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The Effects of Chloro Linoleate Acylal Prodrugs on the Inositol Biosynthetic Pathway in Saccharomyces Cerevisiae

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Honors Thesis

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Abstract

Bipolar disorder is a debilitating mental illness characterized by recurrent episodes of mania and depression. FDA-approved drugs lithium and valproate, are the most common treatments for this disorder. Currently, omega-3 fatty acids are being researched as an alternative treatment, free of the negative side effects valproate and lithium exhibit. The mechanisms by which valproate and lithium work are unclear, however they effectively lower intracellular inositol levels. Valproate and omega-3 fatty acids similarly inhibit yeast cell growth as well as protein kinase C activity. We hypothesize that omega-3 fatty acids can also decrease inositol levels. A prodrug combining structural properties of valproate and omega-3 fatty acids was synthesized and tested on the growth of *Saccharomyces cerevisiae* yeast cells. Results suggest the mechanisms by which valproate and omega-3 fatty acids lower inositol levels are similar.

Introduction

Bipolar disorder is a chronic mental illness affecting approximately 1.3-1.5% of the American population (Azab et al., 2007; Azab and Miriam L. Greenberg, 2006). The disorder is characterized by mood swings ranging from lows of depression to highs of mania. Lithium and valproate are two of the most commonly used drugs for treating the symptoms of bipolar disorder. Although the mechanisms by which they remedy bipolar disorder are not clearly known, studies have shown both valproate and lithium target the inositol biosynthetic pathway, inhibiting steps of inositol synthesis, and effectively lowering inositol levels (Vaden et al., 2001). Inositol a is a chemical compound that forms the basis of many second messenger systems in cells of the human body. As a second messenger molecule, inositol relays signals from the surface of the cell to the targets within that cause changes in cell activity. Inositol is also an essential component of cell membranes.

Lithium and valproate are not always effective in treating bipolar disorder, thus there is a need for more effective treatments. Currently omega-3 fatty acids are being researched as an alternative treatment for bipolar disorder. In combination with other treatments, omega-3 fatty acids effectively reduce symptoms of this illness without the adverse side effects exhibited by lithium and valproate (Ross et al., 2007). Interestingly valproate (Murray and Greenberg, 2000) and omega-3 fatty acids similarly inhibit growth in yeast cells (Murray, unpublished) and also the activity of protein kinase C (PKC) (Mirnikjoo et al., 2001), an enzyme involved in the inositol biosynthetic pathway. Therefore we hypothesize both compounds suppress inositol levels in yeast by similar mechanisms of action. My first goal in this research project is to synthesize a chloro linoleate acylal (α-chloropropyl linoleate) in a one-pot reaction involving linoleic acid, a model system for omega fatty acids. Finally, I would like to determine the effects of the prodrug on the inositol biosynthetic pathway by

measuring the growth of wild type (SMY7) and mutant (SH1 and SH7) *Saccaromyces cerevisiae* yeast in the presence and absence of the synthesized compound. *Saccaromyces cerevisiae* yeast serves as a model system for the inositol biosynthetic pathway in the human body and its cell growth is dependent on inositol levels. SMY7 yeast produce normal levels of inositol whereas SH1 yeast under produce inositol and SH7 yeast over produce inositol. The prodrug has structural properties of both valproate and omega-3 fatty acids. Understanding how it affects the inositol biosynthetic pathway will shed light on the mechanisms by which both compounds work and contribute to evidence for the possible use of omega-3 fatty acids in treatment of bipolar disorder.

Methodology

PRODRUG SYNTHESIS:

Trial 1

A 50 ml round bottom flask and spin vane were dried in the oven overnight. The subsequent day the flask was placed in an ice bath over a magnetic stir plate, and capped with a calcium chloride drying tube. Cyclohexane, linoleic acid and thionyl chloride were added to the flask and the calcium chloride drying tube replaced with a water-cooled condenser. The mixture was refluxed 30 minutes to create linoleyl chloride. Reflux involves boiling the solution with minimal evaporation and continual cooling of the vapor which is returned to the flask as a liquid. The linoleyl chloride was allowed to cool to room temperature after which the condenser was replaced with the drying tube. Propanal and zinc oxide (catalyst) were added to the flask and stirred at room temperature for 90 minutes. The solution was extracted three times using fresh dichloromethane and deionized water each

time. The solution was dried with anhydrous sodium sulfate for three days, rotovapped for 20 minutes, and placed on a vacuum line for 24 hours to ensure the removal of solvent from the desired product. The density and actual yield of the product was determined. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) and infrared (IR) spectroscopy were performed to analyze the product in relation to the starting materials, propanal and linoleic acid. Table 1 summarizes the amount of each compound used this trial.

