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Evaluating mitochondrial uncoupling potentials of A7E and DNP in *Saccharomyces cerevisiae* : implications for human obesity

Roger Vaughan

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Roger Alan Vaughan
Candidate

Nutrition
Department

This thesis is approved, and it is acceptable in quality
and form for publication:

Approved by the Thesis Committee:

Carole A. Conn, Ph.D., R.D., C.S.S.D.

Chairperson

Donna Lockner, Ph.D., R.D.

Armand E. K. Dichosa, Ph.D., M.S.

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**Evaluating Mitochondrial Uncoupling Potentials of A7E and DNP in
Saccharomyces cerevisiae: Implications for Human Obesity.**

BY

Roger Alan Vaughan

PREVIOUS DEGREES

Bachelor of Science: Nutrition and Dietetics

THESIS

Submitted in Partial Fulfillment of the
Requirements for the Degree of

Master of Science

Nutrition

The University of New Mexico
Albuquerque, New Mexico

May, 2011

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DEDICATION

I dedicate this work to my parents. Many people were vital to the success of this work; however, my parents are my source of inspiration. Mom, the support you have given me throughout my life has been immense. Your tireless effort to please the people in your life is amazing. You are truly remarkable. I also appreciate all of our conversations and the humor and happiness you have created in my life. You are everything that I love about the world. Thank you!

Dad, you are the best man I know. I appreciate all of the listening and advice you have given me over the years. I thank you for the work ethic and values you have instilled in me. The world would be a better place if more men were like you. Thank you. You truly are my best friend.

Collectively, you two have been the perfect team for my personal and professional development. You are the two most wonderful people I know. Thank you both very much for everything. I love you more than words describe. No child ever had better parents. I love you.

Love Bud

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**Comparing Evaluating Mitochondrial Uncoupling Potentials of A7E and
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ABSTRACT OF THESIS

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by

Roger Alan Vaughan

B.S., Nutrition and Dietetics, University of New Mexico, 2008

M.S., Nutrition, University of New Mexico, 2011

ABSTRACT

Obesity is one of the most prevalent maladies in the United States and is a major cause of preventable death. Weight loss supplements frequently claim uncoupling as a mechanism of action. Uncoupling agents could be used for weight loss because they disrupt mitochondrial metabolism thereby reducing adenosine triphosphate (ATP) yield. Consequently, metabolic efficiency diminishes increasing basal metabolic rate. 2,4-Dinitrophenol (DNP) successfully uncouples but was banned in 1938 because of a narrow window between efficacy and toxicity. **PURPOSE:** To measure the ability of a blend that reportedly contains 17-dihydroxy-delta-5-etiocholane-7-one and *p*-methylcarbonylphenol and other substances (A7E), a purported uncoupling agent, to interfere with oxidative phosphorylation in *Saccharomyces cerevisiae* as evidenced by lower ATP production. **METHODS:** Timed culture studies of *S. cerevisiae* were performed using two separate agents, A7E (a purported uncoupling agent) and DNP (a known uncoupling agent) at three doses (DNP: Low, Moderate, High; A7E: Low, Moderate, High), and an ethanol-treated control to detect interference with mitochondrial

coupling. Microbial staining was used to ascertain cell viability and any changes in cell densities. ATP production was estimated by measuring luminosity generated in culture supernatants using the ATP Bioluminescence Kit (Sigma St. Louis, MO.). **RESULTS:** Luminosity measurements estimating ATP production revealed statistically lower ATP in agent-treated supernatants than in control supernatants ($p < 0.001$) except for low dose A7E versus control ($p > .05$), suggesting that both A7E and DNP acted by a mechanism of uncoupling. Luminosity values were measured in relative luminosity units (RLU). Treatments with A7E at Low, Moderate, and High doses generated group mean luminosity values of 24,596, 16,038, and 6,969, respectively. Treatments with DNP at Low, Moderate, and High doses generated group mean luminosity values of 17,191, 11,901, 767 RLU respectively. The control group mean was 31,645 RLU. Culture studies had no statistical difference ($p > 0.0167$ *adjusted*) in total and viable cell densities between the control and A7E and DNP treatments. **CONCLUSION:** A7E is effective at reducing ATP levels in this assay, as is known uncoupling agent DNP, supporting the hypothesis that A7E may also uncouple oxidative phosphorylation. Because A7E requires a higher dosage than DNP to equivalently disrupt mitochondrial metabolism, it may have a wider range of therapeutic doses. This suggests that A7E should be studied further for safety and efficacy with respect to metabolic efficiency and weight loss.

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Chapter 1 Introduction

Obesity has rapidly become one of mankind's most prevalent preventable maladies. According to the U.S. Department of Health and Human Services, roughly 60% of adults in the United States are overweight, and approximately 30% of them are considered obese [1]. In 1994, when the CDC had recorded values for all 50 states, 35 of the states had obesity prevalence between 10 and 14% and only 15 states had prevalence greater than 14% but less than 20%. In 2004 the CDC estimated that over 34% (approximately 72 million Americans) of adults age 20 and over were obese [2]. In 2007, the CDC reported that only one state in America (Colorado) had prevalence of obesity lower than 20% and, that 6 states (Alabama, Mississippi, Oklahoma, South Carolina, Tennessee, and West Virginia) had a population with more than 30% obesity prevalence [2]. As a result of its prevalence, obesity is one of the leading causes of preventable death. The obesity epidemic in America increases healthcare costs by an estimated \$147 billion annually [3].

