Differentiation Paradigms and STAT Protein Expression in Neuro-2A Cells

by

Cody E. Keating

A Thesis Submitted in Partial Fulfillment

of the Requirements for the Degree of

Master of Science in Biology

Middle Tennessee State University

2013

Thesis Committee:

Dr. William Stewart, Thesis Advisor

Dr. Anthony Farone

Dr. Matthew Elrod-Erickson

Abstract

One of the most recent and exciting potential treatments for cancer is differentiation therapy which is inducing the differentiation of cancerous cells into normal functioning somatic cells. An excellent candidate for differentiation therapy is the neuroblastoma. The compounds examined in this experiment to induce differentiation in the N2A murine neuroblastoma cell line while performing morphometrics and western blot analysis in this project are: mevinolin, dibutyryl cAMP, all-trans-retinoic acid, ceramide, and perillyl alcohol. Morphometric study data: 12h study, perillyl alcohol exhibited the greatest differentiation at 63%; 24h study, perillyl alcohol exhibited the greatest again at 62%; 48h study, perillyl alcohol was again the highest differentiator, but this time at 68% differentiation. STAT proteins were found to be expressed in all treatments. Acetylcholinesterase was expressed in most of the treatments. NeuN was present in all treatments.

Acknowledgements

I am very thankful for the help in my research guidance provided by my research advisor, Dr. William Stewart, as well as my committee members Dr Matthew Elrod-Erickson, and Dr. Anthony Farone. I appreciate all the help and knowledge instilled in me during short period of time that I had with Dr. Jerry Reagan. I would like to thank the MTSU Bio Department and the research scholarships provided for funding this research. I would also like to thank Dr. Brian Robertson for the use of his imaging equipment. I am thankful to the other members of my lab, Dani Millay & Paul Hollis, for helping out on pieces of the project and Eric Vick for loaning me materials needed from time to time. I would also like to thank my friends and family for their understanding of my time and support through this endeavor.

Table of Contents **Page**

List of Tables, Charts, and Figures

Tables Page

Charts

Figures

Introduction

One of the most recent and exciting potential treatments for cancer is differentiation therapy. This is the process of inducing the differentiation of cancerous cells into normal functioning somatic cells by manipulating signal transduction pathways. Differentiation therapy can potentially be used as a new way of targeting only cancerous cells without causing harm to the normal cells. This would be unlike what routinely happens in the chemotherapy and radiation therapy commonly used as cancer treatments today (Ton Cao, 2005).

An excellent candidate for differentiation therapy is the neuroblastoma. Neuroblastomas are common and deadly tumors found in young children. These neuroendocrine tumors arise from migratory neural crest cells which normally make up the adrenal glands, but fail to differentiate into normal neural cells. Neuroblastomas are one of the rare human malignant cancers to spontaneously regress from an undifferentiated cancerous state to a completely benign neuronal cell (Brodeur, 2003). The identity of this differentiation trigger in vivo has yet to be determined, but several chemicals have been identified that can induce this differentiation in cell culture. The compounds examined in this experiment for their ability to induce differentiation in the N2A murine neuroblastoma cell line are: mevinolin, dibutyryl cAMP, all-trans-retinoic acid, ceramide, and perillyl alcohol.

The Janus Kinase/ Signal Transducers and Activators of Transcription (JAK/ STAT) second messenger pathway is known to play a major role in cell

differentiation, proliferation, and apoptosis by regulating transcription of various proteins within various cell types. Numerous cytokines, working through their receptors, will activate the Janus kinase, which in turn activates STAT proteins by phosphorylation of specific tyrosine residues within the STAT proteins. These newly activated STAT proteins translocate to the cell's nucleus and activate the transcription of various tissue-specific genes leading to the production of tissue specific proteins. As of today, seven mammalian STAT proteins have been described in the literature: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6 (Rawlings *et al.*, 2004). STAT1 & STAT3 are known to be involved in gp130 cytokine signal transduction in adipocytes (White *et al.*, 2011) whereas STAT5A enhances adipogenesis in fibroblast cells (Stewart, *et al.,* 2011). The JAK/STAT signaling pathway is a potential candidate for the differentiation of neuroblastomas similar to what has previously been shown in adipocyte differentiation (Stewart *et al.*, 2011 & White *et al.,* 2011).

Mevinolin is a statin compound that works as a hydroxymethylglutaryl-coenzyme A reductase inhibitor which blocks the synthesis of cholesterol. Statins are widely used compounds for treating hypercholesterolemia and are also associated with lower levels of malignancies (Corsini *et al.,* 1995). The treatment of neuroblastoma cells with statins is shown to up-regulate synthesis of the neuronal marker protein, NeuN, and induce neurite outgrowth (Evangelopoulos *et al.,* 2009). The differentiation induced by statins has been shown to be reversible with the addition of mevalonate to the media after differentiation has been achieved (Maltese,

1984. Maltese *et al.* 1985*)*. Apoptotic proteins Bcl-2 and Bcl-XL have been shown to be suppressed by statins (Agarwal *et al.* 1999. Park*, et al.* 1999. Dimitroulakos*, et al.* 2000. Garcia-Roman *et al.,* 2001. Blanco-Colio, *et al.* 2002. Blanco-Colio *et al.,* 2003). The expression of proapoptotic protein Bax and the activity of caspase-7 have each been shown to be upregulated by statins (Agarwal *et al.* 1999. Marcelli *et al.* 1998).

