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How Does 2,4-Dinitrophenol Compare to Triclosan as a Mitochondrial Uncoupler?

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HOW DOES 2,4-DINITROPHENOL COMPARE TO TRICLOSAN AS A MITOCHONDRIAL UNCOUPLER?

by

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Abstract

2,4-Dinitrophenol (DNP) is a well-documented mitochondrial uncoupler that was widely used as a dieting drug in the 1930's. It was later banned in 1938 due its negative side effects which included extreme weight loss, the formation of cataracts, skin rashes, and death. Triclosan (TCS) is a common antimicrobial agent that is a component in soaps, toothpastes, and other household products. In addition to its antimicrobial role, TCS has been found to alleviate skin inflammation and dermatitis. However, TCS has also been linked to several health issues including increased cases of asthma and allergy, developmental problems, and decreased fertility. Previous studies in the Gosse laboratory found that TCS inhibits the primary functions of mast cells, key players in the immune system. More recent studies have found that in addition to increasing oxygen consumption, TCS significantly inhibits ATP production in rat basophilic leukemia cells, clone 2H3 (RBL-2H3) at non-cytotoxic doses with an EC_{50} of 7.5 μM to 9.6 μM. These results strongly indicate that, like DNP, TCS is a mitochondrial uncoupler.

Since DNP is a known, dangerous mitochondrial uncoupler, it serves as a useful comparison to assess the potential danger of TCS. In this study, cultured RBL-2H3 cells were exposed to increasing concentrations of DNP and were assessed for ATP production and cytotoxicity. DNP was found to significantly inhibit ATP production at non-cytotoxic doses with an EC_{50} of 389 μM to 677 μM. These results indicate that TCS is ~60-fold more potent as a mitochondrial uncoupler in RBL cells than in DNP. More related, comparative research with DNP is needed to fully explore triclosan's mitochondrial toxicity.

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1. Introduction

1.1 2,4-Dinitrophenol

2,4-Dinitrophenol (DNP) is a known mitochondrial uncoupler that was widely used as a weight loss drug in the 1930's[1]. As a dieting drug, it was found to increase the rate of metabolism in patients by 50% or more[1]. In the 1930's, it was discovered that DNP caused several negative side effects and there were several deaths associated with its use[1]. DNP was banned as a dieting drug in the U.S. in 1938[2]. DNP is currently sold over the internet as a workout powder or tablet, commonly marketed to body builders. However, there have been many cases of patients dying of hyperthermia in the emergency room as a result of using those DNP products[3-6]. In addition to its use as a weight loss drug, DNP is also used as a component of dyes, wood preservatives, explosives, herbicides and fungicides[1,2,4].

DNP has been found to cause hyperthermia[3-6]. Also DNP has also been found to cause a variety of other health problems. Before it was banned, it was discovered that DNP could cause skin rashes[1] and the formation of cataracts[7]. Increasing concentrations of DNP were found to cause potassium accumulation in rat kidneys, as well as inorganic phosphate accumulation throughout their bodies[8]. In humans, DNP was found to cause kidney injury[10]. Prolonged exposure in humans can cause a yellow discoloration of the skin and urine[8]. Other reported problems associated with continued use include deafness, confusion, and coma[8]. DNP has been found to cause reproductive and development issues in rats[9]. It has also been found that exposure to DNP via skin contact and inhalation is toxic[11].

Recently, there has been a lot of research done investigating possible positive application of DNP. At controlled doses, DNP was found to prevent obesity in mice[12]. A derivative of DNP, designed specifically to function in the liver, was found to reverse hypertriglyceridemia, fatty liver, and whole-body insulin resistance in rats that were fed a high fat diet[13]. DNP has the ability to stimulate signaling responses in the cerebral cortex, and may have some potential therapeutic applications[14]. DNP can lower the levels of reactive oxygen species (ROS) in mitochondria[15]. This feature of DNP has been found to help protect cells from buildup of ROS in response to heat stress[16], cold stress[17], and exposure to sodium nitroprusside[18].

Limited research has been done on the effect of DNP exposure on mast cells, the cell type primarily studied by the Gosse laboratory. Mast cells are key players in the immune system that, through the process of degranulation, release pro-inflammatory mediators in response to antigen or allergen. One study found that DNP exposure increased the degranulation in mast cells[19]. Two other studies came to the opposite finding[20,21]. Based on the information availaboratoryle, no conclusion can be made as to how DNP affects mast cells at this time.

1.2 Triclosan

Triclosan (TCS) is a broad spectrum, antibacterial agent[22,23] used in a wide variety of consumer products such as soaps, toothpastes, cleaners, and many other household products at concentrations of roughly 10mM[23,24]. The use of TCS in those products is currently not regulated by the U.S. government. TCS has been found in both land[25] and aquatic[26] environments, most notably in wastewater treatment plants[25,26]. TCS has also been detected in a variety of aquatic animals [27,28].

TCS has also been detected in humans[29]. In one study the human test subjects who had ingested roughly 1 tablespoon of TCS-containing mouthwash were later found to have TCS in their blood streams at an average concentration of 1μM[30]. Another study found TCS in the milk of nursing mothers who had used TCS-containing personal care products[31]. Based on TCS detection in urine samples, a 2003-2004 study estimated that 75% of the United States population is likely exposed to TCS[32]. TCS can be absorbed both orally[33,34] and through skin exposure[35-37].

As stated earlier, the primary function of TCS is to prevent bacterial growth by inhibiting the bacterial fatty acid synthesis. During bacterial fatty acid synthesis in *E. coli*, TCS binds to both NAD^+ and the enol-acyl carrier protein reductase (FabI) at its substrate site to form a non-covalent bi-substrate[38]. This prevents the process of fatty acid synthesis from going forward and thus prevents bacterial colonies from expanding as new cell membranes cannot be made.

Studies have found that certain bacteria are, or can become, resistant to TCS. One study found that TCS usage was associated with a high risk of developing resistance in *Staphylococcus aureus* and *E.coli*[39]. Another study found that a mutation in the *fabI* gene of E.coli led to a change in the substrate binding site of the FabI protein, the mutated gene coding for FabI[G93V] protein[38]. TCS had a significantly lower affinity for the mutated binding site on FabI[G93V] reducing its inhibition of fatty acid synthesis[38]. Yet another study found that, in vivo, *Salmonella typhimurium* exposure to TCS could lead to variants of the bacteria that were both resistant to TCS and other antibiotics[40]. A review study went so far as to postulate that risks of TCS resistant bacteria developing from usage of TCS containing soap outweighed the benefits of TCS's antimicrobial

properties[41]. Use of TCS soap in an Italian hospital led to an outbreak of *Pseudomonas aeruginosa*[42], which is intrinsically resistant to TCS[43]. *Pseudomonas aeruginosa* is resistant to TCS due to the presence of a MexAB-OprM efflux system[44]. Exposure to TCS can lead to variants that hyper-express that efflux system and thus have higher resistance to other antibacterial drugs[44]. A review article focusing on TCS use in regular consumer products did not find any correlation between TCS presence and antibacterial resistance, but also stated that more intensive studies were needed[45].

TCS is small, hydrophobic, and ionizable, with a pKa around 7.9. In laboratory experiments it has been found to be stable at up to 200°C[46]. TCS in its deprotonated form can be broken down during prolonged exposure to heat and sunlight in the environment[47]. In its protonated form, TCS is considered toxic to aquatic animals due to its ability to cross cellular membranes[48]. However, more studies need to be done to assess TCS's role in mammalian biology and how it affects mammalian health.

1.3 Triclosan Epidemiology

Triclosan (TCS) is used for a wide array of medical applications. TCS, used as a component of toothpaste, has been found to be effective at preventing gingivitis and plaque buildup[49-51], and has been shown to limit the progress of periodontitis[52]. In skin care use, TCS has been shown to prevent skin inflammation caused by sodium lauryl sulphate[53], nickel sulphate[54], and histamine[55]. TCS has been shown to be a safe and effective option for treating dermatitis[57], thought to be due to it preventing *Staphylococcus aureus* growth[58]. In one study, patients who used topical steroids, along with TCS, to treat dermatitis required less steroid use ($P < 0.05$) than a control group to treat their condition[56]. TCS has also been shown to be very effective at treating both acute

eczematous dermatitis[59] and acute dermatomycoses[60]. TCS is also commonly used to sterilize surgical sites and tools and has been found to be quite effective at preventing surgical site infections[61].

However, TCS exposure has also been linked to a variety of health problems. TCS has been associated with increased occurrence of allergies and asthma[62-64]. One study found that reported atopic asthma increased 34% among individuals exposed to TCS[63]. Another study found that there was a statistically significant increase in reported allergy or hay fever among individuals who had TCS in their urine and were under the age of eighteen[64]. TCS was also associated with an increased chance of food allergies in male test subjects[62].

