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The Effects of a Valproate Fatty Acid Prodrug on the Inositol Biosynthetic Pathway

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Abstract

Bipolar disorder is characterized by alternating episodes of mania and extreme depression. Compounds such as omega-3 fatty acids and valproate individually treat symptoms of bipolar disorder, one of which is elevated inositol levels. Lithium and valproate are known to reduce inositol levels. The goal of this project is to combine linoleic acid (an omega-3 fatty acid substitute) with valproate and observe the prodrug's effects on the inositol biosynthetic pathway using *Saccharomyces cervisiae* yeast as the model system. Since inositol is required for growth, the effects of the prodrug on the inositol biosynthetic pathway was determined by comparing its impact on the growth of wild type yeast cells and yeast cells that overproduce or lack the production of inositol.

Background

Bipolar disorder was originally known as manic-depressive insanity. It is distinguished from other mood disorders based upon whether or not there are varying stages of mania or elevated mood and depression. It affects 1-2% of the population (Vaden, et al. 2001). There are many drugs that are currently used to treat bipolar disorder such as lithium, valproate, antidepressants and recently omega-3 fatty acids.

Valproate is a mood stabilizer and one of the ways in which this compound may function is through its interaction with the inositol biosynthetic pathway. Similar to lithium, valproate targets certain parts of the pathway and lowers the levels of inositol, though by a different mechanism (Vaden, et.al 2001). Inositol provides the basis of the formation of many second messengers in the body. Second messengers themselves have a significant role in relaying signals from outside the cell as they bind to receptors to inside the cell. Signals come from various sources and once the signal reaches the inside of the cell via a second messenger, it prompts a change in cell activity. Omega-3 fatty acids are still currently under research and the mechanism as to how they affect the pathway is yet unknown. These fatty acids are used as a supplement to bipolar disorder treatments.

Because valproate and omega-3 fatty acids are known to individually lower inositol levels, this project seeks to determine what would happen if they were combined. It is unknown whether or not the inositol levels would decrease, stay the same or increase. A plausible hypothesis could be that the prodrug would work to lower the levels of inositol in a given system. For the purposes of this project, specifically, an omega-3 fatty acid substitute (linoleic acid, an essential fatty acid used as it is much less expensive) would be combined with valproate

via mixed anhydride methodology. This method combines the two compounds with covalent enzymatically-cleavable acylal linkages. Essentially during the organic synthesis portion, valproate will be added to the linoleic starting material resulting in a valproate fatty acid prodrug in the form of an oil.

In this project, *Saccharomyces cerevisiae* (strain SM7) yeast was used as the model system with its inositol biosynthetic pathway being of primary concern. Yeast is easy to culture and the inositol biosynthetic pathway is similar in both yeast and humans. A wild type strain as well as two mutant strains was used. One of the mutant strains had a mutation that causes the yeast to overproduce inositol and thus was called the "overproducer mutant". The wild type and this overproducer mutant were both grown in media that lacks any inositol as each of the strains has to ability to synthesize inositol on its own. The other mutant strain had a mutation that causes an underproduction of inositol and thus was called the "underproducer mutant". Because it synthesizes so little inositol it was grown in a media with a small amount of inositol in order to see any growth at all. Yeast growth is dependent upon the production of inositol and thus based upon the number of colonies that grow after the drug is applied, one was able to determine the effects of the prodrug on the inositol biosynthetic pathway.

Methodology

PART ONE: ORGANIC SYNTHESIS

Trial 1:

A dry 50 mL round bottom flask with a spin vane was dried in the oven for 20 minutes and then was cooled in an ice bath for 5 minutes. The flask was capped with a $CaCl_2$ drying tube and placed over a magnetic stir plate. Cyclohexane, linoleic acid and thionyl chloride were added to the flask. The drying tube was replaced with a water-cooled condenser and the contents of the

flask were refluxed for 60 minutes. After reflux, it was cooled to room temperature and the drying tube was placed back onto the flask. Sodium valproate was added to the flask and stirred at room temperature for 30 minutes. After this time, propanal and zinc oxide were added to the flask and stirred at room temperature for 180 minutes.[1] The solution was then added to a separatory funnel. 25 mL of water was added and the solution was extracted 3 times with 20 mL of CH_2Cl_2 each time. 20 mL of NaCl was added to remove the emulsion of the solution. The organic portion of the solution was collected in a beaker and was dried with approximately 3 scoops of a spatula of Na_2SO_4 and gravity filtered by pouring the solution through filter lined with filter paper. The solution was then rotovapped to evaporate the liquid solvent from the desired oil product. Infrared (IR) spectroscopy and nuclear magnetic resonance (NMR) was performed to analyze the product. Table 1 provides the amount of materials used in the first trial of this experiment.

