal evaporation of the reactants, products and solvent. The valproyl chloride was then cooled in an ice bath and octyl aldehyde and zinc oxide (catalyst) were added to the mixture. This solution was stirred at room temperature for approximately two hours. The final mixture was gravity filtered and then rotovapped. Gravity filtration is used to isolate the desired product dissolved in the solvent away from the solid zinc oxide catalyst.

Rotary evaporation or rotavap is then used to ensure the removal of the solvent away from the product. Nuclear magnetic resonance (NMR) spectroscopy was then performed to analyze the resulting product. Table 1 provides the amounts of materials used in this trial.

Table 1

Compound	Moles	Volume (mL)	Grams	Density (g/mL)	Molecular weight (g/mol)
VPA	0.008	1.28	--	0.90	144.21
Octyl aldehyde	0.008	1.25	--	0.82	128.22
Thionyl chloride	0.009	0.66	--	1.61	118.97
Dichloromethane	--	20	--	--	--
Anhydrous zinc oxide	0.002	--	0.273	5.60	81.41

Trial 2

A 100 mL round bottom flask and spin vane were dried in the oven for about 15 minutes. VPA and thionyl chloride were refluxed in dichloromethane for one hour to produce valproyl chloride. Reflux involves boiling the solution with minimal evaporation of the reactants, products and solvent. The valproyl chloride was then cooled in an ice bath and octyl aldehyde and zinc chloride (catalyst) were added. The solution was stirred at room temperature for approximately three hours. The final mixture was gravity filtered and then rotovapped. Gravity filtration is used to isolate the desired product dissolved in the solvent away from the solid zinc oxide catalyst. Rotary evaporation or rotavap is then used to ensure the removal of the solvent away from the product. After performing an NMR of the product, further purification was done through column chromatography. The solvent chosen was hexane and alumina gel was used as the adsorbent. The resulting fractions were rotovapped and analyzed using NMR to determine which fraction was

structurally similar to the desired product. Infrared (IR) spectroscopy was performed on the chosen fraction along with the starting materials (octyl aldehyde and VPA) for further analysis. Table 2 provides the amounts of materials used in this trial.

Table 2

Compound	Moles	Volume (mL)	Grams	Density (g/mL)	Molecular weight (g/mol)
VPA	0.024	3.846	--	0.90	144.21
Octyl aldehyde	0.024	3.744	--	0.82	128.22
Thionyl chloride	0.027	1.990	--	1.61	118.97
Dichloromethane	--	50	--	--	--
Anhydrous zinc chloride	0.006	--	0.665	--	110.86

BIOLOGICAL TESTING:

Liquid media for the yeast was prepared using the ingredients in Table 3. Two types of media were made: one with inositol (I+) and one without inositol (I-). The ingredients for 500 mL of media were mixed into two 1 L Erlenmeyer flasks, labeled either I+ or I- depending on the contents. These flasks were then covered with aluminum foil and autoclaved for 25 minutes along with two empty 250 mL Erlenmeyer flasks each labeled either I+ or I-. Then, 100 mL each of the I+ and I- media was poured into its corresponding 250 mL flask. This was then inoculated with plated SMY7 yeast cells and allowed to grow for 24-30 hours in a shaker bath (150 rpm; 30 °C). After this, 1000 µl of stock I+ media was placed in a cuvette using a pipette as a control, 900 µl of stock I+ media was placed in a second cuvette as well as 100 µl from the I- overnight flask, and 900 µl of stock I+ media was placed in a third cuvette as well as 100 µl from the I+ overnight flask. A concentration reading was then taken with the spectrophotometer to confirm that there was enough growth to continue on with next step (at least 0.1).