Perillyl alcohol is a monocyclic monoterpene which is used in breast cancer research and therapy which has been shown to cause a complete regression in mammary carcinoma without adversely affecting the host (Shi, W. & Gould, M.N., 1995. Ren *et al.,* 1997). Success has also been seen in colon cancer research with perillyl alcohol (Bardon *et al.,* 2002*)*. Perillyl alcohol is in a class of compounds called isoprenoids which also include geraniol and farnesol. These isoprenoid compounds have been shown to inhibit angiogenesis and increase sensitization to radiation (Loutari *et al.,* 2004 & Samaila *et al.*, 2004). Perillyl alcohol can be found in ginger, lemongrass, cherries, mint, lavender, sage, tea leaves, and vegetables (Garcia *et al.*, 2010, Skou, J.C., 1998, & Wiseman *et al.*, 2007). This compound, like mevinolin, also works within the cholesterol synthesis pathway, but does so in a few different ways. One way is by activating allyl phosphatase which is an enzyme responsible for synthesizing farnesol. Farnesol is the transcriptional down-regulator for the synthesis of the same HMG CoA reductase enzyme that mevinolin inhibits. Monoterpenes can inhibit the conversion of latherosterol to cholesterol as well as prevent the synthesis of ubiquinone and the isoprenylation of 20-30 kDa proteins as

well by inhibiting type I geranylgeranyl-protein transferase and farnesyl-protein transferase (Ren, Z. and Gould, M.N., 2004 & Bardon, S. *et al.,* 2002*)*. Protein isoprenylation is a post transcriptional modification of proteins within cells (Ren, Z. *et al.,* 1997). Therefore, inhibitors of protein isoprenylation such as compounds like perillyl alcohol which block geranylgeranyl-protein transferase, the enzyme responsible for modifications to Rac and Rho, as well as block farnesyl-protein transferase, the enzyme responsible for modification of Ras, are being studied as potential cancer therapies (Ren, Z. et al., 1997). Ubiquinone synthesis has been shown to be reduced by 86% by 1mM perillyl alcohol while cholesterol synthesis is reduced by 74% (Ren, Z. and Gould, M.N., 1994). Na/K ATPase pumps have been shown to be inhibited by perillyl alcohol in a concentration dependent manner (Garcia, D.G. *et al.,* 2010*)*. The phosphorylation of extracellular signal regulated kinase (ERK) has been shown to be inhibited by perillyl alcohol at 1mM after as little as 10 minutes suggesting a mechanism for this compound to lead to cell apoptosis (de Saldanha da Gama Fischer, J. *et al.,* 2010*)*. As such, perillyl alcohol has been shown to upregulate levels of proapoptotic proteins BCL-2 and Bak in a concentration dependent manner (Garcia, D.G. *et al.*, 2010 & Burke, Y., *et al.,* 2002).

Chemicals causing differentiation in Neuro-2A cells have been shown to be associated with increased intracellular concentrations of cAMP or will activate a cAMP-driven pathway (Prasad, K.N., 1991). Intracellular levels of cAMP can be induced by stimulating ß2 adrenergic receptors which stimulates the production of cAMP or by using phosphodiesterase inhibitors which will inhibit the break down of

cAMP (Arnold, D.E *et al.,* 2010). An increase in intracellular cAMP has been shown to activate protein kinase A and thus lead to intracellular phosphorylation events such as phosphorylating nuclear transcription factors to transcribe for specific genes. This occurs through the binding of these factors to the cAMP response element which is found inside the promoter region of the these specific genes (Cho-Chung, Y.S. *et al.,* 2002*)*. The CRE binding protein, the CRE modulator, and the activating transcription factor I are each phosphorylated by protein kinase A. Activators of CRE binding protein have been shown to control cell survival, growth, and differentiation. Nerve growth factor-dependent survival of neurons and the synthesis of the enzyme tyrosine hydroxylase used to create dopamine rely on the binding of the DNA by the CRE binding protein (Long, F. *et al.,* 2001 & Lewis-Tuffin, L.J. *et al.* 2004*)*. Differentiation of cells has been shown with the treatment of cells with cAMP (Dimitroulakos, J. *et al.,* 2004 & Lipskaia, L. *et al.* 1998*)*. Individually, 0-10μM lovastatin and 1mM dbcAMP has been shown to increase expression of proteins driven by the CRE promoter by five to ten fold respectively. However, when used in combination, the expression increases by forty fold (Arnold, D.E. *et al.*, 2010).