There have been several conflicting studies done on TCS's role as an endocrine disruptor and the possible health effects caused by its endocrine disruption. One study found that among females aged 6-8, there was a slight inverse relationship between TCS exposure and pubic hair development[67]. Several studies have found that TCS has negative effects on human fertility, likely due to it being an endocrine disruptor[65]. The results of one study found that among participants in the highest quartile of TCS urine concentrations (>71.7 ng/ml) there was a decreased rate of fecundity[65]. A laboratory study found that preimplantation exposure to TCS could lead to implementation failure in mice[66]. Another study found that among individuals in Japan who had suffered a spontaneous abortion, 28.3% had significantly higher concentrations of TCS in their urine as compared to normal pregnacies[68]. This effect of high TCS concentration causing spontaneous abortion was then replicated in laboratory mice[68]. However, other studies have not found any association between TCS exposure through health care products and

negative health effects caused by endocrine disruption[69-71]. One study found that 0.3% TCS in toothpaste did not cause any notable negative effect on thyroid function[70] while another did not find any short term impact of TCS exposure on hormone function[71]. It should, however, be noted that those previous two studies were conducted by a group of researchers affiliated with the University of Queensland (New Zealand). This school is closely associated with Colgate Pamolive, and receives both scholarships[72,75] and corporate sponsorship[73,74] from the company. Colgate Pamolive manufactures and markets the toothpaste Colgate Total, which contains TCS.

1.4 Mitochondrial Uncoupling and Oxidative Phosphorylation

Mitochondria are organelles that have important roles in a wide variety of cellular functions[76]. One of those functions is the energy producing process of oxidative phosphorylation. During oxidative phosphorylation, adenosine diphosphate (ADP) is phosphorylated by the enzyme ATP synthase to adenosine triphosphate (ATP), the energy currency of the cell. H_2O is also produced as a byproduct[77]. ATP synthase derives its energy from the proton gradient maintained by the proton pumps of the electron transport chain, which pump protons from inside the mitochondrial matrix to the intermembrane space while also shuttling electrons into reaction with O_2 to produce H_2O. Proton pumping produces a proton gradient, in which more protons are on the outside of the inner mitochondrial membrane than on the matrix side. In typical, healthy mitochondria, the only way a proton can flow back into the matrix is through a pore in the ATP synthase complex. The flow of a proton through ATP synthase, down its concentration gradient releases free energy, which ATP synthase uses to catalyze the

phosphorylation of ADP. This process is the primary way in which human cells produce energy, with less efficient alternatives including anaerobic lactate formation[77].

Mitochondrial uncouplers interfere with oxidative phosphorylation by providing a way for protons to flow down their concentration gradient, back into the matrix, without passing through ATP synthase. In an uncharged, protonated state, proton ionophore mitochondrial uncouplers can pass through the inner mitochondrial membrane. In general, more potent mitochondrial uncouplers have a pKa above 6[79]. Once inside, the uncoupler can dissociate a proton, thereby disrupting the proton gradient and decreasing ATP production[78]. The dissipation of the proton gradient is detrimental to oxidative phosphorylation and can be lethal to the cell, as it will have to find other ways to produce energy. Uncoupling also leads to an increase on O_2 consumption as the electron transport chain tries to re-establish the proton gradient[77]. Thus the main ways of evaluating mitochondrial uncoupling are measuring O_2 consumption and ATP production[80,81].

1.5 Health Effects of Mitochondrial Uncouplers

As stated before, use of DNP as a dieting drug can have severe effects on human health. When used as a dieting drug, DNP uncouples mitochondria, causing them to dissipate the potential energy produced by the electron transport chain as heat instead of using it for ATP production. This causes the human body to quickly burn through its fat stores as it tries desperately to get mitochondrial proton gradients re-established. In many cases, this results in death by hyperthermia.

Human cells that are part of adipose brown tissue (brown fat) are actually able to take advantage of mitochondrial uncoupling to produce heat by using the inner

mitochondrial membrane uncoupling protein thermogenin[82]. In laboratory research, the process of thermogenesis in brown fat was found to be critical to the energy regulation of laboratory rats and potentially had a role in the prevention of obesity[83].

The mitochondrial uncouplers CCCP and carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) have been shown to have effects on rat basophilic leukemia (RBL) mast cells, the main cell type used in this study[84-86]. One study showed that CCCP exposure both decreases RBL ATP production and significantly inhibits its primary function in media that did not contain glucose (the presence of glucose allows cultured cells to adequately produce energy through glycolysis, it is common to use media not containing glucose to force cultured cells to rely on oxidative phosphorylation)[85].

1.6 Mast Cells

Previous research in the Gosse laboratory has found that TCS both uncouples mitochondria in mast cells and inhibits their primary function[114]. Mast cells are key players in the immune system, with roles in type I hypersensitivity[104] and allergic immune response[115]. Their primary function is to release pro-inflammatory mediators from their many intracellular granules. Mast cells are ubiquitous in the body and, in addition to their role in allergic responses, have roles in immune responses to infectious disease and carcinogenesis, as well as neurological disorders such as autism and anxiety[84,87,88]. According to recent research, mast cells may also play a role in modulating behavior[89]. Mast cells are a good model for studying the effects of TCS and DNP because they are so ubiquitous (located in the mouth, skin, and connective tissue[116-118]) and thus would very likely be exposed to TCS, used in epidermal and oral care, and DNP, which is ingested orally.

Human mast cell development starts in the bone marrow from CD34+/CD117+/CD13+ pluripotent precursor cells[90]. From the bone marrow, immature mast cells travel through the blood stream to peripheral connective or mucosal tissue. This is where they complete their development. Next KIT is activated after stem cell factor (SCF)-induced KIT dimerization and auto-phosphorylation[119]. This key step enables mast cell maturation. SCF plays an important role in the maturation process, controlling mast cell survival and cell adhesion to either other cells or substrates. SCF also allows for the proliferation and differentiation of mast cells[91].

Being present throughout the body, mast cells are often the first responders to viruses, bacteria, and parasites[104]. Mast cells are important to both the innate and adaptive immune systems. The role of mast cells in the immune system is to release pro-inflammatory mediators from their internal granules. These mediators include histamine, tryptase, serotonin, and chymase[92]. Of those, histamine is the most well-known, significant effector[93] In addition to the release of those mediators, mast cell activation also stimulates the synthesis of lipid mediators such as leukotrienes and prostaglandins, cytokines, and chemokines[94]. As well as initiating the inflammatory response, these mediators also have roles in angiogenesis, tumorigenesis, and cellular hyperplasia. Degranulation usually occurs as a result of IgE receptor crosslinking, however, mast cells can also be activated by a variety of different mechanisms[121-124].

Research suggests that the distribution of mediators among granules is heterogeneous and that the release of specific mediators depends on the tissue microenvironment in which a mast cell is located[95]. The particular physiological role of a mast cell is dependent on the mediators contained in its granules. Mast cells rely on

surface receptors to sense their environment. These receptors include Fcε, Fcγ, TLRs, major histocompatibility complexes (MHC) classes I and II, KIT, and various interleukins[96]. In type I allergic responses, mast cells are activated by IgE crosslinking to the FcεRI surface receptor.

1.6.1 RBL-2H3 Cell Line

Rat basophilic leukemia cells (RBL) cells, clone 2H3 (RBL-2H3) are a commonly used mast cell model. They are a continuous cell line that was isolated from rat leukemia cells treated with β-chlorethylamine[98]. They are functionally homologous to human mast cells[97,98] and have the same core signaling machinery[99,100]. As a biosensor, RBL cells have been used to test the environmental toxicity of various chemicals[101]. Substantial research has been done with RBL cells to study IgE-FcεRI interactions and degranulation signaling pathways[102].

This study, as well as previous studies in the Gosse laboratory, makes use of RBL-2H3 cells both because the line serves as good model for human mast cells and because a large number of monoclonal RBL-2H3 cells can be produced by basic cell culture technique. RBL-2H3 cells give a dose responsive curve after stimulation by DNP-BSA antigen[103], indicating that they are a good model for studying degranulation. A similar response has also been reported in human basophils[120].

1.7 Mast Cell Degranulation

As stated earlier, the primary function of mast cells in the immune system is to release pro-inflammatory mediators through the process of degranulation (the release of mediators from intracellular granules). Degranulation begins when allergen or antigen

bind to a sensitized mast cell[104]. Degranulation ends when the cells intracellular granules have undergone exocytosis. Many of the key steps of degranulation, including ATP priming steps, vesicles docking at release sites and fusing with the membrane, and the release of the mediators, have been successfully imaged and analyzed using internal reflection microscopy[105].