Table 1

Reagents	**Molecular Weight (g/mol)**	**Density (g/cm^3)**	**Moles**	**Grams**	**Volume (mL)**
Linoleic Acid	280.45	0.9	0.0046	-------	1.42
Thionyl Chloride	118.97	1.638	0.0079	-------	0.574
Sodium Valproate	166.20	--------	0.0046	0.765	--------
Propanal	60.10	0.81	0.0046	-------	0.341
Zinc Oxide	81.408	5.606	0.00046	0.08	
Cyclohexane	84.16	0.779	-------	-------	15

Trial 2:

The procedure for trial 2 is identical to the first trial with a few exceptions. One of these exceptions were that new, fresh propanal was used in hopes of removing some of the supposed

[1] This period of time is twice as long as intended. However, the analysis showed that this extended period of stirring did not affect the final product.

impurities that resulted in the spectroscopy of the first trial. Another exception was that the period of time when the solution was being stirred after the propanal and the zinc oxide were added changed from 180 minutes to 90 minutes, the latter period of time being the original amount of time that it should have been stirred. Table 1 provides again the amount of materials used in this trial.

PART TWO: BIOLOGICAL TESTING

Trial 1:

Two types of liquid media were prepared using the ingredients in Table 2. The difference between the two types of media was that one had inositol (I^+) and the other did not (I^-). 500 mL of each media was prepared using two 1-liter Erlenmeyer flasks. YPD agarose gel plates were also prepared using the ingredients in Table 3. The flasks with media were covered with aluminum foil and autoclaved for 25-30 minutes along with three empty 250 mL Erlenmeyer flasks. After autoclaving 100 mL of the I^+ media was added to one of the 250 mL flasks and the other two flasks were filled with 100 mL each of the I^- media. The flask with the I^+ media was inoculated with plated SMY7 underproducer mutant yeast cells. One of the flasks with the I^- media was inoculated with plated SMY7 wildtype yeast cells and the remaining flask was inoculated with plated SMY7 overproducer mutant yeast cells. These three flasks were then placed in a shaker bath (150 rpm; 30 °C) for 24-30 hours to allow growth of the cells. After this period of time, cell growth was estimated. To determine the effect of the prodrug on growth, cells from these cultures were then used to inoculate media containing the prodrug. Equating the O.D. reading of the spectrophotometer to cell concentration, cell cultures were inoculated to a staring O.D. of 0.1.

Trial 2:

The same procedure was used with the same concentration of the prodrug.

Table 2

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 3

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

Results

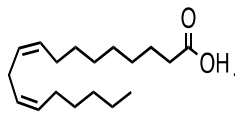
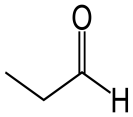
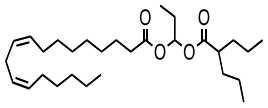
PART ONE: ORGANIC SYNTHESIS

IR and NMR Analysis

Table 4 lists the important spectral features of the product as well as comments as shown by the IR, the ^{1}H NMR and the ^{13}C NMR spectroscopy. Though there were two trials the results for the

spectroscopy came out to be essentially the same revealing that an identical product was synthesized for both products.

Table 4

Compounds	IR (cm^{-1})	^{1}H NMR	^{1}H NMR Comments	^{13}C NMR	^{13}C NMR Comments
Linoleic Acid 	C-H_2 (3009.63) C-H_3 (2927.23) O=C-OH (1710.69)	H_a (5.35) H_b (0.9) H_c (2.5)	H_a = H's bonded to diene carbons H_b = methyl group H's at opposite end of the acid H_c = alpha H's next to carbonyl	C_1 (180.0) C_2 (130.0)	C_1 = acid carbon C_2 = diene carbons
Propanal 	-------	H_d (9.75) H_e (2.4) H_f (1.1)	H_d = H attached to carbonyl H_e = alpha H's H_f = methyl group H's	-------	None was performed
Valproate Fatty Acid Product 	C-H_3 (2854.85) O-C=O (1760.78) O-C=O (1708.12)	H_g (6.38)	H_g = H bonded to carbon that is single bonded to two oxygens *Disappearance of the H_d peak (aldehyde peak)	C_3 (77.0) C_4 (26.0)	Cannot say conclusively