Ceramide is thought to be an intracellular mediator in neural cell differentiation due to an increase in this molecule when cells are administered known differentiation inducing compounds such as retinoic acid resulting in neurite outgrowths and freezing the cells in G0 (Riboni, L. *et al.,* 1995; Tettamanti, G. *et al.*, 1996; Dobrowsky, R.T., *et al.*, 1994). Serine/threonine phosphatases have been shown to be part of the differentiation process mediated by ceramide (Prinetti, A., *et*

al., 1997*)*. Studies have shown that administering ceramide inhibitors will decrease the differentiating ability of known differentiaton inducing compounds such as retinoic acid. Sphingolipid and sphingoid molecules are suggested to be involved in neural development regulation (Riboni, L. *et al.*, 1995). Ceramide levels have been shown to increase in differentiating cells, maintain at a high level when in the differentiated state, then decrease when dedifferentiating. Ceramide increases in differentiating cells via ceramide synthesis and sphingomyelin degradation (Tettamanti, G. and Riboni, L., 1994).

All-trans retinoic acid is known to bind the retinoic acid and retinoid X receptors with high affinity (Allegretto, E.A. *et al.*, 1993) to induce differentiation in leukemia, cervical, melanoma, and breast cancer cells (Kizaki, M. *et al.*, 1993; Sakashita, A. *et al.*, 1993; Kizaki, M. *et al.*, 1994; Anzano, M.A., *et al.*, 1994; Anzano, M.A., *et al.*, 1996; Gottardis, M.M., *et al.*, 1996; Lotan, R., 1980) mediated through nuclear receptors (Irving, H. *et al.*, 1998). Time lapse studies of the retinoic acid treatment of neuroblastoma cells have shown large numbers of neurite outgrowths in less than 48 hours. This differentiated morphology remained even after the removal of the drug (Lotan, R., 1980). Retinoic acid's differentiation has been shown to occur through Skp2's, an F-box protein, degradation and an increase in p27, a cyclin-dependent kinase inhibitor (Sidell, N., 1982). Accumulation of Cdh1 was found in the nucleus of the retinoic acid treated cells which corresponded with elevated cytosolic levels of expressed p27 and degradation of Skp2. Retinoic acid was shown to downregulate the nuclear transport factor, Rae1. Rae1 modulates

APC^{Cdh1} activity during mitosis. Therefore, this downregulation of Rae1 leads to APC^{Cdh1}'s breakdown of Skp2 leading to differentiation. (Cuende, J. *et al.*, 2008). Cyclooxygenase-1 and cyclooxygenase-2 are normally found in neuronal cells (Schneider, N. *et al.*, 2001). Cycloxygenase-1 has been shown to be involved in the autonomic functions in the ovine brain (Breder, C.D. *et al.*, 1992). Cyclooxygenase-1 was found to be localized to hippocampal neurons, some pyramidal neurons, and neocortex neurons in humans (Yermakova, A. *et al.*, 1999). It has also been shown to be upregulated by retinoic acid in differentiated neuroblastoma cells. Retinoic acid combined with dexamethasone showed even higher levels of upregulation of cyclooxygenase-1 than retinoic acid alone (Schneider, N. *et al.*, 2001). Retinoic acid has also been shown to induce apoptosis (Sidell, N., 1982; Lotan, R., 1980).

Murine neuroblastoma cell cultures were treated with compounds shown by the literature to induce differentiation through different pathways and then compare the differentiation patterns noticed at different time intervals post-treatment (12 hours, 24 hours, and 72 hours). The hypothesis for the project is to not only to determine STAT protein expression, but to also find varying levels between each of the treatments in the differentiated neuroblastoma cells. Once STAT protein expression has been determined, future work can explore the activation of STAT proteins and the potential use of STAT protein activators to induce differentiation of neuroblastoma cells potentially leading to a clinically administered differentiation cancer therapy for the neuroblastoma.

Methods and Materials

Cell Culture

The cells used were PC-12 Pheochromocytoma rat Neuro-2A cells obtained from American Tissue Culture Collection (Manassas, VA). The media used for culturing was Mediatech-Cellgro's Dulbecco's modified Eagle's medium/Ham's F-12 50/50 with MEM vitamins, penicillin-streptomycin, and finally heat inactivated fetal bovine serum from Atlanta Biologicals (Lawrenceville, GA) was added to make up 10% of the final volume (from here on referred to as 10% FBS media; recipe in appendix A). Mediatech-Cellgro's MEM vitamins, penicillin-streptomycin, and trypsin were obtained from Fisher Scientific (Pittsburgh, PA). The cells were grown as a monolayer in 75cm^2 vented angle neck flasks obtained from Fisher Scientific (Pittsburgh, PA) with humidified incubation of 37 °C and 5% CO2.