In RBL-2H3 cells, degranulation can begin when either the receptor FcεRI is activated by IgE bound to antigen, or when calcium is mobilized within the cell[84]. FcεRI has both and α chain, a γ subunit, and a β chain, which spans the membrane four times[106]. When IgE binds FcεRI, crosslinking occurs, spurring the phosphorylation of tyrosine residues on the β and γ subunits by Lyn[107]. Syk then binds to the γ subunit of the receptor and undergoes a conformational change. Syk then acts as a catalyst for a number of phosphorylation reactions. This leads to the activation of the linker for T-cell activation (LAT) and PI3K[108]. LAT recruits PLCγ1 to the membrane, where Syk phosphorylates it. There, PLCγ1 serves as the catalyst for the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) to inositol-1,4,5-trisphosphate (IP3) and diacylglycerol (DAG)[109]. DAG activates protein kinase C (PKC) while IP3 sets off an influx of intracellular calcium by binding to the endoplasmic reticulum (ER). The ER then releases its store of calcium. As the amount of calcium in the ER stores decreases, a conformational change in STIM1 is spurred. STIM1 then interacts with calcium release activated calcium (CRAC) channel/Orai1 in the plasma membrane, which allows for calcium to influx into the cytoplasm[110]. With the use of Sarco/endoplasmic calcium ATPase (SERCA) pumps, the ER stores of calcium are replenished, while the new higher level of intracellular calcium is sustained. This is the key signal that leads to mast cell degranulation[111].

Mitochondria, which have a high calcium buffering capacity and specific tools for calcium uptake and efflux, play a major role in the regulation of this signal[112].

PKC phosphorylates the protein myosin, which stimulates the cytoskeleton rearrangements in the cell that are needed for degranulation[125]. Intracellular calcium, along with PKC, activates both isoforms of PLD[121]. PLD1 is involved in the movement of granules within the cell, while PLD2 plays a role in the granule fusion to the cell membrane[126]. All together, these many steps lead to mast cell degranulation.

1.8 Previous Research

Previous research done in the Gosse laboratory has shown that TCS disrupts antigen and thapsigargin-stimulated degranulation in RBL-2H3 cells and calcium ionophore-stimulated degranulation in human mast cells clone 1.2 (HMC-1.2), at non-cytotoxic doses[113,114]. TCS was also found to disrupt antibody- and calcium ionophore-stimulated degranulation in RBL-2H3 cells[113]. Based on its small molecular structure, its hydrophobic properties, and its ionizable proton, it was theorized that TCS was a mitochondrial uncoupler, and that a decrease in ATP production could be responsible for the decreased degranulation response. Further research was done using a Toxglo assay (Promega) to assess the ATP production of cells exposed to TCS. The research found that, in glucose free, galactose-containing media, TCS significantly inhibited ATP production in RBL-2H3 cells with an EC_{50} of 7.5 to 9.6 μM (95% confidence interval)[114]. Similar studies done with HMC-1.2 cells found a similar effect with an EC_{50} of 4.2 to 13.7 μM (95% confidence interval)[114]. These effects were not observed in Toxglo assays done in glucose media, where cultured cells can produce adequate energy from glycolysis. ATP and degranulation response experiments were then done with triclosan-

methyl (mTCS), a derivative of TCS that have a methyl group in place of TCS's ionizable proton. mTCS, which again does not have an ionizable proton, was not found to have any significant effect on either ATP production or degranulation[114]. In addition it was found that TCS exposure led to an increase in oxygen consumption in RBL-2H3 cells[114]. All these pieces of evidence strongly suggested that TCS in a mitochondrial uncoupler.

If TCS is a mitochondrial uncoupler, then it could pose a serious health risk to those who come in contact with it. As mentioned earlier, the vast majority of U.S. citizens are likely exposed to TCS. The purpose of my research was to investigate the potential danger of TCS by comparing its mitochondrial uncoupling effect to that of the known mitochondrial uncoupler DNP. This study focuses on characterizing DNP's effect on RBL-2H3 ATP production in the same conditions where TCS inhibited ATP production. The Promega Toxglo assay, as well as several cytotoxicity assays, were utilized. If TCS has a similar, or more potent, mitochondrial uncoupling effect in RBL-2H3 cells, it could indicate that there is a serious health risk with using this antibacterial product.

2. Materials and Methods

2.1 Cell Culture

RBL-2H3 cells (A gift from D. Holowka, Cornell University, Ithaca, NY, USA) were cultured using the methods of Hutchinson *et al*[127].

2.2 Galactose Media Preparation

Glucose free, galactose containing media was used to plate cells 1-h before exposure to DNP each day of an experiment. Galactose media contains 8.3 g l^{-1} Dulbeccos modified Eagle's medium (DMEM; Sigma) without glucose, L-glutamine, phenol red, sodium pyruvate or sodium bicarbonate. 10mM D-(+)-galactose (Sigma), 0.5842 g l^{-1} L-glutamine (Lonza, Alpharetta, GA, USA), and 3.7 g l^{-1} sodium bicarbonate were added to this DMEM to complete the media. The media was then brought to a pH of 7.4 using a pH electrode and adding small amounts of sodium hydroxide and hydrochloric acid. Bovine serum albumin (BSA; Calbiochem) was then added.

2.3 DNP Media Preparation

DNP was purchased from Sigma-Aldrich (St. Louis, MO, USA) (≥98%, moistened with water 15%, CAS no. 51-28-5). A starting DNP stock was freshly prepared each day of an experiment by dissolving DNP into cell culture, along with 3.7 g l^{-1} sodium bicarbonate buffer (VWR), before bringing the pH of the solution to 7.4 using a pH electrode and small amounts of sodium hydroxide and hydrochloric acid. This dilution method avoids the use of organic solvents. After adjusting the pH, the DNP stock was run through a 0.22µm filter to remove any particulate matter. The exact concentration was then determined using ultraviolet-visible (UV-vis) spectrophotometry and the Beer-Lambert equation. Absorbance was measured at 400 nm using a 1 cm path length and an extinction coefficient of 12,100 M^{-1} Lcm^{-1} [128,129]. The components of galactose media were then added in the same concentrations as in galactose media. BSA was then added.

2.4 Adenosine Tri Phosphate Production Assay

ATP production was measured using a Promega (Madison, WI, USA) Toxglo kit, following the methods described by Weatherly *et al* [114] with one exception. Based on the suggestion of a Promega scientist that the cytotoxicity reagent was not necessary for ATP measurement, the cytotoxicity reagent was not added. The cytotoxicity reagent from this particular kit works by reacting with proteases released by lysed cells to produce a fluorescence signal. Since DNP is a fluorescence quencher[130], meaningful cytotoxicity data could not be measured using this kit component.

2.5 Trypan Blue Cytotoxicity Assay with a 6-Well Plate

Cytotoxicity was measured using a trypan blue exclusion assay and a 6-well plate (Grenier). This assay uses trypan blue dye (Lonza) which can penetrate an RBL cell's membrane. A healthy, living RBL cell will be able to pump the dye out, while an unhealthy or dead cell will not. When examined under a microscope after trypan blue exposure, healthy cells appear as distinct clear circles, while unhealthy cells appear as dark indistinct blobs. With use of a hemocytometer and a microscope, it is thus possible to assess the number of healthy cells in a sample that has been exposed to trypan blue dye.

At the start of each experiment DNP media and galactose media were freshly prepared as described earlier. A 6 mM stock of DNP media was then made using the DNP and galactose media. From this stock, 3.5 mL dilutions were made of each concentration of DNP that was to be tested.

Cells were then harvested from a cell culture flask that had been prepared at least two days prior to the experiment and plated on 6-well plates using galactose media. Cells were plated at a density of 2 million to 3 million cells per well (depending on the number of cells that were harvested from the flask), in 1 mL of sterile galactose media. Two wells were plated for each concentration of DNP that was being tested. The cells were then put in a 37°C, 5% CO_2 incubator for 1-h in order to adhere to the plate.

After the hour, the plates were checked under the microscope in order to make sure cells were alive and had adhered to the plate. Then 1 mL of each DNP dilution were added to their designated well. The plates were then put back in the incubator for a 1-h exposure period. After the exposure period, the DNP and media were removed from each well. The wells were then washed with 1 mL of trypsin before another 1 mL of trypsin was added to each well. The plate was then placed in the incubator for another 10 minutes in order to allow the trypsin to separate the cells from the plate. The cells were then harvested, collected into samples, and quenched with galactose media. Next, 50 µL of each sample was then added to 50 µL of trypan blue dye. From the resulting cell solution, 50 µL was then taken and added to a hemocytometer. The number of living cells was then counted by looking at the hemocytometer under the microscope. The total number of living cells in a sample could then be calculated using the following equation.