A combination of factors has contributed to the current co-morbidities that Americans face. Many healthcare professionals agree that a combination of food overabundance, sedentary lifestyle, and genetic predisposition has resulted in the obesity pandemic that exists today. Obesity is generally characterized by an excessively high fat mass, usually accompanied by a high ratio of body weight to height. Most health care professionals utilize a systematic classification system known as the body mass index (BMI). The BMI measurements are calculated by dividing the subject's weight (kg) by the subject's height (m^2). BMI classifications include:

- Obese - Values ≥ 30

- Overweight - Values between 25 and 29.9
- Ideal weight - Values between 18.5 and 24.9
- Underweight - Values < 18.5

Obesity has many contributing factors including increased food availability, increased food intake, sedentary lifestyles, and potential genetic predisposition. It is hypothesized that many Americans have what is known as a “thrifty gene”. In actuality, the thrifty gene is not one gene, but a combination of genetic and environmental factors that allows individuals in this set of circumstances to be more efficient in their use of energy, resulting in reduced energy need. During times of famine it was advantageous to be efficient at storing energy in adipose tissue; however, when there is more than enough to eat and people can survive with limited movement, the thrifty gene promotes fat storage to a detrimental degree [4].

It has also been established that people with low socioeconomic status have a greater probability of becoming obese in developed nations with plentiful food. Minority groups have greater prevalence of obesity than non minority groups and low socioeconomic status may play a more profound role in these groups [5].

Obesity is an individualized disease, and it is beneficial to have many diverse treatment approaches. There are a variety of current weight management approaches that are used. Individuals may use any number of treatments including medically or non-medically supervised calorie restriction (dieting), meal replacement pack supplements, group therapy sessions (e.g., Overeaters Anonymous), low-calorie prepackaged foods (e.g., Jenny Craig), bariatric surgery, liposuction, over-the-counter (OTC) supplements, and several other options. In addition, physicians may prescribe pharmaceutical

interventions (e.g. appetite suppressants or macronutrient absorption inhibitors). Current pharmaceutical agents include phentermine, sibutramine, orlistat, and diethylpropion [6]. These agents, although somewhat effective at appetite reduction or lipid mal-absorption, are not effective for everyone and have unwanted side effects. Moreover, many of these drug interventions have not been approved for use longer than 12 weeks. Uncoupling agents like dinitrophenol (DNP) [6-9] have shown positive results in animal models as an anti-obesity agent but can have extreme toxic effects [6, 10]. It is clear that no single treatment or combination of treatments is correct for every person. Therefore, it is essential to explore and pursue every option until obesity is constrained.

Purpose

This work specifically seeks to determine if the active ingredients in the blend (A7E) that comprises the over-the-counter supplement HOTROX™ specifically uncouple mitochondrial membranes interfering with oxidative phosphorylation. The blend A7E purportedly contains 17-dihydroxy-delta-5-etiocholane-7-one and *p*-methylcarbonylphenol (believed to be the active uncoupling agent/s) as well as many other ingredients.

Hypothesis

I hypothesized that the A7E uncoupling agent alters mitochondrial metabolism in *Saccharomyces cerevisiae* by interfering with oxidative phosphorylation by means of proton translocation and that A7E is less effective at uncoupling mitochondria than DNP (positive control). In order to address this hypothesis, I investigated the effects of both A7E and DNP on the process of oxidative ATP production in the eukaryote *S. cerevisiae*

by comparing A7E and DNP at varying doses on the growth and ATP production in *S. cerevisiae* with a control treated with ethanol alone.

Testable Hypotheses

Following treatment of *S. cerevisiae* at defined doses and incubation:

1. Null- There is no difference in cell density between untreated yeast cells and yeast cells treated with either A7E and DNP
2. Null- There is no difference in ATP production between controls and those treated with varied amounts of DNP
3. Null- There is no difference in ATP production between controls and those treated with varied amounts of A7E
4. Null- There is no difference in ATP production between those treated with DNP and those treated with A7E

See discussion for individual statistically analyzed testable hypotheses

Definitions

Adenosine Triphosphate (ATP) - a phosphorylated nucleotide $C_{10}H_{16}N_5O_{13}P_3$ composed of adenosine and three phosphate groups that supplies energy for many biochemical cellular processes by undergoing enzymatic hydrolysis especially to adenosine diphosphate (ADP)

β -oxidation- catabolism of fatty acids in which two-carbon chains are removed from the carboxyl end of the fatty acid. Conversion of fatty acids to acetyl Co-A

Basal Metabolic Rate- oxygen cost of energy expenditure measured under basal (resting) conditions

Calorie Deficit- Energy intake less than energy expenditure resulting in a state of catabolism

Cellular Respiration- any of various energy-yielding oxidative reactions in living matter that typically involve utilization of oxygen and production of carbon dioxide and water as end products

Cytochrome- any of several intracellular hemoprotein respiratory pigments that are enzymes functioning in electron transport as carriers of electrons

Glycolysis- the enzymatic breakdown of a monosaccharide resulting in the metabolic end product pyruvate. Conversion of glucose, fructose or galactose to pyruvate

Mitochondria- any of various round or long cellular organelles of most eukaryotes that are found outside the nucleus which produce energy for the cell through cellular respiration and oxidative phosphorylation

Mitochondrial intermembrane space- Space between the inner and outer mitochondrial membranes where proton concentration gradients accumulate

NADH- the reduced form of NAD, a vitamin co-factor that acts as an electron carrier in many biochemical reactions, including many of those required for energy yield

Oxidative Phosphorylation- the synthesis of ATP by phosphorylation of ADP for which energy is obtained by electron transport and which takes place in the mitochondria during aerobic respiration