DMSO, ceramide, mevinolin, perillyl alcohol, and retinoic acid were obtained from Sigma-Aldrich (St. Louis, MO). Dibutyryl cAMP was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). All stock solutions of chemicals were diluted with Dulbecco's modified Eagle's medium/Ham's F-12 50/50 along with MEM vitamins plus penicillin-streptomycin was used for treatment solutions (0% FBS media) before being placed into the dishes. The mevinolin was dissolved in DMSO. This stock solution was stored at 4°C and cells were treated with it at a concentration of 10μM. The perillyl alcohol was used at a concentration of 2mM. The dibutyryl

cAMP was dissolved in 0% FBS media, stored at -20°C, and used at a concentration of 100mM. The ceramide was dissolved in DMSO, stored at -20°C, and used at a concentration of 25μM. The retinoic acid was dissolved in DMSO and the cell treatment used was 20μM. All of the work involving retinoic acid was performed in the dark, the dishes were covered in foil before placed into the incubator, and the stock solution conical was wrapped in foil before being stored at -20°C.

Morphometrics

N2A cells were examined for neurite outgrowths by using microscopy photographs. 1.5 x 10^4 cells were seeded per 33mm dish each containing 2ml of 10% FBS media and a glass cover slip. The cells were then allowed 24 hours to adhere to the cover slips before treating them with the compounds which will be added into the culture media already present in the dishes. After incubation with the appropriate compound, the media and drug was removed from the dish. The cover slip was removed and placed upside down on a glass microscope slide which could be viewed under an Olympus BX60 microscope. Digital photographs were taken with an Olympus DP71 camera mounted onto the Olympus BX60 microscope to allow for the counting of the number of differentiated cells from the total number of 200 cells per experimental group to arrive at a percentage of differentiation per treatment group. The definition of a differentiated cell in this instance will be a cell which possesses a neurite extension the length of two cell bodies or one cell with multiple neurite extensions of one cell body length.

Preparation of lysates

Western blot analysis was used to look at protein expression with these cells. Cell lysate creation was done by plating two 100x20mm dishes per treatment and allowing them to grow to eighty percent confluency before treating them with the various compounds which was typically 61 hours after plating. At 12, 24, and 48 hours post-treatment, the cells were washed twice in ice cold Cellgro phosphate-buffered saline which was obtained from Fisher Scientific. The cells were then harvested in the ice cold buffered saline by scraping the dishes using a rubber policeman and then transferring the solution to a microcentrifuge tube. The tubes were centrifuged at 10,000xg in a Hermle Labnet Z233MK refrigerated centrifuge and the supernatant was drawn off before freezing the cell pellet at -80°C. The cells were resuspended in a CelLytic M Cell Lysis buffer with Protease Inhibitor Cocktail each obtained from Sigma-Aldrich, sodium vanadate and a chelating agent, EDTA from Fisher Scientific, to protect the proteins once they are removed from the cells. The cell membranes were broken up by needling the cells in the microcentrifuge tube allowing the proteins to spill out into the buffer solution. These tubes were then centrifuged at 10,000xg again and the supernatant was kept as the cell lysates.

Protein assay

A Pierce BCA kit from Fisher Scientific was used to ensure equal protein loading between lanes on the polyacrylamide gels. The directions that came with the

kit were followed for mixing reagents, creating standards, and then samples were loaded in triplicates into a Falcon U-Bottom Tissue Culture 96-well microplate from Fisher Scientific which was then read on an Optimax Tunable Microplate Reader.

Gel electrophoresis

Protein samples were loaded at 50µg per lane along with either at 2x or 4x concentration of loading buffer (appendix A) which consisted of deionized water plus a solution of 20% sodium dodecyl sulfate solution, 0.5M Tris (ph 6.8) solution, and 100% glycerol all obtained from Fisher Scientific. Beta mercaptoethanol from Fisher Scientific was added to the buffer just prior to use then the samples were boiled for 5 minutes before being cooled and loaded into each of the wells. SDS PAGE was run using 7% polyacrylamide gels in 1x running buffer before transferring the proteins onto PVDF membranes. The polyacrylamide gels were mixed (recipe in appendix A) using deionized water, ultra pure protogel from National Diagnostics (Atlanta, GA), TEMED, sodium dodecyl sulfate, and ammonium persulfate from Fisher Scientific before being loaded into an Amersham Biosciences 15 Watt electrophoresis apparatus. An Amersham rainbow marker from Fisher Scientific was loaded into a designated lane at 10µL on each gel as the standard protein ladder for comparing protein sizes. Each gel was run at a constant 30 volts (18milliamps and 1 watt at the start) for 20 hours using a Consort EV265 power supply.

Western blotting

The proteins were transferred from the polyacrylamide gel to PVDF membranes obtained from Fisher Scientific through the use of a horizontal electrophoretic transfer using a Bio Rad Trans-Blot Cell with an EC250-90 power supply at a constant 75 volts for 3 hours. Before beginning this process, the PVDF membrane was soaked in methanol for 20 seconds then equalized in the transfer buffer (appendix A). These membranes were then stained with a pre-made Ponceau red solution, obtained from Fisher Scientific, and the gels were stained with Coomassie blue (appendix A) to ensure full protein transfer with the aforementioned horizontal electrophoretic transfer protocol. At this point, any unstained excess PVDF was cut away. First, the membranes were dried using 95% ethanol then the membranes were cut into upper and lower sections to allow two probings per blot. The upper portion was used for looking for STAT or neuronal proteins. The lower part of the membrane was used for looking at the load control of total protein so it was probed for ERK-1. The membranes had to be reactivated with methanol as done before the initial step of the western blotting procedure. These membranes which are now blotted with proteins and cut to specification were then blocked overnight at 4°C in a 4% dry milk 1x TBS-T solution (appendix A).