PART TWO: BIOLOGICAL TESTING

Table 5 displays results from Trial 1:

Table 5

Type of Media	**Number of Colonies**
Wild Type (without Prodrug)	401
Wild Type (with Prodrug 5mM)	0
Overproducer Mutant (without Prodrug)	4500
Overproducer Mutant (with Prodrug 5mM)	2

Underproducer Mutant (without Prodrug)	180
Underproducer Mutant (with Prodrug 5mM)	0

Table 6 displays results from Trial 2:

Table 6

Type of Media	Number of Colonies
Wild Type (without Prodrug)	780
Wild Type (with Prodrug 5mM)	0
Overproducer Mutant (without Prodrug)	1000000 +
Overproducer Mutant (with Prodrug 5mM)	8
Underproducer Mutant (without Prodrug)	769
Underproducer Mutant (with Prodrug 5mM)	0

Discussion

PART ONE: PRODRUG SYNTHESIS

Linoleic acid was used instead of an actual omega-3 fatty acid because it is a less expensive alternative that has a similar structure. The results of the synthesis in trial 1 proved to be somewhat successful. Though the solution was stirred at one point for twice as long as it was meant to (180 minutes instead of 90 minutes) this did not have an effect on the synthesis of the product and still yielded a decent amount of the product. The structure of the product from trial 1 showed a similar structure to the desired product but the analysis had evidence of some impurity. There were more peaks than should be seen at some points of the analysis. This was thought to be because one of the starting materials, propanal, was decomposing and not very fresh. Thus the main difference between the two trials was the use of fresher, newly ordered propanal for the second trial. The second trial was successful as well with a good percent yield. However, the results of the analysis when compared with the analysis of the first trial proved that the two products were essentially the same and that the use of newer propanal had little to no effect.

IR and NMR Analysis

The IR of the product differed from the IR of the starting material linoleic acid in that the COOH peak that is indicative of an acid was missing. This peak was found at 1710.69 cm^{-1} on the linoleic acid IR. The product also had two peaks at 1760.78 cm^{-1} and 1708.12 cm^{-1}. These peaks are characteristic of esters (O-C=O) and this change from an acid to an ester was exactly what was desired. The ^{1}H NMR of the product showed evidence of a hydrogen that is bonded to a carbon single bonded to two oxygens (6.38 ppm). This as well as the disappearance of the aldehyde peak (which was at 9.75 ppm and came from the starting material propanal) also helped to verify that obtained the desired product was obtained. However, not all of the hydrogens of the product could be seen on the ^{1}H NMR. The ^{13}C NMR of the linoleic acid showed the diene carbons and the carbon of the acid portion. Although one is able to identify peaks on the product ^{13}C NMR, nothing can be said conclusively as to what product was formed.

PART TWO: BIOLOGICAL SYNTHESIS

Trials 1 and 2 (as shown in tables 5 and 6) exhibited essentially the same results and do not support the hypothesis that the prodrug will inhibit the inositol biosynthetic pathway. One would expect the overproducer with the prodrug to grow much more than just a small number of colonies. It seems that the prodrug simply inhibits growth. The controls for the three types of yeast for these two trials grew as expected. The overproducer mutant plate had the greatest number of colonies as it produces the most inositol; the wildtype plate had an intermediate number of colonies and the underproducer mutant plate at the smallest number of colonies on the plate as it produces very little inositol. The variables with, in this case, the 5mM concentration of the drug also showed satisfactory results. The prodrug inhibited growth of the wildtype and the underproducer mutant altogether while the overproducer mutant, whose mutation causes the yeast to grow profusely, a small number of colonies: 2 and 8, respectively for trials 1 and 2.