Proton Translocation- Process of protons reentering the mitochondrial matrix from the mitochondrial intermembrane space independent of ATP synthase (Complex V)

Redox Reaction- a chemical reaction in which one or more electrons are transferred from one atom or molecule to another

Socioeconomic Status- the position of an individual on a social-economic scale that measures such factors as education, income, type of occupation, place of residence, and, in some populations, heritage and religion

Uncoupling Agents- substances that allow protons that have accumulated in the inter-mitochondrial space to translocate back into the mitochondrial matrix

Chapter 2 Background

Mitochondrial cell uncoupling agents have been used to assist in weight loss as fat burners [11, 12]. The addition of such compounds to a consistent diet and exercise program is suspected to notably reduce obesity. The potential uncoupling agent A7E is available commercially in a product called HOTROX_{TM} produced by Biotest. Biotest states that A7E up-regulates fat burning, mobilizes fat stores for energy requirements, and decreases fat storage during occasional overeating. It is also stated that HOTROX_{TM} may induce uncoupling [13]. A7E may be functionally similar to the known uncoupling agent DNP. There are no published studies that reveal a relationship between A7E and an interference with mitochondrial respiration, and uncoupling. My investigation will therefore be the first experiment to address these issues.

Function and Biochemistry

Tri Carboxylic Acid Cycle

The body has many metabolic pathways that transform macronutrients into energy via metabolic intermediates and vitamin cofactors, including glycolysis and β -oxidation. The end products of these reactions eventually enter the tri-carboxylic acid cycle (Kreb's Cycle). The cycle is initiated with a 6-carbon citrate molecule which undergoes several reactions releasing CO₂. This cleavage allows the molecule to undergo redox reactions involving electron transfer ultimately yielding reduced co-enzyme products, NADH⁺H⁺ and FADH₂ [14]. These electron carriers continue oxidative metabolism by entering the electron transport chain (ETC).

Electron Transport Chain

If oxygen is present, NADH^+H^+ and FADH_2 will enter the ETC. The electron transport chain is subdivided into five complexes. In Complex I, NADH^+H^+ donates electrons directly to electron donor flavin mononucleotide (FMN) which donates through an iron/sulfur base which reduces ubiquinol to ubiquinone. During this process, four protons are released into the intermembrane space and two are consumed in the redox reaction. This process takes place independent of Complex II. In Complex II, succinate dehydrogenase oxidizes succinate forming fumarate, a process that reduces FAD to FADH_2 which ultimately reduces ubiquinol to ubiquinone. Electrons are transferred to Complexes I and II (Figure 1) in the form of two protons.

Figure 1. Complexes I and II of ETC

In Complex III, Cytochrome C is reduced and ubiquinol becomes oxidized (Figure 2). Cytochromes are iron-based electron transferring proteins that are unable to accept protons. This is significant because there is a sequential transfer of four protons that are transported into the intermembrane space.

Figure 2. Complex III of ETC

Cytochrome C is then transferred to a large integral protein with many membrane spanning regions forming Complex IV or Cytochrome C Oxidase (Figure 3). Cytochrome C is oxidized in Complex IV and the electrons are propelled back into the matrix where they combine with 2 protons and oxygen forming metabolic water [15].

Figure 3. Complex IV (Cytochrome C Oxidase)

During Complexes I, III, and IV, protons are pumped against the concentration gradient through the inner mitochondrial membrane into the inter-membrane space. This yields an inequality between internal and external electrical charges and proton concentration. Protein channels facilitate diffusion of protons across the inter-membrane and back into the matrix. These channels are paired protein aggregates existing in two sectors, F_1 and F_0 . F_1 is the catalytic sector located in the matrix comprised of three alpha

and three beta subunits. The beta subunits are the active site for ADP phosphorylation. The force of proton ions flowing back into the matrix drives the attachment of adenosine diphosphate (ADP) and inorganic phosphate group (Pi) forming ATP, a reaction catalyzed by ATP synthase. This process is oxidative phosphorylation which is initiated by transport protein aggregates working sequentially to form Complex V (Figure 4) [15].

Figure 4. Complex V of ETC ATP Synthase (F-Type ATPase)

The initial concentration gradient is important because if ion disequilibrium is not great enough then there will not be sufficient energy to produce ATP. Thus, electron transport and oxidative phosphorylation are said to be coupled. Electron transport and oxidative phosphorylation are illustrated in Figure 5.

Figure 5. Electron Transport Pathway

Mitochondrial Uncoupling Agent Function

Uncoupling agents work against ATP production by reducing the proton concentration gradient using proton translocation by allowing protons to seep across the mitochondrion membrane independent of Complex V (ATP synthase), an essential step in ATP synthesis (Figure 4). This action inhibits ATP synthesis by channeling the proton concentration gradient through the inner mitochondrial membrane independent of ATP synthase [15]. The electronegative effect of the oxygen in the intracellular matrix drives the redox reactions that create the proton gradient responsible for ATP synthesis. Whenever protons are allowed to seep back into the matrix, oxygen accepts protons to form metabolic water that satisfies the electronegative driving force and inhibits oxidative phosphorylation (Figure 6).

Figure 6. Uncoupled Mitochondrial Electron Transport

Figure 7 illustrates the chemical similarities of known uncoupling agent 2,4-dinitrophenol and a proposed structure of one of the purported ingredients in the A7E blend, *p*-methylcarbonylphenol.

Figure 7. Chemical structure of DNP and A7E. A7E purportedly contains p-MCEP and Figure 7 is a proposed structure.