STAT3, STAT5A, STAT5B, and acetylcholinesterase primary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The NeuN primary antibody was obtained from Millipore (Billerica, MA). An antibody for mitogen

activated protein kinase, ERK1, was obtained from Santa Cruz Biotechnology (Santa Cruz, CA) to use as a loading control to ensure even loading of protein in each of the lanes of the gel. These primary antibodies were all used at a concentration of 1:1000 in 1% dry milk 1x TBS-T for 90 minutes on the rocker at room temperature. Three washes of 1X TBS-T were performed on the blots at 15 minutes each. The secondary antibodies were goat anti-mouse and goat anti-rabbit each conjugated with a horse radish peroxidase enzyme were obtained from Jackson Immuno (West Grove, PA). The secondary antibodies were used at a concentration of 1:10,000 in 1% dry milk 1x TBS-T for 90 minutes on the rocker at room temperature. Three washes of 1X TBS-T were performed on the blots for 10 minutes each. An ECL kit was obtained from Perkin Elmer (Waltham, MA) and used at 0.125ml per cm^2 of PVDF membrane in a half and half solution to visualize the antibody probes on the blot to look for specific banding with the use of a Bio Rad ChemiDoc MP Imaging System and Image Lab Software v 4.0. The blots were kept at -20°C for later strip and reprobes. Antibodies probing for the presence of STAT proteins and neuronal protein markers were used.

Stripping and reprobing blots

To save on materials, protein sample used, and time, a blot was stripped and reprobed then compared the image quality to that of a fresh blot. Once the method was proven effective as shown by the use of our imaging equipment, the blots which had been kept at -20°C were reprobed for other proteins. To accomplish this, first, the Ponceau red was allowed to sit on the blot with gentle agitation using the rocker at

room temperature for 15 minutes. The blot was then rinsed with deionized water at 30 second intervals with gentle manual agitation until all of the Ponceau had been rinsed away. The blot was then covered with 0.1M sodium hydroxide (appendix A) for 30 seconds with gentle manual agitation. The blot was then rinsed for 3 minutes with deionized water on the rocker. The blot was then ready to be taken into the blocking steps followed by probing and visualization.

Results

Morphometrics

N2A cells were counted and labelled as differentiated if they exhibited a neurite outgrowth of two cell body lengths or if the cell has multiple neurite outgrowths of a single body length and were then calculated as a differentiation percentage for each of the cell treatment groups. The percentages of differentiated cells were then compared between each of the treatment groups. In the 12 hour time study, the ceramide treatment group exhibited the least amount of differentiation at 9% while perillyl alcohol exhibited the greatest at 63%. Control cells had 13% differentiation, dibutyryl cAMP treated cells showed 42% differentiation, Mevinolin treated cells had 37% differentiation, and retinoic acid treated cells had 45%. In the 24 hour time study, the control group showed the least differentiation with 16% while perillyl alcohol exhibited the greatest again, but only 62% this time. The dibutyryl cAMP cells were 46% differentiated, the ceramide cells were 40% differentiated, the mevinolin cells were 58% differentiated, and the retinoic acid cells were 32%

differentiated. In the 48 hour study, mevinolin had the lowest percentage of differentiation at 0% because all cells were dead or in the process of dying whereas perillyl alcohol was again the highest differentiator, but this time at 68% differentiation. Control cells were 17% differentiated, dibutyryl cells were 60% differentiated, ceramide cells were 30% differentiated, and retinoic acid cells were 46% differentiated. The comparison of these differentiated percentages can be seen in table form in Table 1 & graphically in Chart 1. The morphometric examples of each of the treatment compound groups and their respective time frames can be found in Figure 1 through Figure 6.

Western Blotting

The western blot data can be seen in Figure 7 through Figure 14. STAT proteins of interest were found to be expressed in all treatments. Acetylcholinesterase was expressed in most treatments. Ceramide and mevinolin treatments failed to show expression in the 12 hour study where as retinoic acid and serum withdrawal failed to show expression in the 24 and 48 hour studies. NeuN was present in all treatments with only a few samples not showing all three of the expected NeuN protein bands while still containing at least one of them. In Figure 7, the 3 bands of interest seem to be nearly the same expression levels regardless of the treatment used. In Figure 8, the 10μM mevinolin 48 hour lane has a fainter 66kD band compared to others and is missing the 48kD band. The rest of the samples look the same in terms of expression of the 66kD band. The 48kD and 46kD bands show differences in concentration

between treatments. Figure 9 shows that the 10μM mevinolin 12 hour lane is missing the 82kD band, the 25μM ceramide lane as well as the control lane are showing faint 82kD bands, while the rest of the lanes are showing the band well. In Figure 10, the 24 hour control and 100mM dibutyryl cAMP lanes, the 82kD bands are very faint. The 24 hour 10μM mevinolin lane and the 48 hour 100mM dibutyryl cAMP lanes are missing the 82kD bands. Figures 11 and 12 illustrate that all lanes are expressing the 94 and 92kD bands. The 48 hour 10μM mevinolin lane had the most expression of the 94 and 92kD bands while the rest of the lanes were roughly the same. Figures 13 and 14 show that all lanes expressed the 91 and 86kD bands at roughly the same concentrations.