Total number of cells = (dilution factor)(average number of living cells per square millimeter)(10,000)(total volume of solution in mL)

2.6 Trypan Blue Assay with a 96-well plate

Several attempts to measure cytotoxicity were done using a trypan blue exclusion assay on a non-adherent, non-tissue culture treated, 96-well plate (Grenier, Falcon). This assay uses the same principle as stated above.

At the start of each experiment DNP media and galactose media were freshly prepared as described earlier. A 2 mM stock of DNP media was then made using the DNP and galactose media. From this stock, 0.5 mL dilutions were made of each concentration of DNP that was to be tested.

Cells were then harvested from a cell culture flask that had been prepared at least two days prior to the experiment and plated on a 96-well plate using galactose media. A concentration of 50,000 cells, in 50 μL of sterile galactose media, were then plated, in random order, in each well. Three wells were plated for each concentration of DNP that was being tested, as well as three wells for untreated control, and several mock wells. The cells were then put in a 37°C, 5% CO_2 incubator for 1-h.

After the hour, the plates were checked under the microscope in order to make sure they were alive. Then 50 μL of each DNP dilution were added to their designated well. The plates were then put back in the incubator for a 1-h exposure period. After the exposure period, the DNP and media were removed from each well. After this hour the plate was again looked at under the microscope to see if the cells were alive. Then 50 μL of cells/media was removed from each well and added to 50 μL of trypan blue dye. 50 μL was then taken from the resulting solution and then added to a hemocytometer. The number of living cells was then counted by looking at the hemocytometer under the

microscope. The total number of living cells in a sample could then be calculated using the following equation.

Total number of cells = (dilution factor)(average number of living cells per square millimeter)(10,000)(total volume of solution in mL)

2.7 Crystal Violet Assay

A crystal violet assay was done after a failed attempt to do a trypan blue assay using a 96-well plate. The crystal violet assay is done using components of the FACE PI3 Kinase ELISA kit (Active Motif, Carlsbad, CA). This crystal violet component of this kit is designed to bind and stain the nuclei of cells, producing an absorbance signal at 595 nm meters that is proportional to the total number of cells.

Cells were plated and exposed to DNP as described in that assay's subsection. Following the discovery that the RBL cells had adhered to the plate, the remaining media and DNP in the plate was dumped. Each well was then washed twice with 200 µL of the kit's wash buffer and then another two times with 200 µL of 1X PBS buffer (Lonza). The plate was then air dried for five minutes. After the five minutes 100 µL of crystal violet dye was then added to each well and the plate was incubated for 30 minutes at room temperature on a shaker. After the half hour, the crystal violet solution was dumped and the each well was washed with 200 µL of 1X PBS buffer three times. During each of these washes, the plate was placed on a plate shaker for five minutes. These washes are designed to remove any crystal violet that is not bound to cells. After the third wash, 100 µL of 1% sodium dodecyl sulfate (SDS) solution was added to each well. In this assay SDS solution is used to bring the cells with bound crystal violet dye into solution and

then release the dye, providing an absorbance signal that can be read by the plate reader. The plate was incubated on the plate shaker at room temperature for 1-h. After the hour, the absorbance of each well at 595 nm was measured using the plate reader (BioTek).

2.8 Lactate Dehydrogenase Assay

A lactate dehydrogenase (LDH) assay was attempted in order to assess the cytotoxicity of DNP. The assay was done using a kit from Roche Applied Science. This assay works adding a dye solution and catalyst to RBL cells in a plate. The dye solution reacts with LDH released by dead cells and produces an absorbance signal at 490 nm that can be measured using a plate reader. The more dead cells there are, the higher the absorbance will be. In addition to cells being exposed to varying concentrations of DNP, there are also low control cells (unexposed to any toxicant) and high control cells (cells exposed to a lysis solution).

At the start of each experiment DNP media and galactose media were freshly prepared as described earlier. A 2 mM stock of DNP media was then made using the DNP and galactose media. From this stock, 0.5 mL dilutions were made of each concentration of DNP that was to be tested. Aliquots of kit components were thawed on the day of each experiment.

Cells were then harvested from a cell culture flask that had been prepared at least two days prior to the experiment and plated on a 96-well plate using galactose media. A concentration of 50,000 cells, in 50 μL of sterile galactose media, were then plated, in random order, in each well. Three wells were plated for each concentration of DNP that was being tested, as well as three wells for low control and three wells for high control.

The cells were then put in a 37°C, 5% CO_2 incubator for 1-h in order to adhere to the plate.

After the 1-h, 50 µL of the varying DNP dilutions were added to the corresponding wells on the plate. Plain galactose media was added to the control wells. The plate was then placed back in the incubator for 45 minutes. After the 45 minutes the plate was removed from the incubator and 5 µL of lysis solution was quickly added to three high control wells. The plate was then placed back in the incubator for another 15 minutes. During that time the dye solution and catalyst were brought into solution with each other to make a reaction mixture. After the 15 minutes, the plate was removed from the incubator and 100 µL of reaction mixture was added to each well. The plate was then incubated for 30 minutes at room temperature. Following the 30 minutes, 50 µL of stop solution was added to each well. The absorbance at 490 nm was then measured for each well using the plate reader.

2.9 Data Analysis

Data from the ATP production assay were analyzed by first averaging the luminescence value of the three replicates of each sample from one experiment. Those averages were then compared to the average of the untreated control replicates. This comparison was expressed as a percentage of the untreated control. Those data were then entered into GraphPad Prism software to produce a figure. The data were analyzed using a one-way ANOVA followed by Tukey's post-hoc test to determine statistical significance.

Data from the trypan blue assay were analyzed first by calculating the total number of cells from each sample. The total number of living cells from each DNP replicate was then averaged together. That number was then compared to the average of 0 mM DNP (serving as the low control) and that comparison was the expressed as a percentage. Those data were then entered into GraphPad Prism software to produce a figure. The data were analyzed using a one-way ANOVA followed by Tukey's post-hoc test to determine statistical significance.

Data from the crystal violet assay were analyzed by averaging the absorbance at 595 nm each of the replicates for each sample. These averages, along with the averages for the control, were then entered into GraphPad Prism software to produce a figure.

Data from the LDH assay were analyzed first by averaging the absorbance at 490 nm from each of the replicates for each sample, as well as the high and low controls. Those averages were then compared to the average of the untreated control replicates. This comparison was expressed as a percentage of the untreated control. Those data were then entered into GraphPad Prism software to produce a figure. Additionally, to assess whether an assay's results were valid, the average of the high control was divided by the average of the low control. If this ratio was at least 20, then the results were considered valid[131].

3. Results

3.1 Determining the Most Effective Technique for Dissolving DNP

In order to perform any experiments with DNP, it is necessary to get DNP into a uniform solution. Literature suggested that DNP was soluble in water at concentrations up to 15-30 mM[132,133]. Initially DNP was brought into solution simply by weighing out a 0.037 g of DNP, adding it to 20 mL of cell culture water (Lonza), and then vortexing and inverting the solution. The resulting solution should be 8.5 mM DNP (DNP supplied by Sigma is 15% wetted with water). The exact concentration was determined using UV-Vis spectrophotometry and an extinction coefficient (12,100 $M^{-1}Lcm^{-1}$) from Peralta *et al*[129]. However, this initial technique only resulted in solutions that were around 4 mM. A more effective way of dissolving DNP was needed.

In order to determine a more effective way, four dissolving techniques were tested: changing the pH to 7.4 (physiological pH), sonicating for 90 minutes, stirring for 90 minutes at room temperature, and stirring for 90 minutes at 50°C. An approximately 8.5 mM DNP solution was prepared as described earlier before each method was tested. Exact concentration was determined before and after each technique was used. Changing the pH of DNP in solution by itself did not work because DNP has a low pKa of 3.9[134]. Sonicating for 90 minutes resulted in a solution that was 4.73 mM, stirring the solution at room temperature for 90 minutes resulted in a 3.69 mM solution, and stirring the solution at 50°C resulted in a 3.90 mM solution. These results indicate that none of these techniques were particularly effective.

It was then decided to try adjusting the pH of DNP in the presence of a buffer. Sodium bicarbonate buffer was chosen since it was the same buffer used in the galactose media that was prepared for Toxglo assays, and because it has a pKa of 6.4, which is much closer to the target pH of 7.4. Using UV-Vis spectrophotometry it was determined that sodium bicarbonate did not produce absorbance in the same range as DNP, so it would not interfere with determining the concentration of DNP. To test this technique, an 8.5 mM solution of DNP was again prepared as described earlier, but this time in the presence of 44 mM sodium bicarbonate. The pH was measured using a pH electrode meter, and the pH was adjusted adding small amounts of sodium hydroxide and hydrochloric acid with a Pasteur pipette. The resulting solution was 5.78 mM. This result, compared with the previous results, indicated that this method of dissolving DNP was by far the most effective, and was the dissolving technique that was used for all the following experiments.