Table 1

Compound	Moles	Grams	Volume (mL)	Density (g/mL)	Molecular weight (g/mol)
Cyclohexane	_	_	10	0.779	84.16
linoleic acid	0.0064	_	2	0.9	280.45
thionyl chloride	0.0084	_	0.62	1.64	118.97
Propanal	0.0064	_	0.46	0.81	58.08
zinc oxide	0.00128	0.104	_	5.61	81.41
dichloromethane	_	_	60	_	_

Trial 2

A 100ml round bottom flask was dried in the oven overnight. The flask was placed on an ice bath over a magnetic plate and capped with a calcium chloride drying tube. Cyclohexane, linoleic acid, thionyl chloride and a spin vane were added to the flask. The drying tube was replaced with a water condenser and the mixture refluxed for 45 minutes at

80°C. The linoleyl chloride was allowed to cool to room temperature. The water condenser was again replaced by the drying tube. Propanal and zinc oxide (catalyst) were added to the flask and stirred at room temperature for 120 minutes. The mixture was extracted three times using fresh dichloromethane and deionized water, then dried with anhydrous sodium sulfate to remove excess water. After a few minutes of drying, the solution was rotovapped. In the midst of the rotary evaporation the flask containing the solution broke in the apparatus. The solution was collected as best as possible, reextracted, dried with anhydrous sodium sulfate and rotovapped for 10-20 minutes. ^{1}H and ^{13}C NMR of the product were performed. The product was placed on a vacuum line for futher purification and ^{1}H and ^{13}C NMR were performed again for analysis of the product in relation to the starting materials (propanal and linoleic acid). Table 2 summarizes the amount of each compound used in this trial.

Table 2

Compound	**Moles**	**Grams**	**Volume (mL)**	**Density (g/mL)**	**Molecular weight (g/mol)**
Cyclohexane	_	_	20	0.779	84.16
linoleic acid	0.0128	_	4	0.9	280.45
thionyl chloride	0.0168	_	1.24	1.64	118.97
Propanal	0.0128	_	0.92	0.81	58.08
zinc oxide	0.0026	0.208		5.61	81.41
dichloromethane	_	_	40	_	_

BIOLOGICAL TESTING:

Trial 1

Liquid media for the yeast was prepared using the ingredients listed 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- . Media in the I+ labeled flask included myo-inositol whereas the flask labeled I- did not. These flasks were covered with aluminum foil and autoclaved for 25 minutes along with three empty 250 mL Erlenmeyer flasks, two labeled I- and one labeled I+. Then, 100 mL each of the I+ and I- media was poured into its corresponding 250 mL flask. The I+ flasks was then inoculated with plated SH1 yeast and labeled as such. One of the I- flasks was inoculated with plated SMY7 yeast and the other with plated SH7 yeast and labeled respectively. The inoculations were allowed to grow for 24-30 hours in a shaker bath (150 rpm; 30 °C). After this, 1000 μl of stock I+ media was pipetted into a cuvette as a control, 900 μl of stock I+ media was placed in a second cuvette as well as 100 μl from the I+. 1000 μl of stock I- media was pipetted into a cuvette as a control, and for each I- flask 900 μl of stock I+ media was placed in a cuvette as well as 100 μl from the overnight flask. Concentration readings were then taken with the spectrophotometer to confirm that there was enough growth to continue on with next step (at least 0.1).