Treatments	Differentiated cell %
Control 12hr	13.8
Control 24hr	16.6
Control 48hr	17.8
cAMP 12hr	42.1
cAMP 24hr	46.4
cAMP 48hr	59.3
Ceramide 12hr	9.4
Ceramide 24hr	40.6
Ceramide 48hr	30.3
Mevinolin 12hr	37.5
Mevinolin 24hr	58.3
Mevinolin 48hr	0
Perillyl Alcohol 12hr	63.8
Perillyl Alcohol 24hr	62.4
Perillyl Alcohol 48hr	68.3
Retinoic Acid 12hr	45.9
Retinoic Acid 24hr	32
Retinoic Acid 48hr	46.5

Table 1. Cell treatments and differentiation percentages. N2A cells were treated with 100mM dibutyryl cAMP, 25μM ceramide, 10μM mevinolin, 2mM perillyl alcohol, and 20μM retinoic acid. The cells were counted at different time frames post treatment and a differentiation percentage was calculated for each.

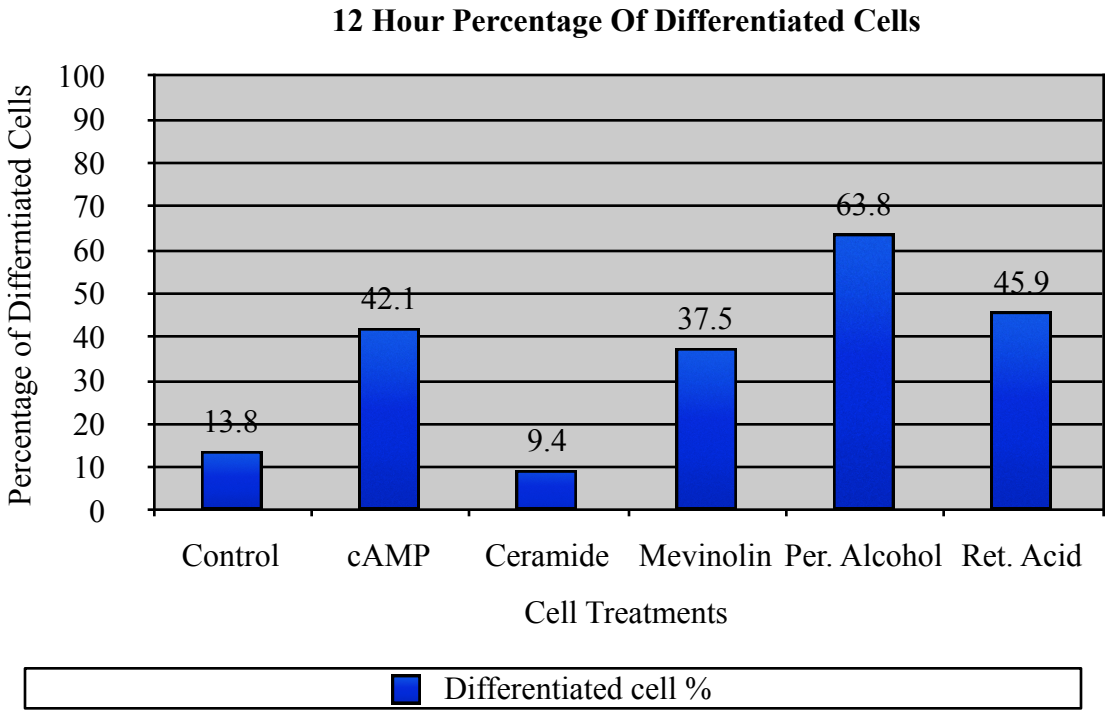

Chart 1. 12 hour differentiation graph. N2A cells were treated with 100mM dibutyryl cAMP, 25µM ceramide, 10µM mevinolin, 2mM perillyl alcohol, and 20µM retinoic acid. The cells were counted at 12 hours post treatment and a differentiation percentage was calculated for each. This color coded bar graph shows the differences in differentiation between each of the treatment groups during this time frame.