3.2 DNP Effect on Cellular ATP production

ATP production was measured in RBL-2H3 mast cells (RBLs) using the techniques mentioned in Materials and Methods. Combined data from at least four different experiments for each concentration indicate that DNP inhibits ATP production in RBL cells significantly, with an EC_{50} between 389 μM and 677 μM (median 533 μM) as seen in Figure 1. Cytotoxicity experiments (see below) indicate that the concentrations of DNP used are non-cytotoxic, indicating that the decrease in ATP is due to mitochondrial uncoupling and not cell death. Previous research done under the same conditions from Weatherly, *et al*[114] indicate that TCS inhibits ATP production with an

EC_{50} of 8.6 μM. Together, these results show that TCS decreases ATP production in RBL cells at concentrations that are 62 times lower than DNP's EC_{50} for ATP production. These data suggest that TCS is a much more potent mitochondrial uncoupler of mast cells than DNP.

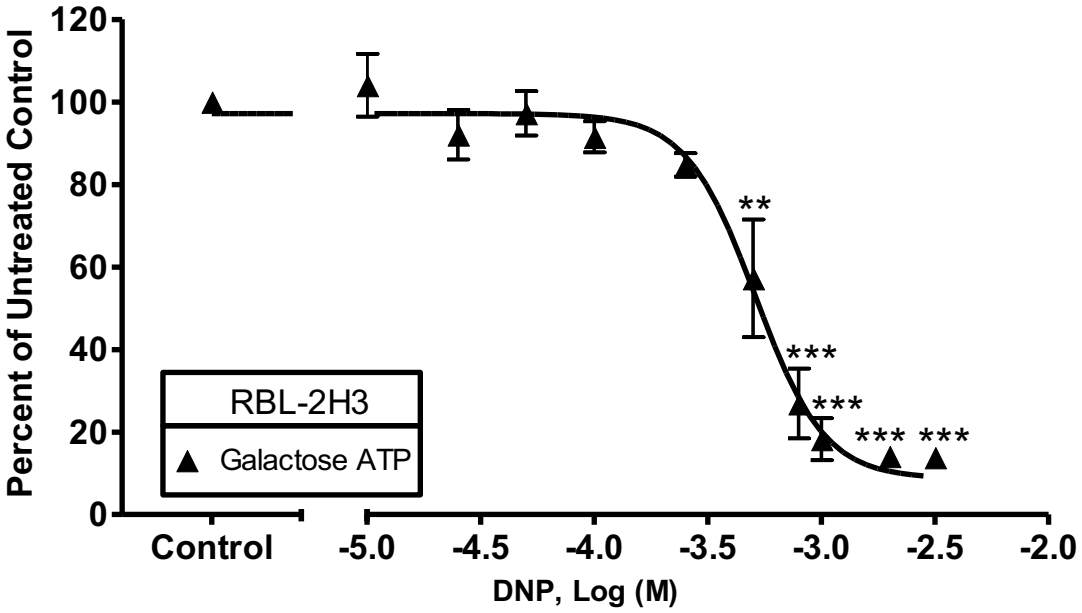

Figure 1. ATP production in RBL cells exposed to increasing concentrations of DNP. ATP production was measured in RBL cells after exposure in galactose media for one hour. Percent of untreated control for ATP production is determined by dividing the luminescence value of the sample by the average of the untreated control. The values presented are the means ±SEM of at least four different experiments; each with three replicates. Statistically significant results, as compared to untreated control, are indicated by **P<0.01, and ***P<0.001 as determined by one way ANOVA followed by Turkey's *post-hoc* test. RBL, rat basophilic leukemia (mast cell), DNP, 2,4-Dinitrophenol.

No-cell control experiments done to test whether DNP disrupts background luminescence levels in the Toxglo™ assay indicate no significant effect of DNP on these values (Figure 2A). Control experiments done to test whether DNP interferes with the ATP reagent also indicated no disruptive effect of DNP (Figure 3A). These data indicate that the decrease in luminescent signal in Toxglo experiments is due to decreased ATP production and not signal interference.

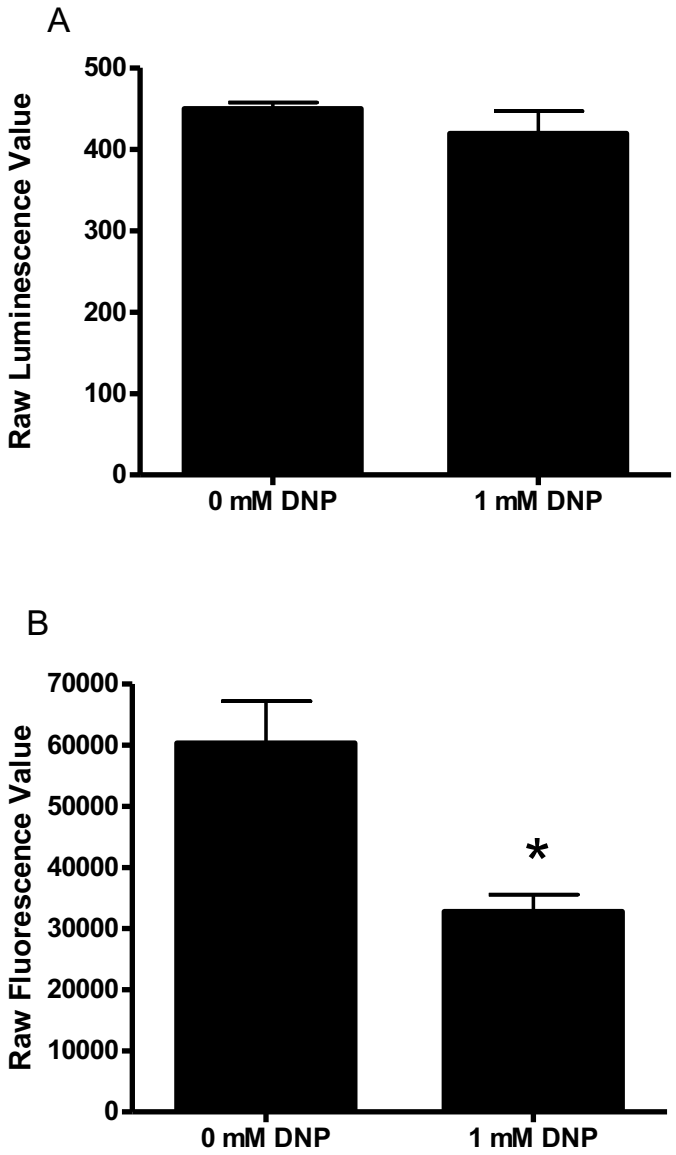

Figure 2. Controls to examine whether DNP disrupts background luminescence levels (**A**) and/or background fluorescence (**B**) in the Toxglo^{TM} assay. All steps were from the Toxglo assay described by Weatherly, *et al*. were followed, with the lone modification being that no cells were plated. (**A**) Raw luminescence values, following treatment with ± 1mM DNP. The values are low, due to the lack of cells, and thus, lack of ATP production. DNP does not change these values. (**B**) Raw fluorescence values from the control experiment, ± 1mM of DNP. These values are moderate, likely due to background cleavage of the cytotoxicity substrate, even in the absence of cells. These data show that DNP dampens fluorescence at the wavelength used for the assay, meaning that use of this particular assay to assess the cytotoxicity of DNP is not viable. The values presented are means ± SEM of three independent experiments, each with three replicates. Statistically significant results, as compared to 0 mM DNP, represented by *P<0.05, are determined by one-way ANOVA followed by Tukey's *post-hoc* test. DNP, 2,4-Dinitrophenol.