Once sufficient uncoupling agents are present in the mitochondrial, oxidative metabolism normally resulting in ATP production will become less efficient resulting in additional exogenous fuel demands to produce a given amount of ATP. This increased oxidative metabolism without ATP production dissipates significant energy as heat. In an attempt to compensate for the increased fuel utilization to produce a given amount of ATP, cells will have an ongoing requirement for increased fuel creating a whole body energy deficit if food intake does not increase compared to the heightened cellular fuel requirement for a given amount of ATP production. Humans lose weight as a result of calorie deficit; therefore, the use of uncoupling agents with a consistent diet and exercise program will theoretically yield greater weight loss than diet and exercise alone. Loss of fat has been demonstrated as a result of diminished ATP synthesis [11].

Brown Adipose Tissue

Brown adipose tissue (BAT) is a topic of recent interest due to the presence of uncoupling protein 1 (UCP1), an uncoupling protein imbedded in the membrane of mitochondria specific to BAT [16-18]. BAT plays a role in energy homeostasis by using UCP1 for thermogenesis [16-18]. Until recently it was presumed that BAT was only present and functional in certain mammals and human infants [16, 18]. Recently it was identified that human adults do possess functional BAT in measurable quantities [16]. BAT appears to be more prevalent in women than men and appears to be inversely related to BMI [16]. Similarly, a comparative study of BAT distribution in cold-exposed adult men found that obesity was associated with reduced BAT activity [18]. Ectopic portions of BAT have been isolated in white adipose tissue (WAT) in a process known as recruitment, which might increase thermogenesis and subsequently play a role in energy

balance [17]. It has been documented that chemical agents such as fucoxanthin from sea weed and a novel plant extract from *Cirsium oligophyllum* can increase expression of BAT in rat studies [19, 20]. It is postulated that if adult humans have active BAT present, then stimulating the uncoupling provided by UCP1 could play a vital role in the treatment and prevention of obesity.

DNP

DNP is a confirmed uncoupling agent [7-9, 21] that has been used for weight loss [11, 12]. Various research suggests that DNP and related uncoupling agents up-regulate energy consumption via heat dissipation by mitochondria in cells. It has been postulated that, through the increase of mitochondrial proton leak, a decrease in the efficiency of oxidative phosphorylation could result, combating obesity by enabling fatty acids to be oxidized with a decrease in ATP yield [6, 21]. If more energy is dissipated as heat as a result of less efficient ATP production [22] during cellular respiration, then a larger energy-deficit could be attained thus resulting in greater weight loss [6]. DNP was originally researched at Stanford University using animal models to assess the effect of the nitrophenols on basal metabolic rate (BMR). The studies concluded DNP could increase BMR by 40% [6] and the drug was widely introduced to the public in 1933 despite warnings that the drug should only be used experimentally [6]. This rise in BMR is proportional to the dosage of uncoupling agent administered. DNP was removed from sale in 1938 as a result of its severe side effects [10], which include severe fever / brain damage, dermatitis, agranulocytosis, hepatotoxicity, visual impairment, and death.

Because DNP has high lethality resulting from its strong uncoupling, it is not an appropriate agent for therapeutic weight loss, however an uncoupling agent that is less

efficient at performing proton translocation could be an effective addition to obesity management [21, 23]. Conceivably, a weaker uncoupling agent would create a less wasteful oxidative mitochondrial metabolism, while simultaneously widening the therapeutic dose of the drug, reducing toxicity. Uncoupling agents have also been shown to dynamically decrease fatty acid synthesis while increasing fatty acid oxidation [24].

A7E

To date, there is no published literature examining A7E's roles as an uncoupling agent. However, A7E might elicit a therapeutic mitochondrial interference with a dose response that could be used effectively by humans for weight loss. This work will demonstrate what effect, if any, A7E has on oxidative phosphorylation.

Test Organism

Saccharomyces cerevisiae is the model organism for my study because it has been used as the test organism in previous examinations of DNP [25, 26] and other uncoupling agents [25, 26]. Uncoupling agents have been shown to reduce production of ATP in *S. cerevisiae* [27]. The eukaryote *S. cerevisiae* is a model organism for estimating human ATP production because the mitochondria found in *S. cerevisiae* have identical cytochrome sequences to mitochondria present in human cells and share identical functions, which can be inhibited by several chemical agents [28]. *S. cerevisiae* has also been used as a model organism for evaluating other mitochondrial metabolism, including the decrease production of reactive oxygen species when treated with DNP or energy restriction [26]. Because of these organelle similarities, *S. cerevisiae* has been used since the late 1960's for making inferences about potential effects in other eukaryotic cells,

such as mammalian tissue [28]. Lastly, *S.cerevisiae* is ideal to use in cellular experiments because of its single cell simplicity making it easier for the researcher to control variables.

Chapter 3 Methods and Materials

Laboratory Materials

- 1.5 ml Eppendorf (Sigma Genesis Catalog # Z376787 tubes St. Louis, MO)
- 1- 500 ml beaker (n = 1)
- Glass luminosity tubes (Sigma Genesis Catalog # Z217638 St. Louis, MO)
- Luminometer model # LUMAT9501 (Berthold Detection Systems, Black Forest, Germany)
- Hemocytometer (Reichert New York City, NY)
- *Saccharomyces cerevisiae* (Wards catalog # 85w5010; Rochester, NY.)
- Trypan Blue (Sigma Genesis catalog # T8154; St Louis, MO)
- Adenosine 5'-triphosphate (ATP) Bioluminescent Assay Kit (Sigma Genesis catalog # FLAA; St Louis, MO)
- CellLytiċa Yeast Cell Lysis/Extraction Reagent (Sigma Genesis catalog # C 4482; St. Louis, MO)
- 2,4-Dinitrophenol (Sigma Genesis Catalog # 198501; St. Louis, MO)
- HOTROX_{TM} (Biotest Laboratories Lot #10007177, Exp. 1/2012; Colorado Springs, CO.)
- Centrifuge (capable of 7000 and 12000 x g)
- Nutrient Broth
- 99.9999 % Ethanol Anhydrous Sigma Genesis Catalog # 459836; St. Louis, MO)
- Vortex