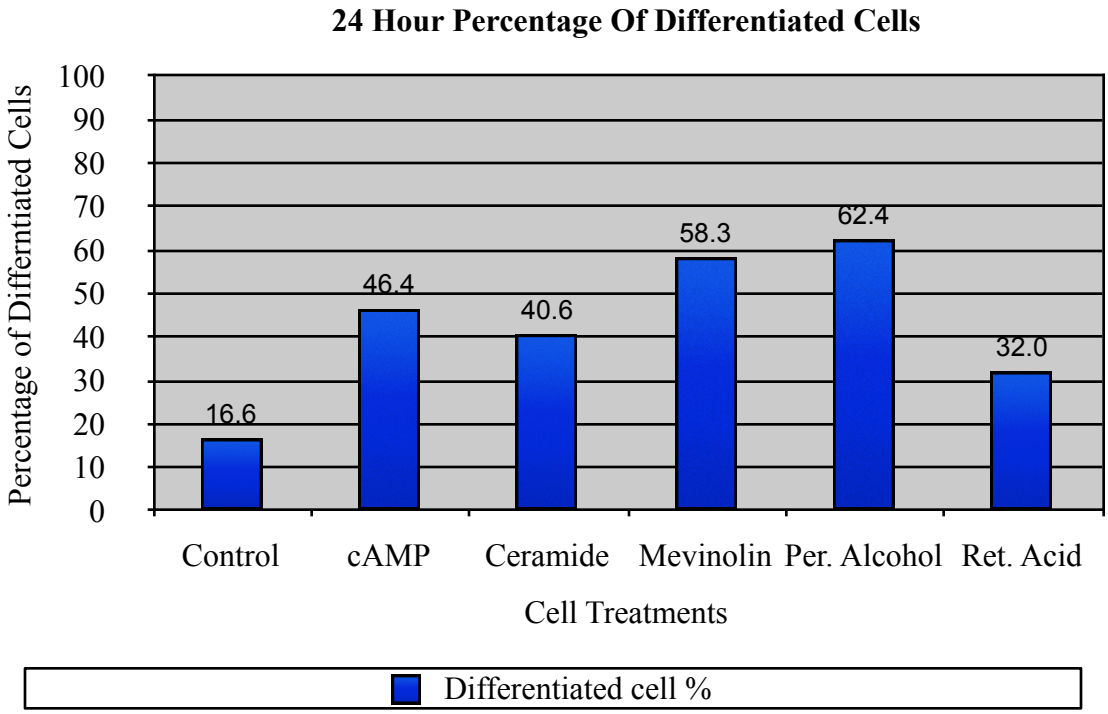

Chart 2. 24 hour differentiation graph. N2A cells were treated with 100mM dibutyryl cAMP, 25µM ceramide, 10µM mevinolin, 2mM perillyl alcohol, and 20µM retinoic acid. The cells were counted at 24 hours post treatment and a differentiation percentage was calculated for each. This color coded bar graph shows the differences in differentiation between each of the treatment groups during this time frame.

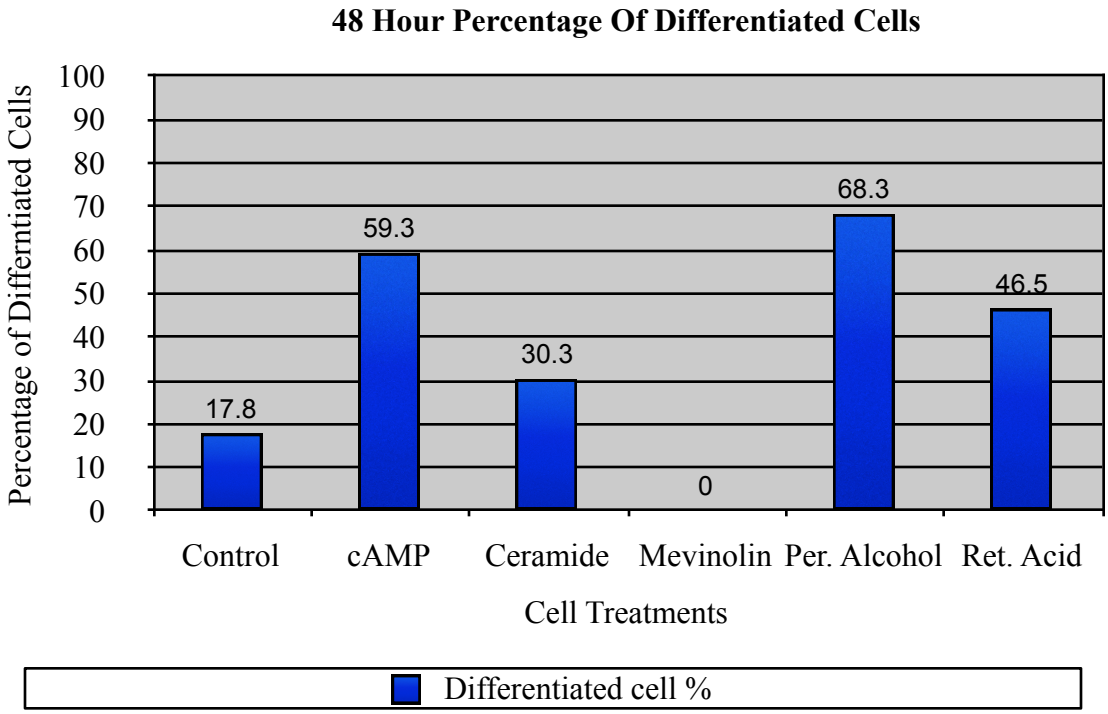

Chart 3. 48 hour differentiation graph. N2A cells were treated with 100mM dibutyryl cAMP, 25µM ceramide, 10µM mevinolin, 2mM perillyl alcohol, and 20µM retinoic acid. The cells were counted at 48 hours post treatment and a differentiation percentage was calculated for each. This color coded bar graph shows the differences in differentiation between each of the treatment groups during this time frame. ***10µM mevinolin at 48hrs is "0" due to no cell viability.