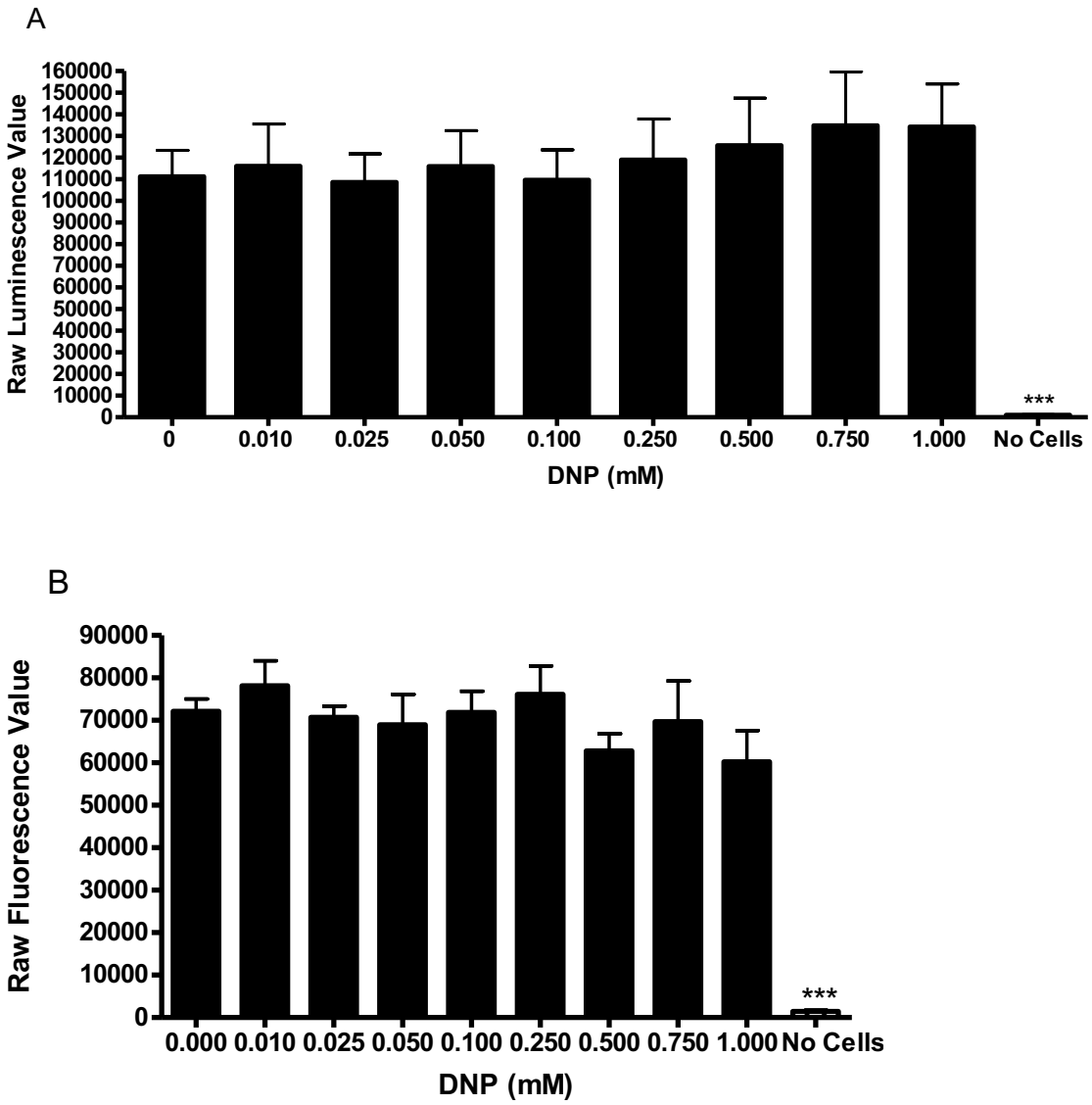

Figure 3. Controls to determine whether DNP interferes with either the ATP reagent (**A**) cytotoxicity reagent (**B**). (**A**) For the ATP reagent controls, the Toxglo™ procedure described by Weatherly, *et al*. was followed with the following changes: Cells were incubated for 1-h with plain media, not containing DNP. After discarding the media, ATP reagent, which lyses the cells, was added, followed immediately by the varying DNP concentrations. This was done to test whether DNP affects the ATP luminescence reaction without affecting cellular ATP production. Results indicate that DNP does not significantly affect this reaction. (**B**) For the substrate-protease control, the steps described by Weatherly, *et al*. were followed with two exceptions. The first being that black, clear bottom plates (Grenier) were used, and the second being that digitonin, which lyses cells, was added to all wells before DNP exposure. Fluorescence was then measured to determine whether DNP interfered with the substrate protease reaction. Results show that DNP does not significantly alter this reaction, in contrast to the no cell control experiments (**Figure 2**). Values presented are means ±SEM of three independent experiments, each with three replicates. No significance, as compared to untreated control, was determined by one-way ANOVA, followed by Tukey's *post-hoc* test. DNP, 2,4-Dinitrophenol.

3.3 DNP Interference with Cytotoxicity Measurement

In order to determine the potency of DNP as a mitochondrial uncoupler, it is necessary to be able to assess its cytotoxicity, in order to determine if any decrease in ATP production is due to inhibition of oxidative phosphorylation, or cell death. Initially, cytotoxicity was assessed using a proprietary cytotoxicity reagent provided within the Toxglo assay kit (Promega). This reagent reacts with a protease released only by lysed (dead) cells[135]. This reaction produces a fluorescent signal[135]. Initial experiments yielded cytotoxicity data like those seen in Figure 4. Note that the cytotoxicity shows a linear regression as the concentration of DNP increases. This would indicate that the cytotoxicity of DNP actually *decreases* at higher DNP concentrations. These results were surprising, given that DNP is a known dangerous compound.

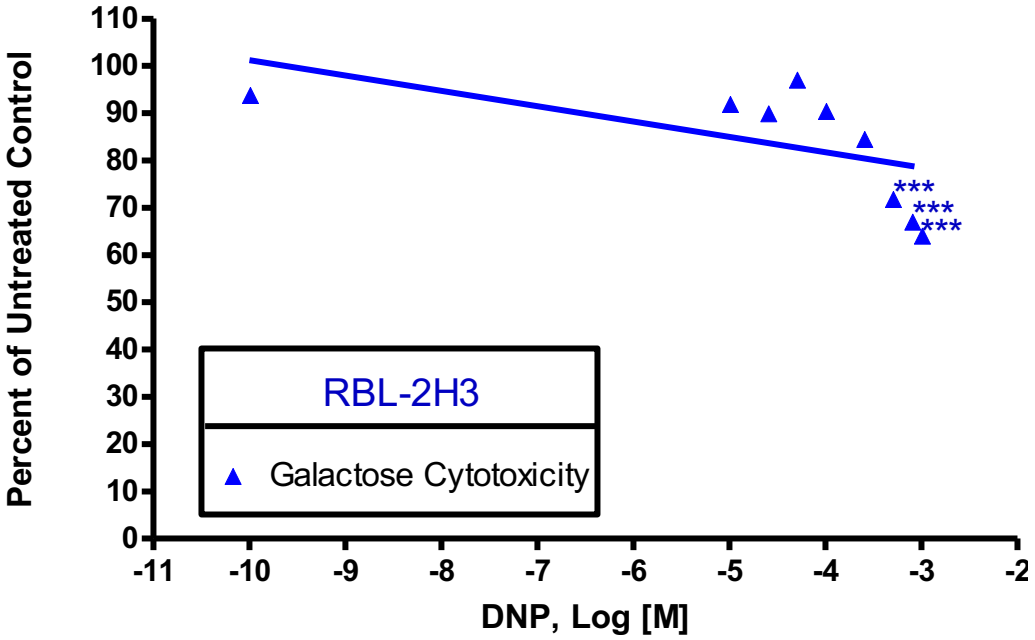

Figure 4. Cytotoxicity of RBL cells exposed to increasing concentrations of DNP in galactose media, measured by the Toxglo™ assay. Percent of untreated control for cytotoxicity is determined by dividing the fluorescence value of the sample by the average of the untreated control. The values presented are means ±SEM of three replicates from one experiment. Statistically significant results, as compared to untreated control, are indicated by ***P<0.001, as determined by one way ANOVA followed by Turkey's *post-hoc* test. RBL, rat basophilic leukemia, DNP, 2,4-Dinitrophenol.

Control experiments were done to determine whether DNP interferes with the cytotoxicity reagent-protease reaction. Control experiments done in the presence of cells indicated no significant interference (Figure 3B). However, no-cell control experiments indicated significant interference at the high concentrations of DNP utilized (Figure 2B). Further investigation of DNP in the literature led to the published fact that DNP is a

fluorescence quencher[130]. This revelation, coupled with the data from initial and control experiments, indicated that a new way of determining DNP cytotoxicity was needed.

3.4 Determining the Cytotoxicity of DNP

A trypan blue exclusion assay using a 96-well plate was initially used to determine cytotoxicity. RBL cells were plated on a clear, 96-well, non-adherent, non-tissue culture treated plate and exposed to varying concentrations of DNP as described in Materials and Methods. However, when samples were collected from the plate and counted after the DNP exposure period, there were almost no cells. This was true of not only the samples from wells exposed to DNP, but also of the control wells that were not exposed to any toxicant. The plate was then observed under a microscope, and a fairly confluent lawn of cells was observed in all the wells that had had cells plated in them. While looking at them under the microscope, the cells remained stagnant even when the plate was shaken. These same observations were noted in two separate experiments. These observations indicated that, in spite of the plate being designed to prevent cell adherence, the RBL cells had adhered to the plate.