After autoclaving five new empty 250 mL Erlenmeyer flasks, each flask was labeled I+, I-, PD1, PD2, or PD3 (PD corresponds to prodrug). 100 mL of I+ media was added to the new I+ flask and 100 mL of I- media was added to each of the other four flasks. Based on calculations using the formula C1V1 = C2V2, specific volumes of the I+ and I- overnight solutions were added to the corresponding flasks containing new media. The prodrug flasks contained both I- overnight solution as well as certain concentrations of prodrug. PD1 had a prodrug concentration of 0.5 mM, PD2 had a concentration of 2.5 mM, and PD3 had a concentration of 5.0 mM. These five flasks were placed in a shaker bath (150 rpm; 30 °C) for 24-30 hours. Another concentration reading was taken of the resulting cells using the spectrophotometer. A 10^{-5} dilution was then performed on the resulting solutions. 1 mL of each solution was placed in a cuvette, 10 µl of that was placed in another cuvette with 990 µl of water, 10 µl of that was placed in a third cuvette with 990 µl of water, and finally 100 µl of that was spread on a previously made YPD plate (see Table 4 for recipe). The plates were incubated for 48 hours and then the resulting yeast colonies were counted.

Table 3

Ingredient	Amount
Vitamin Free Yeast Base	0.345 mL
Ammonium sulfate	1.005 mL
Glucose	10 g
100x vitamins	10 g
Myo-inositol	5 mL
Adenine	7.5 mL
Arginine	1 mL
Histidine	1 mL
Leucine	2 mL
Lysine	1 mL
Methionine	1 mL
Threonine	8 mL
Tryptophan	1 mL

Uracil	8 mL
Deionized H_2O	500 mL

Table 4

Ingredient	Amount
Yeast extract	10 g
Bacto-peptone	20 g
Glucose	20 g
Agar	20 g
Deionized H_2O	1 L

Results

NMR and IR ANALYSIS:

Table 5 summarizes the important spectral features that resulted from IR, ^{1}H-NMR, and ^{13}C-NMR spectroscopy. The number of peaks refers either to the number of different hydrogens or carbons depending on the type of NMR performed.

Table 5

	Important Spectral Features		
Compound	**IR (cm^{-1})**	**^{1}H-NMR (ppm)**	**^{13}C-NMR (ppm)**
Octyl aldehyde	C=O (1727)	H_1 (9.7) H_a (2.4) # of peaks* = 8	C_α (44.0) C_8 (14.0) # of peaks** = 8
VPA	C=O (1706) O-H (2961) C-O (1215,1253)	H_b (2.35) # of peaks* = 5	C_1 (184.0) C_2 (45.0) # of peaks** = 5
α-chlorooctyl valproate (product)	C=O (1758) C-O (1110)	H_a (6.4) H_b (2.35) # of peaks* = 12	C_1 (174.5) C_2 (45.0) C_α (84.0) C_8 (14.0) # of peaks** = 13

BIOLOGICAL TESTING:

Trial 1

The following table shows the results of colony growth in Trial 1:

Table 6

Type of media	Number of colonies
With inositol (I+)	82
Without inositol (I-)	101
I- and Prodrug (0.5 mM)	1060
I- and Prodrug (2.5 mM)	517
I- and Prodrug (5.0 mM)	184

Trial 2

The following table shows results of colony growth in Trial 2:

Table 7

Type of media	Number of colonies
With inositol (I+)	85
Without inositol (I-)	8
I- and Prodrug (0.5 mM)	4
I- and Prodrug (2.5 mM)	18
I- and Prodrug (5.0 mM)	9

Trial 3

The following table shows results of colony growth in Trial 3:

Table 8

Type of media	Number of colonies
With inositol (I+)	468
Without inositol (I-)	167
I- and Prodrug (5.0 mM)	84

Discussion

PRODRUG SYNTHESIS:

The main differences between Trial 1 and 2 were the amounts of materials, the time required, and the catalyst used. The amount of moles used in Trial 1 were tripled for Trial 2 because there was not enough material produced from Trial 1 and therefore the experiment needed to be scaled up. As a result, the time required for each step needed to be altered to accommodate the greater volume of solutions. Also, the catalyst used in Trial 1 was zinc oxide while the catalyst used in Trial 2 was zinc chloride. The reason that zinc oxide was originally used was because it is easier to produce and not easily contaminated. Zinc chloride, on the other hand, is a strong catalyst but it requires more preparation and therefore is more susceptible to contamination. The product that resulted from Trial 1 was not exactly the desired product. This could have been due to the fact that the zinc oxide catalyst was not efficient enough to drive the reaction to completion. When the catalyst was switched to zinc chloride in Trial 2, the product came out with a structure similar to the expected product. Because Trial 1 resulted in a poor product, there was no further purification done. That is why column chromatography only appears in Trial 2.