Experimental Design

This experiment cross compared the effects of A7E and DNP at varying doses on the growth and ATP production in *S. cerevisiae* with a control treated with ethanol alone. The control samples received 75 μ l ethanol anhydrous while the experimental groups received the same volume of ethanol with specific quantities of one of either test drugs. After each sample received its respective treatment, all samples were incubated for 6 hr under identical conditions. Observations from both the A7E test group and the DNP positive control group were analyzed in comparison to control group. The dose relationship of both the A7E and the DNP was assessed to identify similarities.

Instrumentation

Cell counts were taken to ensure that any decreased ATP observed was not an artifact of any cytolytic effect of the uncoupling agents. Cell counts were counted using hemocytometry while simultaneously measuring cell death using trypan blue exclusion staining. Counts were performed using three quartiles of the hemocytometer per sample to ensure accuracy. Luminosity in relative luminosity units (RLU) was measured using 10 sec measurement intervals. Because the reagent in the ATP bio-luminescence kit was designed specifically to measure ATP, luminosity is a direct function of ATP present. Luminosity has been used in previous experiments to measure diminished ATP resulting from DNP treatment [22]. The following two reactions summarize the overall reaction of ATP in the presence of luciferin (ATP bio-luminescence kit, Sigma) to generate light:

1. $\text{ATP} + \text{Luciferin} \rightarrow \text{Adenyl-luciferin} + \text{PPi}$
2. $\text{Adenyl-luciferin} + \text{O}_2 \rightarrow \text{Oxyluciferin} + \text{AMP} + \text{CO}_2 + \text{light}$

Statistical Methods

ANOVA was used to test for group mean difference in luminosity. Tukey's correction was used to protect for potential familywise error resulting from multiple pairwise comparisons. χ^2 analyses were used to measure differences in cell counts and cell viability. Bonferroni's correction was used to control for familywise error from the multiple comparison procedures. Alpha was set at 0.05 for luminosity for ANOVA analysis, luminosity pairwise comparisons, cell count and viability χ^2 .

Experimental methods

Creating DNP Treatment Solution

DNP stock solution was created containing 0.1334 mg DNP blend / μ l of ethanol. From the DNP stock solutions 2 other solutions were made which contained 0.0333 mg DNP blend / μ l of ethanol and 0.0666 mg DNP blend / μ l of ethanol. These solutions contribute 2.5, 5, or 10 mg of DNP suspended in 75 μ l of ethanol anhydrous.

Creating A7E Treatment Solution

A7E stock solution contained 1.0667 mg A7E blend / μ l of ethanol. Further solutions created from stock solution created treatments providing 0.222 mg A7E blend / μ l of ethanol and 0.444 mg A7E blend / μ l of ethanol. These dilutions provide 16.6, 33.3, or 80 mg of A7E blend for each aliquot.

Group Assignment

Group titles were assigned as follows: Control- treated with ethanol, DNPHigh- 10-mg DNP treatment, DNPMid- 5-mg DNP treatment, DNPLow- 2.5 mg DNP

treatment, A7EHigh- 80 mg A7E treatment, A7EMod- 33.3 mg A7E treatment, A7ELow- 16.6 mg A7E treatment.

***Saccharomyces cerevisiae* Culture**

S. cerevisiae was inoculated into an autoclaved 500 ml beaker in sterile nutrient broth, capped and incubated at 37° C with stir bar. The yeast grew for 7 days (168 hr).

Drug Exposure and Incubation

Once drug treatment dilutions were created, the stock yield was distributed into the first set of numbered 1.5 ml Eppendorf tubes (identified as tubes numbered 1 to 36). Each Eppendorf tube received 1 ml of stock cell culture while shaking. After stock yield was distributed into the Eppendorf tubes the tubes were divided into seven groups:

- Control group receiving 75 µl ethanol anhydrous ($n = 6$)
- Three DNP groups receiving 75 µl assigned drug dilutions in five replicates per dilution ($n = 15$ total)
- Three A7E groups receiving 75 µl distinguished dilutions in 5 replicates per dilution ($n = 15$ total)

36 total subjects were tested. See Table 1.

Table 1. Group Compositions

Group	Number of Subjects
CNT	6
DNP 2.5	5
DNP 5	5
DNP10	5
A7E 20	5
A7E 40	5
A7E 80	5
$n =$	36

All tubes containing drug exposures were consistently vortexed for 6 sec at approximately 1000 RPM just prior to treatment administrations. After the cultures were treated they were incubated at 37° C while shaking for 6 hr.

Cell Lysis and ATP Recovery

The cells were lysed following 6 hr of uncoupling agent treated incubation following protocol modified from CelLytic[®] bulletin. The tubes were centrifuged at 7000× g for five min in a refrigerated room. Because the centrifuge only held 18 tubes, the subjects were centrifuged in two sets one immediately following the other. Cells were pelleted and the supernatant discarded. The cells then received 1 ml of CelLytic[®] yeast lysing agent. The tubes were placed on a vortex for 15 min at approximately 500 RPM to allow the tubes to shake. Immediately following vortexing, the tubes were centrifuged at 12000 × g for ten min to pellet the cellular debris. After centrifugation, 1 ml of the ATP containing supernatant was pipetted and placed in a new, sterile and cooled 1.5 ml Eppendorf tube that was numbered to its corresponding supernatant donor. The samples were kept on ice and transported to Cancer Research Facility (CRF) laboratory, located at North Campus of the University of New Mexico. This transport took approximately 15 min total.