After the second failed trypan blue assay, a crystal violet assay was done using the adhered cells from the second trypan blue experiment using the techniques described in Material and Methods. Data from the crystal violet assay indicate that there is no significant change in DNP cytotoxicity at concentrations of up to 100 μM (Figure 5). This experiment included three replicates for each concentration of DNP assessed. These results were promising, but not definitive. The trypan blue assay was not working, so another assay was attempted.

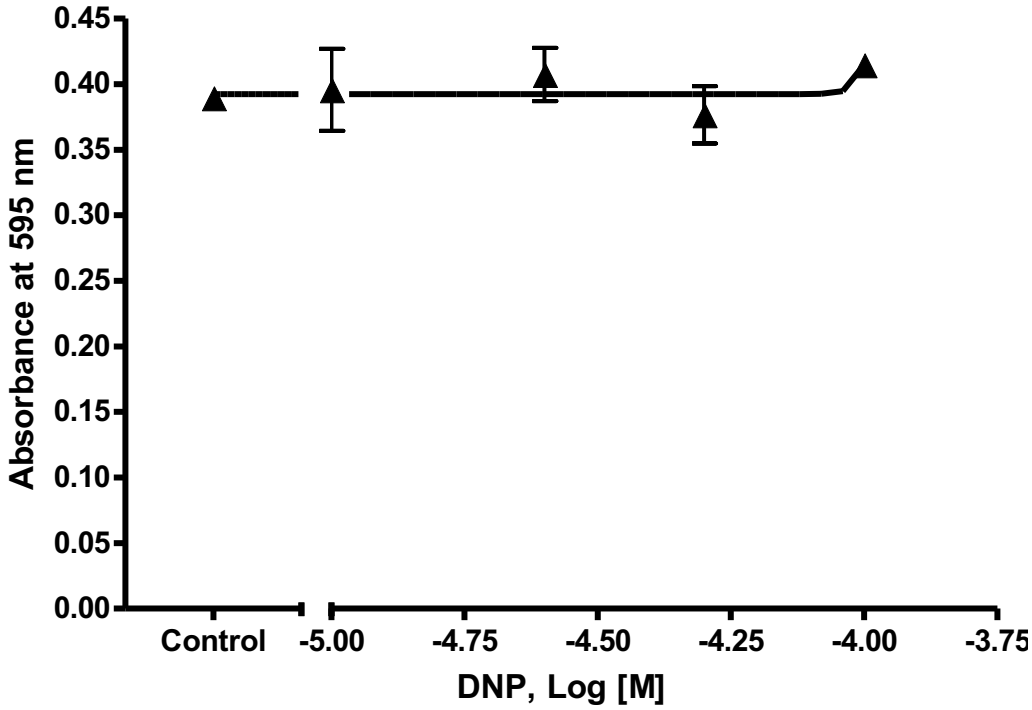

Figure 5. Cytotoxicity of 1-h DNP exposure assessed by absorbance at 595nm after staining with crystal violet dye. After plating cells on a clear, non-adherent plate and a 1-h exposure to DNP, an attempt was made to count living cells, collected in samples from the plate, using trypan blue staining. However, before counting was done, it was determined that the RBL cells had adhered to the non-adherent plate, preventing samples from being collected from the plate. The adhered cells were then stained using crystal violet dye, and cytotoxicity was assessed as described in Materials and Methods. No significant changes in cytotoxicity were observed among the concentrations of DNP used. Values presented are the means of three replicates from the plate. DNP, 2,4-Dinitrophenol.

A lactate dehydrogenase (LDH) assay was used next performed as described in Materials and Methods. The data from three independent LDH assays (Figure 6), each with three replicates, show a decrease in cytotoxicity for every concentration tested, as well as a couple of very large decreases at higher concentrations. As with the cytotoxicity

measured using the Toxglo reagent, these results did not seem to make sense. The validity of the results was then tested by measuring the ratio of absorbance from the high control (fully lysed cells) and low control (untreated cells) samples. A valid test would have an absorbance ratio of at least 20. The three experiments that I ran had ratios that ranged from 1.5 to 2.5. A graduate student (Lisa Weatherly) then performed the same experiment under the same conditions and got similarly confusing results (not shown). Combined, these results indicated that the assay was not working properly and, yet again, another way of measuring cytotoxicity was needed.

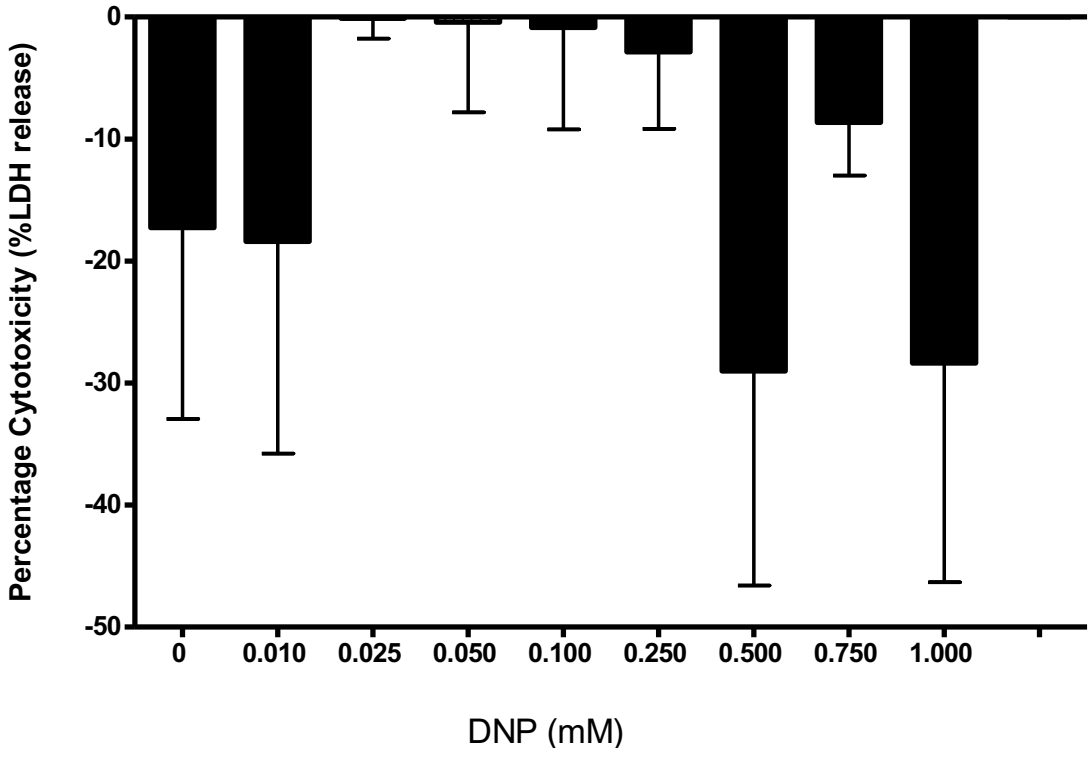

Figure 6. Cytotoxicity of 1-h exposure to DNP measured using Cytotoxicity Detection Kit (LDH) from Roche. Values Presented are means ±SEM of three different experiments. Based on three very low ratios of high control (fully lysed cells) to low control (untreated cells), it was determined that all three tests were invalid. The low ratio was most likely due to DNP interfering with the absorbance signal that this assay depends on to measure cell lysis. DNP, 2,4-Dinitrophenol, LDH, Lactate dehydrogenase.

The next method used was another trypan blue assay, but this time using tissue culture-treated 6-well plates (which have much larger well areas than do 96-well plates). This assay was performed as described in Materials and Methods. Initial results (not shown) were only slightly better than the original trypan blue assays, however the cell harvesting technique was gradually improved upon by experience and a workable version of the assay was developed. Data from five viable tests show no significant change in RBL cytotoxicity at DNP concentrations up to 3mM (which was the absolute highest concentration used in the DNP Toxglo experiments). These data indicate that the concentrations used in the Toxglo assay were, in fact, non-cytotoxic. Combined with the Toxglo ATP data, these data indicate that the DNP's inhibition of ATP production was likely due to DNP's mitochondrial uncoupling effect.