NMR and IR Analysis

The IR of the prodrug showed two main diagnostic peaks that indicate the success of the reaction. There was a peak at 1758.42 cm^{-1} and at 1110.32 cm^{-1}, which are both characteristic of an ester and thus the desired product. The ^{1}H NMR of both the prodrug and VPA showed a peak at 2.35 ppm. This is because the specific hydrogen atom in VPA remained in the same position throughout the reaction and was therefore also present in the prodrug. The prodrug also shares a hydrogen atom with octyl aldehyde. However, in

the octyl aldehyde, it produced a peak at 2.4 ppm while in the prodrug, its peak was at 6.4 ppm. This chemical shift was due to the fact that the reaction resulted in the hydrogen atom moving next to an oxygen atom and a chloride atom, both very electronegative groups. Finally, the ^{13}C NMR confirmed the success of the reaction. Most of the carbon atoms in the octyl aldehyde and VPA stayed in relatively the same positions throughout the reaction. The main chemical shift occurred for the alpha carbon atom in the octyl aldehyde. It moved from next to an oxygen atom to next to an oxygen atom and a chloride atom. These electronegative groups attracted the electrons from the carbon atom, causing the shift in ppm.

BIOLOGICAL TESTING:

Ideally, the prediction was that plates containing I+ media should have the largest number of colonies. Cells need inositol to grow and therefore the addition of extra inositol in the I+ media should have led to increased growth. The plates with I- media and the prodrug were predicted to have less growth than the two control plates. This is because the cells would not be getting extra inositol from the media and the inositol made intracellularly should be depleted by the prodrug. Therefore, the lack of inositol should inhibit large amounts of growth. Increasing the concentration of the prodrug should result in a decrease of cell growth.

As shown in Tables 6, 7, and 8, the results from the plating of the yeast cultures varied from trial to trial. Trial 1 resulted in the plate with cells grown in I- media and 0.5 mM of prodrug having the most growth and the plate with cells grown in I+ media having the least growth. This pattern is most likely due to the fact that the plates were

contaminated before the cells could be counted and therefore cell growth was affected. The plates with the prodrug did, however, display the expected pattern that increasing prodrug concentration results in decreased cell growth. The results from Trial 2 showed that the I+ plate had the most growth, which was expected. Also, the fact that each prodrug plate had less growth than the I+ plate follows the prediction that the prodrug decreases cell growth by depleting intracellular inositol levels. The problem with Trial 2 is that there isn't a linear relationship between prodrug concentration and colony growth. Also, the two plates with the highest prodrug concentration have more growth than the I- plate, which is opposite to what should have happened. Finally, Trial 3 displayed a pattern that was most similar to the predicted pattern. The I+ plate had the most growth and the prodrug plate had the least growth. Unfortunately, there was not enough prodrug left to test three different concentrations so only the concentration of 5.0 mM was used in the trial as it was the most effective.

Conclusion

The prodrug synthesis was determined to be successful based on the NMR and IR analysis. The one problem with the synthesis was that not enough of the drug was produced. A solution for this would be to increase the starting amounts of materials. There would need to be more testing done for the results from the biological testing to be conclusive. Future experiments should include more trials in order to obtain a broader range of data.

Annotated Bibliography

Azab, Abed N., and Miriam L. Greenberg. "Anticonvulsant efficacy of valproate-like carboxylic acids: a potential target for anti-bipolar therapy." Bipolar Disorders 9.3 (2007): 197-205. Print.

This article provides a review of the anti-convulsant properties of valproate-like compounds. Anti-convulsant drugs are most often used in the treatment of epileptic seizures but studies have shown that they can also act as mood-stabilizers. The valproate analogs were tested mainly in vitro and on animals. Since the compounds had anti-convulsant effects similar to that of valproate, they could be effective alternatives for the treatment of bipolar disorder. The information gained from this study is useful to my research because it provides various compounds and mechanisms that can be tested further for their specific effects on bipolar disorder.

Azab, Abed N., and Miriam L. Greenberg. "Lipid connection to bipolar disorder." Future Neurology 1.4 (2006): 505-13. Print.