After autoclaving six new empty 250 mL Erlenmeyer flasks, each flask was labeled wildtype, underproducer, overproducer, wildtype + PD, underproducer + PD, and overproducer + PD (PD corresponds to prodrug). 100 mL of I+ media was added to the underproducer and underproducer + PD flasks 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 the overnight solution and a prodrug concentration of 5.0 mM. These six 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^{-3} dilution was then performed on the resulting solutions For each solution 10 µl of the solution and 990 µl of water were placed in a 1 mL microcentrofuge tube. Each was spread on a previously made YPD plate and labeled (see Table 3). The plates were incubated for 48 hours and then the resulting yeast colonies were counted.

Trial 2

The same steps for preparing the yeast and allotting the prodrug in Trial 1 were repeated. A 10^{-3} dilution was performed on the resulting solutions and plated on YPD plates. The plates were labels and incubated for 48 hours and the resulting yeast colonies counted.

Table 3

Ingredient	**Amount/L**
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
Agar**	10g

*Only used for I+ media for SH1/underproducer yeast
**Only used to make YPD plates

Results

NMR and IR ANALYIS:

Trial 1 yielded a product with a density of .966g/ml and actual yield of 1.9ml. Table 4 summarizes the important spectral features in the IR, ^{1}H NMR, and ^{13}C NMR spectroscopy for propanal, linoleic acid and the product, α-chloropropyl linoleate. The important spectral features for Trial 1 and Trial 2 were the same.

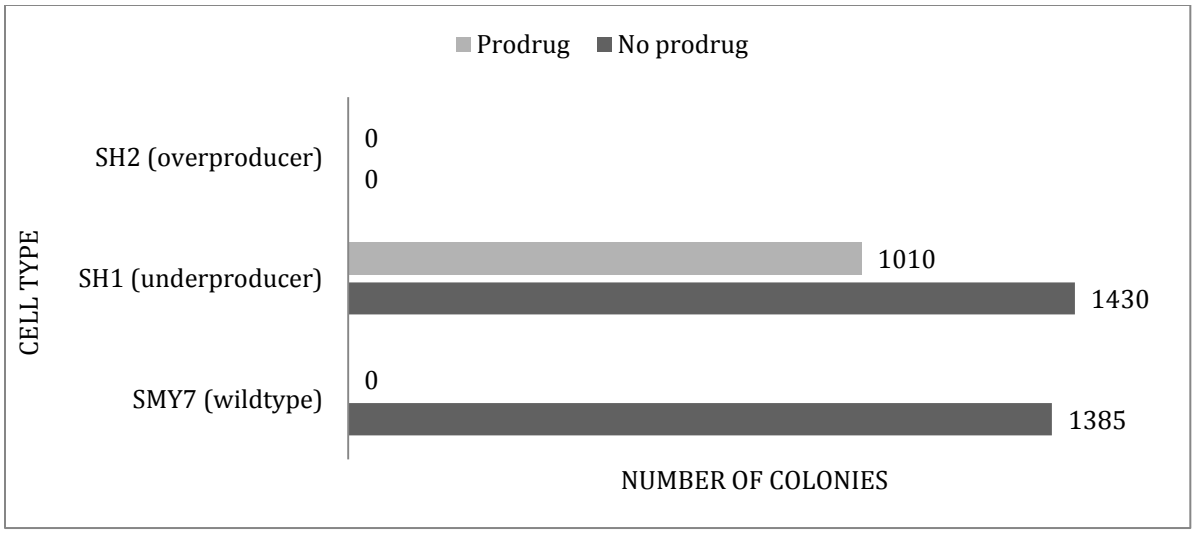
Table 4

	Important Spectral Features		
Compound	**IR (cm^{-1})**	**^{1}H NMR (ppm)**	**^{13}C NMR (ppm)**
Propanal	C=O (1734)	aldehyde H @ 9.8	aldehyde carbon @ 204
Linoleic acid	C=O (1707) O-H (2927, broad)	diene H's @ 5.4	acid carbon @ 180
α-chloropropyl linoleate (product)	C=O (1761) C-O (1136)	acylal H, triplet @ 6.4 present aldehyde H @ 9.8 is gone	Present: acylal carbon @ 85 ester carbon @ 172 Absent: aldehyde carbon @ 204 acid carbon @ 180

BIOLOGICAL TESTING:

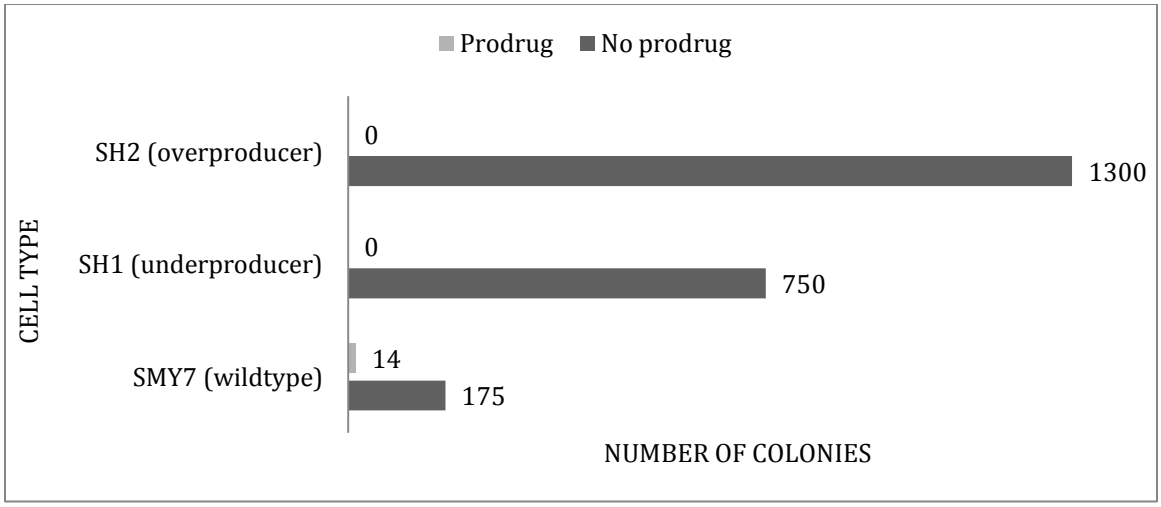
Trial 1

Graph 1: The results of colony growth in Trial 1.



Trial 2

Graph 2: The results of colony growth in Trial 2.



Discussion

PRODRUG SYNTHESIS:

The IR of the prodrug showed two main diagnostic peaks that indicate the success of the reaction. There was a peak at 1761 cm^{-1} and at 1136 cm^{-1}, which are characteristic of C=O and C-O groups respectively. The ^{1}H NMR propanal 9.8 ppm that was absent for the prodrug. Also the characteristic acylal proton showed up as a triplet peak at 6.4ppm for the prodrug. The ^{13}C NMR confirmed the success of the reaction. Most of the carbon atoms in the propanal and linoleic acid stayed in relatively the same positions throughout the reaction. The peak for the aldehyde carbon in propanal (204ppm) as well as the peak for the acid carbon in linoleic acid (180 ppm) were absent in the ^{13}C NMR of the prodrug. Instead a peak for the acylal carbon at 85 ppm and for the ester carbon at 172 ppm were present for the prodrug, indicating we had obtained the desired product.

BIOLOGICAL TESTING:

We hypothesized that the prodrug would effectively lower the inositol levels of the yeast causing a decrease in cell growth. In Trial 1 wildtype (SMY7) yeast treated with the prodrug had no growth. The yeast underproducing inositol (SM1) had a less drastic decrease in growth. Yeast overproducing inositol did not grow with or without the drug, so there may have been a mistake made in plating the cells. In Trial 2 the wildtype yeast treated with prodrug had low cell counts (14) in comparison to the control (175). Both the underproduing and overproducing yeast treated with prodrug had no growth.

Conclusion

Based on the NMR and IR analysis, the prodrug synthesis was successful for both trials, inspite of the accident that could have resulted in contamination for Trial 2. Overall the prodrug is effective at inhibiting growth however the inhibition of growth was the same in all three strains. This suggests the effect of the prodrug is not related to the inositol biosynthetic pathway. However a comparison of cell counts for each yeast cell type in Trial 1 and Trial 2 does not demonstrate similar numbers. Further testing needs to be done for results from the biological testing to be concrete. Future experiments should include more trials and various prodrug concentrations in order to obtain a broader range of data.

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