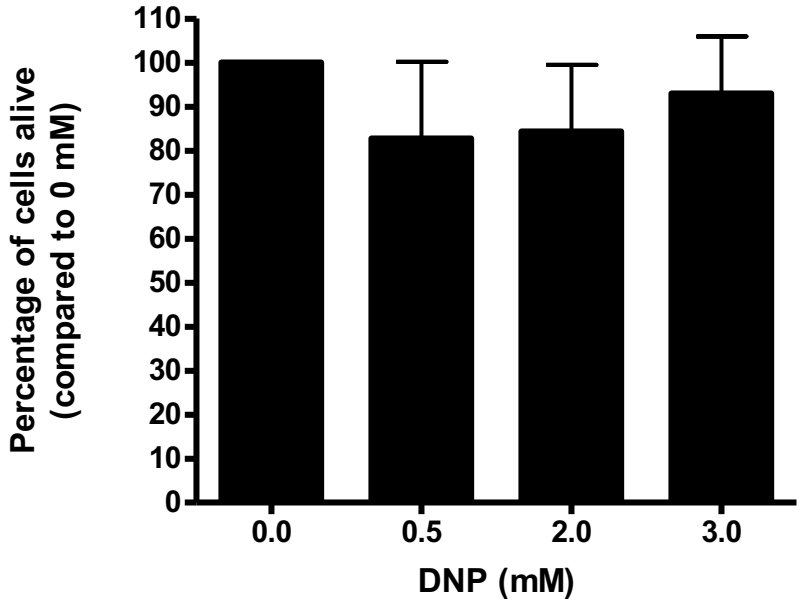

Figure 7. Cytotoxicity of 1-h exposure to DNP in galactose media measured using a trypan-blue exclusion cytotoxicity assay. Percent of cells alive is determined by dividing the number of remaining living cells in a sample by the average number of living cells in an untreated control (0 mM DNP). Values expressed are means ±SEM of 5 independent experiments, each with two replicates per treatment. Statistically significant results (or lack thereof) were determined by one way ANOVA followed by Tukey's *post-hoc* test. DNP, 2,4-Dintrophenol.

4. Discussion

Weatherly *et al.* found that the ubiquitous antimicrobial agent triclosan inhibits ATP production, in a variety of cell types, including in RBL-2H3 mast cells, at non-cytotoxic levels[114]. This finding was part of a larger study which showed that TCS is a proton ionophore mitochondrial uncoupler. In the current study, we have determined the concentrations at which 2,4-dinitrophenol, a canonical mitochondrial uncoupler, inhibits ATP production in RBL cells (Figure 1), in order to allow comparison of the EC_{50} values of TCS and DNP in the RBL cell line. In order to assess if a chemical is a mitochondrial

uncoupler, it is necessary to confirm that the decline in ATP production is not due to cell death. If a decline in ATP production corresponds with an increase in cytotoxicity, then the decline is likely due simply to the toxicant's killing the cells.

In this study, a variety of methods were employed in order to assess the cytotoxicity of DNP on RBL-2H3 cells. Initial experiments done with the fluorescence-based cytotoxicity reagent component from the Toxglo kit yielded data which showed a decline in cytotoxicity due to increasing DNP doses (Figure 4). These data were confusing, and it was later discovered via literature searches that DNP is a fluorescence quencher[130], explaining the decline in fluorescent signal with higher concentrations of DNP (Figure 2B). An LDH assay was also unsuccessful in assessing cytotoxicity due to DNP interference with that kit's reagent (Figure 6). Ultimately a refined trypan-blue exclusion assay generated data that indicated that DNP does not cause cell death, even at concentrations up to 3 mM (Figure 7). These data can be reasonably assumed to be accurate based on both the quantified data of 5 different experiments, none of which indicate an increase in RBL cytotoxicity due to DNP exposure, along with the qualitative observation that there was no visible change in the number of cells exposed to each concentration of DNP. If DNP is not cytotoxic at concentrations ranging from 500 μM to 3 mM, then it is reasonable to assume that the lower concentrations of DNP used to assess ATP production are also not cytotoxic, and that the observed decline in ATP production is very likely due to mitochondrial uncoupling. Since the decline in ATP production, caused via DNP exposure, appears to be due to mitochondrial uncoupling, it can be used as a meaningful comparison to data generated from previous experiments done under the same conditions with TCS.

Mitochondrial uncouplers disrupt ATP production via oxidative phosphorylation by separating the energy produced by the electron transport chain (stored in the proton gradient across the inner mitochondrial membrane) from ATP synthesis[77]. The mitochondrial Toxglo assay (Promega) effectively measures ATP production using a luminescent signal, and is one way of assessing whether a test compound is a mitochondrial uncoupler (when data is paired with corresponding cytotoxicity data)[77]. Previous studies done by the Gosse laboratory have shown that TCS decreases ATP production in a variety of cell lines, including RBL-2H3, in glucose-free, galactose-containing media[114]. In RBL-2H3 cells, the EC_{50} of TCS's ATP production is 7.5 to 9.6 μM. This result, along with data showing that TCS increases oxygen consumption in RBL cells[114] and decreases mitochondrial membrane potential (Lisa Weatherly, personal communication), provide strong evidence that TCS is a mitochondrial uncoupler. In this study, we show that DNP significantly decreases ATP production in RBL-2H3 cells, as would be expected for a mitochondrial uncoupler, but only at much higher EC_{50} than that of TCS. The EC_{50} for DNP's inhibition of ATP production in RBL cells is 389 μM to 677 μM (Figure 1). A comparison of the median EC_{50} values shows that, in RBL mast cells, 62-fold less TCS than DNP is required to inhibit ATP production. These results indicate that TCS could be a much more potent mitochondrial uncoupler than DNP, but more research is needed before anything definitive can be stated. Future studies might investigate how DNP affects the oxygen consumption rate of RBL-2H3 cells under similar conditions and if the same extent of differential TCS vs. DNP EC_{50} values are seen in additional cell types, including primary human cells. Experiments could also be done to see if DNP exposure inhibits RBL cell degranulation at doses similar to TCS.

One thing to note is the fact that DNP is notoriously toxic chemical, yet was found to be both a less potent mitochondrial uncoupler than TCS and non-cytotoxic to RBL-2H3 cells at concentrations up to 3 mM. In a similar experiment utilizing a trypan blue assay, TCS was found to cause significant cytotoxicity at 150 μM[113]. One possible explanation for the seemingly dampened effects of DNP is its low pKa of 3.9[134]. The experiments in this study were done at a pH of 7.4. It is possible that under these conditions, the DNP was highly deprotonated, according to the Henderson-Hasselbalch equation. When deprotonated, proton ionophore mitochondrial uncouplers take on a negative charge and cease to be lipophilic, losing their ability to diffuse across the inner mitochondrial membrane, and thus their ability to disrupt the proton gradient. DNP is actually capable to delocalizing its negative charge across its entire structure when deprotonated, thus maintaining its lipophilic properties and its ability to diffuse across the inner membrane of the mitochondria[77]. However, when it is deprotonated, it no longer can carry a proton, and thus it cannot disrupt the proton gradient. TCS has a pKa of 7.9 and thus would not be nearly as deprotonated under these conditions, and would maintain its mitochondrial uncoupling ability. This theory might explain the results of this experiment, but it fails to account for the fact that ingestion of DNP can cause enough mitochondrial uncoupling to induce hyperthermia in humans, whose natural internal pH is approximately 7.4.

Previous research has been done by Lisa Weatherly with the other canonical mitochondrial uncoupler carbonyl cyanide 3-chlorophenylhydrazone (CCCP) under the same or similar experimental conditions[114]. Her data show that, like TCS, CCCP inhibits RBL degranulation. Her data also show that CCCP inhibits ATP production in RBL cells

with an EC_{50} of 0.8 μM to 1.6 μM, much lower than the comparable TCS EC_{50}[114]. 1 μM of CCCP was also shown to increase oxygen consumption in RBL cells at a similar rate to 10 μM TCS[114]. It is interesting to note that, unlike DNP, the known mitochondrial uncoupler CCCP inhibits ATP production at much *lower* concentrations than TCS and suggests that mitochondrial uncoupling effect of DNP may be decreased in these particular experimental conditions.

Currently there have been some, though not a large body of research into the possible adverse effects of prolonged TCS exposure. Studies have found links between TCS exposure and increased incidence of allergy and asthma[62-64], decreased fertility[65], and developmental issues[67]. Other studies have found no connection between use of TCS-containing products and hormone-related health problems[69-71]. More research is needed to be done to determine whether the use of TCS in personal care products is dangerous, but the findings of this study, along with those from previous studies, indicate that there is a strong possibility that TCS use poses a significant threat to human health.

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Author's Biography

Erik Gerson was born in York, Maine on January 4th, 1994. He was raised in Melrose, Massachusetts where he graduated Melrose High School in 2012. As a biochemistry major, Erik twice received the *Radke Undergraduate Fellowship*. Erik was also an active participant in the *Health Professions Club*, the *Creative Writing Club*, and the *Maine Society of Microbiology*.

Upon graduation, Erik will be moving to Boston, with the hopes of finding work in a research or laboratory based profession.