Conclusion

For trials 1 and 2, a 5mM concentration of the prodrug completely stopped the growth of the underproducer and the wildtype but not of the overproducer. This could suggest that a 5mM concentration is effective in stopping the growth of yeast but this concentration was not too strong to completely inhibit all yeast growth and it is not terribly weak as to allow the growth of some wild type colonies. However, only one concentration was tested and one cannot say conclusively what the effects of the prodrug on the inositol biosynthetic pathway are. Future work must be completed including the testing of a second concentration (2.5mM) simultaneously with the 5mM concentration.

Annotated Bibliography

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

This article is shows evidence that lipids are connected with the mechanism of bipolar disorder. It reviews the interaction of certain mood stabilizers, such as lithium and valproate, with lipids as a possible mechanism that can be worked with to treat the disorder. This is useful to my research because it helps me understand one of the mechanisms that valproate could effect as well as role of fatty acids in bipolar disorder.

Chiu, Chih-Chiang, Shih-Yi Huang, Kuan-Pin Su, Mong-Liang Lu, Ming-Chyi Huang, Chiao-Chicy Chen, and Winston W. Shen. "Polysaturated Fatty Acid Deficit in Patients with Bipolar Mania." *European Neuropsychopharmacology* 13 (2003): 99-103. Print.

This study explored the polyunsaturated fatty acid levels in individuals with bipolar disorder as well as in those who did not. This study concluded and confirmed that individuals who have bipolar disorder and were taking medication have reduced levels of fatty acids such as arachidonic acid and docosahexaenoic acid. This would make sense because, as the authors state, studies were done previously that showed that individuals with bipolar disorder had elevated levels of these fatty acids and mood stabilizers such as lithium and valproate worked to reduced these levels. This is useful to my research because I am working with valproate and linoleic acid and this helps me to gain a clearer picture of the relationship between the two.

Rapoport, Stanley I., Mireille Basselin, Hyung-Wook Kim, and Jagadeesh S. Rao. "Bipolar Disorder and Mechanisms of Action of Mood Stabilizers." *Brain Res Rev* 61.2 (2009): 185-209. *National Institute of Health*. PubMed, 23 June 2009. Web. 8 Nov. 2011.

<http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2757443/>

This article was essentially a review of many other papers that all related to bipolar disorder. It was a good summary of the details of the actual disorder such as possible causes, the extent of its effect on the population and the mechanism in the body that could be targeted to help to cure an individual of the disorder. This mechanism is the arachidonic acid cascade. It also discussed the different treatments that have been explored. It is useful to my research because it gave a good overall picture of bipolar disorder and information on the drugs (antidepressants and mood stabilizers) that are used to treat this disorder. The information on these drugs includes the mechanisms with which they are believed to work and their possible side effects in the body. This gives me a better understanding of where I am coming into with my research.

Stoll, MD, Andrew L., W. Emanuel Severus, MD, PhD, Marlene P. Freeman, MD, Stephanie Rueter, Holly A. Zboyan, Eli Diamond, Kimberly K. Cress, MD, and Lauren B. Marangell, MD. "Omega 3 Fatty Acids in Bipolar Disorder." *Arch Gen Psychiatry* 56 (1999): 407-12. Print.

This article's results show that the ingestion of fatty acids could aid in the stopping of the overactive signal transduction pathways associated with certain compounds (phosphatidylinositol, arachidonic acid). The overproduction of these compounds could be the cause of bipolar disorder as studies have shown that individuals with the disorder have increased levels of these compounds. This is useful to my research because I will be combining linoleic acid with valproate, the latter of which is a commonly used mood stabilizer.

Thompson, Lisa. "*The Effects of Valproate Prodrugs on the Inositol Biosynthetic Pathway in* Saccharomyces cerevisiae *Yeast."* Andrews University Honor's Program (**2011**).

My research was a continuation of Lisa Thompson's work with the same yeast and drug. The difference is that the valproate prodrug that I wish to synthesize has a fatty acid chain on the end. I used Lisa's thesis to help me understand exactly at which point of the research I was coming into, to understand the details of the methodology and how to analyze the results, and also as a foundation to how I would write my own Honors Thesis.

Vaden, Deirdre 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. Print.

This paper proves the point that lithium and valproate, the latter of which is of interest to my research, do indeed reduce inositol levels in yeast. This is important to note because it is this particular biosynthesis pathway that I seek to test using the valproate prodrug that I will synthesize. My results should show that when the prodrug is tested on the yeast, the inositol levels should be reduced.