The goal of this paper is to examine the lipid connection to bipolar disorder since there has been increasing evidence that lipids are associated with the mechanism and pathology. The authors focus on three main molecules and their effect on bipolar disorder: fatty acids, cholesterol, and phospholipids (specifically, the phosphatidyl cycle). This is beneficial to my research because the article explains the mechanisms of these molecules in relation to bipolar disorder.

Degrassi, Giuliano, Lasse Uotila, Rafaella Klima, and Vittorio Venturi. "Purification and Properties of an Esterase from the Yeast Saccharomyces cervisiae and Identification of the Encoding Gene." Applied and Environmental Microbiology 65.8 (1999): 3470-472. Web. <http://aem.asm.org/cgi/reprint/65/8/3470.pdf>.

In this study, an esterase from the yeast Saccharomyces cerevisiae was isolated and found capable of cleaving S-formylglutathione as well as detoxifying formaldehyde. Esterases are enzymes that catalyze the hydrolysis of aliphatic and aromatic esters. The compounds that we will be synthesizing are these types of esters and therefore it is helpful to know what effect the esterase will have on them when they are tested on the yeast cells.

Gillepsie, Nigel. "*Synthesis of valproate prodrugs and their effect on the inositol levels of yeast cells*." Andrews University Honor's Program (**2010**).

Since this was a continuation and improvement on Nigel's research, I used his thesis as a foundation for the experimental methodology and to compare the results of my work with his.

Harwood, A. J. "Lithium and bipolar mood disorder: the inositol depletion hypothesis revisited." Molecular Psychiatry 10 (2005): 117-26. Nature. 23 Nov. 2004. Web. 7 Feb. 2010. <http://www.nature.com/mp/journal/v10/n1/pdf/4001618a.pdf>.

This article presents an overview of the inositol-depletion hypothesis as a proposed mechanism in the treatment of bipolar disorder. The author's goal is to examine the current hypothesis and knowledge of mood stabilizers and how both can be further investigated in the future. Alternatives to the inositol-depletion hypothesis are also mentioned such as neuronal growth cone behavior and lithium inhibition of GSK-3 kinases. This article is very helpful because it describes in detail the actions of current mood-stabilizing drug and also what can be improved on in the future.

Manji, H.K., G. J. Moore, and G. Chen. 2001. Bipolar disorder: leads from the molecular and cellular mechanisms of action of mood stabilisers. The British Journal of Psychiatry **178**: 107-119.

The focus of this article is to explore the molecular mechanisms of the effects of mood stabilizers such as lithium and valproate. It was shown that these drugs modulate the expression of several genes involved in inositol synthesis. Therefore, the regulation of signaling pathways plays a major role in the actions of lithium and valproate. This article is useful to my research because it not only explains the various mechanisms but it also describes the clinical implications of this study.

Shaltiel, Galit, Alon Shamir, Joseph Shapiro, Daobin Ding, Emma Dalton, Meir Bialer, Adrian J. Harwood, Robert H. Belmaker, Miriam L. Greenberg, and Galila Agam. "Valproate decreases inositol biosynthesis." Biological Psychiatry. 56.11 (2004): 868-74.

This article provides further evidence that lithium and valproate decrease cellular inositol by inhibiting either inositol monophosphatase or myo-inositol-1-phosphate (MIP) synthase. This information was obtained through various experiments on yeast cells and rat neurons. The details of the experiments provide a good background for carrying out my own experiments and understanding the underlying mechanisms.

Vaden, Dierdre L., Daobin Ding, Brian Peterson, and Miriam L. Greenberg. "Lithium and Valproate Decrease Inositol Mass and Increase Expression of the Yeast INO1 and INO2 Genes for Inositol Biosynthesis." The Journal of Biological Chemistry 276.18 (2001): 15466-5471.

This article looks at the genetic mechanism behind the decrease in intracellular inositol by lithium and valproate. The goal of the authors is to determine the targets of valproate and lithium in the inositol metabolic pathway and they use the yeast Saccharomyces cerevisiae as a model for this. The study demonstrates that lithium and valproate affect common targets but deplete inositol by different mechanisms.

Since this article focuses on experiments using yeast, my research can benefit from this study by examining its methods and comparing the results to my own.