Measuring Luminosity

Per manufacturer's protocol, 0.1 ml ATP assay solution was added to an empty reaction vial and allowed to stand at room temperature for 3 min. After one min assay acclimation had passed, a second 0.1 ml ATP assay solution was added to an empty

reaction vial and allowed to stand at room temperature for 3 min. This was repeated for each sample to reduce the amount of time necessary for each measurement. Upon completion of the three min assay acclimation, 0.1 ml of the first sample was mixed with the ATP assay reagent, placed in a vortex for approximately six sec and immediately placed into the luminometer for measurement of intensity. The measurements were recorded. This procedure was repeated for each sample. This process took approximately one hour preparing one reagent every minute for measurement (thus, more than one sample was reacting, while the previous sample was being measured) and immediately measuring and recording, then repeating. Supernatants were measured in the order that their respective cells were lysed.

Cell Density

Hemocytometry was used to calculate cell density. *S. cerevisiae* cells were incubated at 37° C for 168 hr while shaking. At baseline (initial introduction of *S. cerevisiae* to treatment) cell density of common yield was measured. After initial measurement, ethanol anhydrous treatment (control) was administered to six control subjects and incubated. The first DNP and A7E dilutions were created using the same protocol as previously described (see “Creating DNP Treatment” and “Creating A7E Treatment”). The first DNP and A7E treatment groups received their assigned treatment 30 min after initial control incubation. These steps were repeated for the second and third treatment of each drug with 30 min displacement between each of the four treatment windows. The 30 min treatment window allowed for accurate cell counts to be taken 6 hr after each culture was incubated. Because six hr presented enough time for significant cell division to occur, I created a no treatment (NT) group. This NT group was incubated

six hr prior to each group control. This allowed us to analyze any differences in cell counts due to culture maturation. After six hr from baseline, the control subjects were measured. Samples of 20 μ l from three of the control vials were combined with 60 μ l of trypan blue in a small plastic tube. The solution was gently mixed and left to stand at room temperature for 5 min. Samplings of 10 μ l of the preparations were counted for stained cells and total cells using a hemocytometer. The procedures were repeated three additional times for each wave of 6 hr incubated cells. All counts were recorded in an Excel spread sheet.

Data Collection and Analysis

Luminosity

Analysis of variance was used to determine if there was a significant difference between group luminosities. ANOVA F statistic was generated using equation 1.

Equation 1. F = Variation among sample means/variation among individuals

Uncoupling agents affect luminosity such that the more effective the uncoupler, the less luminosity present. All groups generated consistent measurements within respective groups. In order to control for the difference in variance between groups the natural log (ln) of each individual measurement was taken and used for the analysis of variance. Pair-wise comparisons were used to assess difference between groups. Tukey's correction factor was used to control for familywise error. Alpha was set to 0.05 for luminosity ANOVA and luminosity pairwise comparisons.

Cell Density

Difference in cell density between groups was analyzed using χ^2 tests. The χ^2 analysis measures how far the observed values of two-way table are from the predicted values. The χ^2 statistic was generated using equation 2.

$$\text{Equation 2. } \chi^2 = \sum (\text{observed count} - \text{expected count})^2 / \text{expected count}$$

χ^2 was performed three times for distinct comparisons between hypothesized relative strengths of DNP, A7E with a control. Alpha was set at 0.05.

Chapter 4 Results

Luminosity / ATP

Mean luminosity for groups DNPLow, DNPMoD, and DNPHigh were lower than the average luminosity of the control group (Figure 8). Mean luminosity for groups A7ELow, A7EMoD, and A7EHigh were all numerically lower than the control group average luminosity. Every treated individual sample generated less luminosity than every control treated individual sample. Figure 9 illustrates transformed luminosity values with log mean, low, first quartile, third quartile and high values for each group.

Figure 8. Group Mean Luminosity

Figure 9. Group Log Luminosities

ANOVA and Pairwise Comparison Results

The ANOVA test revealed that $F(6, 29) = 149.37$ and $p < 0.0001$. Table 2 summarizes individual adjusted p values for each group pairwise comparisons.

Table 2. Group Luminosity Pairwise Comparisons

Groups Compared	<i>p</i> -adjusted
DNPLow-control	0.0014
DNPMMod-control	0.0000
DNPHigh-control	0.0000
A7ELow-control	0.5620
A7EMod-control	0.0006
A7EHigh-control	0.0000
A7ELow-DNPLow	0.1410
A7EMod-DNPMMod	0.1340
A7EHigh-DNPHigh	0.0000

Cell Counts

Cell counts showed that all groups generated average total cell counts less than 50 across the sample sets. Group A7EHigh generated nearly three times the total cell density of any other observed value. All groups generated cell deaths less than 15 cells. Figure 10 illustrates the group means of cell density and cell death and Table 3 is the observed values of both cell density and cell death. See Discussion for description of outlier. Group mean cell counts both total and dead are summarized in Table 4 along with the results from the χ^2 analyses.

Figure 10. Group Mean Cell Counts and Deaths. No Treatment (NT) Tubes were used to compare changes in cell density and/or viability as a result of time progression.

Table 3. Individual Group Cell Counts

Group	Time	Quartile 1*		Quartile 2*		Quartile 3*		Total		Mean	
		Dead	Total	Dead	Total	Dead	Total	Dead	Total	Dead	Total
NT	805am	0	25	1	17	0	16	1	58	0.333	19.3333
NT	836am	5	26	4	39	5	21	14	86	4.666	28.666
NT	924am	5	21	1	13	1	10	7	44	2.333	14.666
NT	950am	2	12	1	10	2	14	5	36	1.666	12
NT	200pm	5	14	3	23	2	15	10	52	3.333	17.333
Control	219pm	2	17	3	12	3	13	8	42	2.333	14
DNPHigh	233pm	1	7	3	9	3	11	7	27	2.333	9
A7EHigh	248pm	5	54	4	39	4	51	13	144†	4.333	48
Control	257pm	3	18	1	19	2	15	3	52	1	17.333
DNPMod	316pm	6	28	3	23	1	23	10	74	3.333	24.666
A7EMod	335pm	1	14	3	14	1	17	5	45	1.666	15
Control	351pm	2	6	1	7	1	4	5	17	1.666	5.666
DNPLow	422pm	1	15	3	18	4	18	8	51	2.666	17
A7ELow	444pm	4	16	4	20	2	22	10	58	3.333	19.333

*Quartile number refers to the area of the hemocytometer.

†Outlier (described in Discussion)

Table 4. Group Mean Cell Counts with Respective χ^2 Analyses p Values

Analysis 1 (High)			Analysis 2 (Moderate)			Analysis 3 (Low)		
Group	Dead	Alive	Group	Dead	Alive	Group	Dead	Alive
CNT	8	34	CNT	3	49	CNT	5	12
DNPHigh	7	20	DNPMod	10	64	DNPLow	8	43
A7EHigh	13	131	A7EMod	5	40	A7ELow	10	48
$p = 0.04541$			$p = 0.374$			$p = 0.4309$		

Chapter 5 Discussion

Luminosity / ATP

There was a dose-dependent lowering of luminosity by both DNP and A7E treatments compared with control. The measurement for tube 5 was abnormally high in luminosity and was therefore repeated two more times. The mean was used as true value for tube 5. The ANOVA test revealed that $F(6, 29) = 149.37$ and $p = 2.2 \times 10^{-16}$ leading to the conclusion that all groups compared to one another were not the same, thus rejecting the null. For pairwise comparisons and respective p -values see Table 2. All groups were significantly different ($p < 0.05$) from the control group with the exception of group A7ELow. All other groups reject their relative null hypotheses with the finding that there is significant difference in luminosity between group DNPLow and control, group DNPMOD and control, group DNPHigh and control, group A7EMOD and control, and group A7EHigh and control. A summary of all results is illustrated in Figure 9. Table 2 summarizes p -values adjusted for familywise error for group comparisons to control. These results are supportive evidence that A7E does act as an uncoupling agent, as does DNP, as predicted in my hypothesis.

A7E Compared to DNP

In order to assess the presence of common dose dependent relationship, A7E was compared to DNP using pair-wise comparisons. The groups were paired by order of dilution; DNPLow and A7ELow, DNPMOD and A7EMOD, and lastly DNPHigh and A7EHigh. There was not a significant difference between the low drug treatment groups, DNPLow and A7ELow, which fails to reject null. Also the medium drug treatments failed to reject the null indicating no difference between the two. There was, however, a

significant difference in luminosity production between groups DNPHigh and A7EHigh, thus, resulting in the rejection of the null. The significance of these findings illustrates that there appear to be uncoupling agents that are less effective by weight than DNP, but still remain effective enough performing proton translocation to have a pronounced affect on metabolism. Moreover, A7E showed increased uncoupling potential with an increase in dose illustrating a dose dependence on effectiveness, suggesting A7E may provide an uncoupling agent with effectiveness and a wider range of therapeutic doses, likely a beneficial characteristic.

Cell Density

χ^2 analysis found no differences between NT groups and control groups showing there was no significant cell growth during the time that passed during cell counts. The first χ^2 analyses compared the strongest drug concentrations plus a control (DNPHigh, A7EHigh and Control). The second analysis included the second dilutions (DNPMod, A7E Mod and Control) and the last analysis compared the weakest concentrations (third dilutions which included DNPLow, A7ELow and Control). The first analysis comparing the high dose showed that the groups generated significantly different cell densities ($p = 0.04541$) which could be a result of cell “clumping”. Based on luminosity values for group A7Ehigh it is unlikely that the cell density was actually twice that of any other group including controls which suggests presence of an outlier. Moreover, when individual χ^2 were performed comparing each pairing of A7EHigh, DNPHigh, and the control with use of Bonferroni’s adjustment to control for familywise error, there was no significant difference between the groups in cell viability or cell density. The second comparison between moderate doses concluded the groups were not significantly

different from one another ($p = 0.374$) demonstrating cell densities were not statistically different. The last analysis compared the weakest drug dilutions (DNPLow and A7ELow) and showed no significant difference in cell densities between groups ($p = 0.4309$). The data fails to reject the null hypotheses, that there is a difference in cell density or viability between group pairings. See Table 4 for cell densities with independent group comparisons and p -values. This data supports the conclusion that treatment with DNP and A7E in the tested quantities did not kill cells or diminish growth (in comparison to control), permitting the assumption that any changes in ATP production (as evidenced by reduced luminosity measurements) were likely due to uncoupling.

Luminosity and Cell Density

The most significant finding was the similar relationship between A7E and DNP in the reduction of luminosity. This follows the prediction that, as uncoupling agent concentration increases in mitochondria, less ATP is produced, thus reducing luminosity. Further, neither agent generated significantly lower cell densities in comparison to the control which suggests that the DNP did uncouple mitochondrial metabolism as documented by previous studies. A7E appeared to function identically to DNP, only in a less efficient fashion. Only the low dose administration of A7E failed to generate significantly less luminosity than the control suggesting 16.6 mg of A7E was not sufficient uncoupling agent to significantly lower ATP production in comparison to the control group. It is, however, important to note that both the mean and all observed values of group A7ELow were numerically lower than both the mean and all values of the control. Also similar to DNP, the A7E did not generate significantly different cell density or viability from control. Because of the greater quantity of agent, these findings

suggest A7E performs mitochondrial uncoupling less efficiently than DNP. Moreover, pair-wise comparisons of drug dilutions against one another between both treatments revealed no statistical difference between DNPLow and A7ELow. The same is true of groups DNPMOD and A7EMOD. Groups A7EHigh and DNPHigh were statistically different most likely because that DNP may be more than eight times more effective than A7E based on $8 \times$ more A7E than DNP by weight. Mean luminosity suggests it is reasonable to hypothesize that DNP is approximately 16 times more effective at mitochondrial uncoupling. Furthermore, with the dilution variance set at eight fold, as DNP approached total uncoupling (no ATP produced), the magnitude of uncoupling significantly surpassed that of A7E. Had the doses been tested at similar weight of DNP but approximately twice as much A7E the luminosity trend slopes of the two agents might have been nearly parallel elucidating a dose related trend. The collective luminosity and cell density data illustrates that A7E and DNP function as uncoupling agents, and that A7E is less efficient than DNP as predicted in my hypothesis.

Conclusion

A7E performs the function of mitochondrial uncoupling and does so less effectively than DNP. It is possible that A7E is an uncoupling agent with a wider range of therapeutic dose, thus, potentially reducing its toxicity and heightening the variety of doses that might benefit people seeking to use uncoupling agents to increase metabolic rate for weight loss. A7E may present a potential option for individuals attempting a weight loss regimen. A7E could augment mitochondrial uncoupling resulting in an increased energy deficit assisting in weight loss.

Limitations to this Study

1. It is difficult to determine if the observations of lower ATP generated from the A7E group resulted solely from mitochondrial interference and not partially due to the reduced ability of the *S. cerevisiae* to absorb nutrients. Cell culture and viability counts were measured to assess replication and death, and it appears that uncoupling is likely the main interference diminishing ATP production.
2. It is also difficult to distinguish if another mechanism resulting in the reduction of ATP production could be occurring (such as that observed in hydrogen cyanide inhibition of Cytochrome C Oxidase).
3. It is difficult to determine which component of the proprietary blend found in HOTROX_{TM} is responsible for the decreased presence of ATP. One of the agents in A7E could be working to degrade or preserve ATP. A7E purportedly contains caffeine which acts as a phosphodiesterase inhibitor which could add to the stability of ATP, which could increase luminosity thereby reducing the observed uncoupling effect.
4. There is also a possibility that the assay could misidentify the nucleotide (e.g. mistake ATP for GTP).
5. This work has not deciphered if A7E is actually less effective than DNP at uncoupling, or if there is some other variable that is limiting the rate at which the A7E can uncouple or is modulating the accumulation of ATP.
6. This work was performed in the eukaryote *S. cerevisiae*. While this microorganism has mitochondria similar to human mitochondria, many variables could change how A7E works in human; for example, absorption in the gut. The

human gastrointestinal tract is much more complex than the cell wall of a eukaryote and could result in further decreased drug efficacy. A7E is readily available over-the-counter and should be tested further in humans.

7. As noted from the observed results it appears that A7E is approximately 16 times weaker at proton translocation than DNP so further work should be done to describe the ratio of efficacy between the two.
8. This work should be repeated in animal models to identify if the effects on ATP observed at the cellular level are transferable to mammals. Mammal experiments could also be used to elucidate other possible effects on metabolism, as well as side effects that are not predicted.

Future Research

Uncoupling proteins and uncoupling agents have a profound history influencing metabolism. The next area that needs to be researched is the effects that uncoupling agents have on the genome. Varied durations of exposure and varied amounts of uncoupling agents may manipulate an organism's genome to have a higher metabolic rate, possess more oxidative mitochondria per cell, and a number of other combinations that may influence humanity well beyond the scope of human obesity. The next series of tests that should be run is the influence that uncoupling agents have on mitochondrial biosynthesis. These tests should be explored first in cell cultures, then in mammals to assess change in weight and mitochondrial density.

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Appendix

Individual tube luminosity measurements

	Tube #	Luminosity Measurement #	Luminosity (RLU)
Control	1	1	30601
	2	2	30806
	3	3	31439
	4	4	29867
	5	5	34722
	6	6	32439
DNPLow	7	7	20425
	8	8	19906
	9	9	18656
	10	10	9512
	11	11	17458
DNPMMod	12	12	24375
	13	13	9861
	14	14	9344
	15	15	7971
	16	16	7955
DNPHigh	17	17	874
	18	18	730
	19	19	944
	20	20	657
	21	21	633
A7ELow	22	22	24520
	23	23	23735
	24	24	24295
	25	25	23411
	26	26	27022
A7EMod	27	27	14433
	28	28	15102
	29	29	19342
	30	30	16215
	31	31	15102
A7EHigh	32	32	7520
	33	33	7699
	34	34	6800
	35	35	7359
	36	36	5470