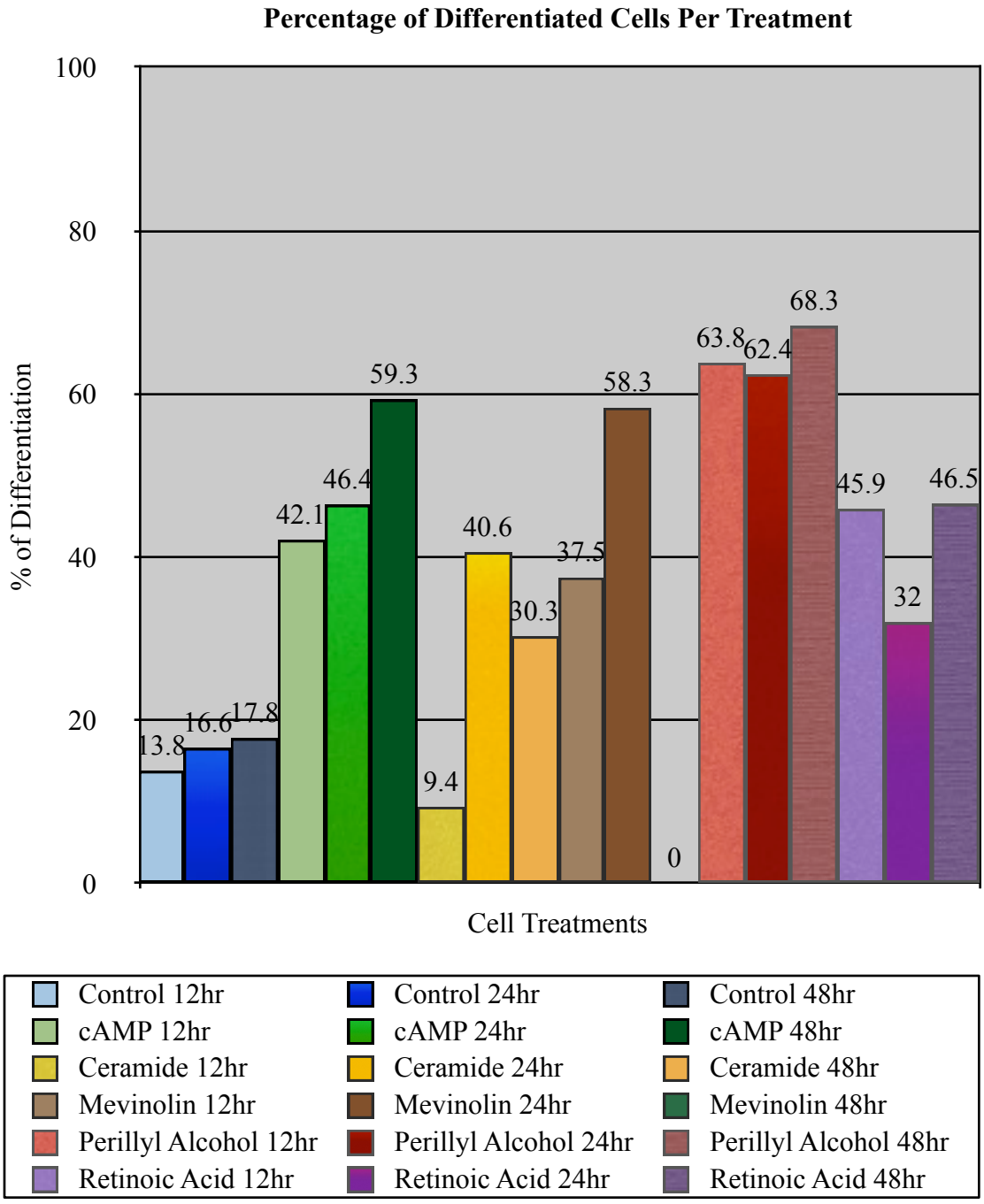

Chart 4. Total differentiation graph. N2A cells were treated with 100mM dibutyryl cAMP, 25µM ceramide, 10µM mevinolin, 2mM perillyl alcohol, and 20µM retinoic acid. The cells were counted at different time frames post treatment and a differentiation percentage was calculated for each. This color coded bar graph shows the differences in differentiation between each of the treatment groups. ***10µM mevinolin at 48hrs is "0" due to no cell viability.

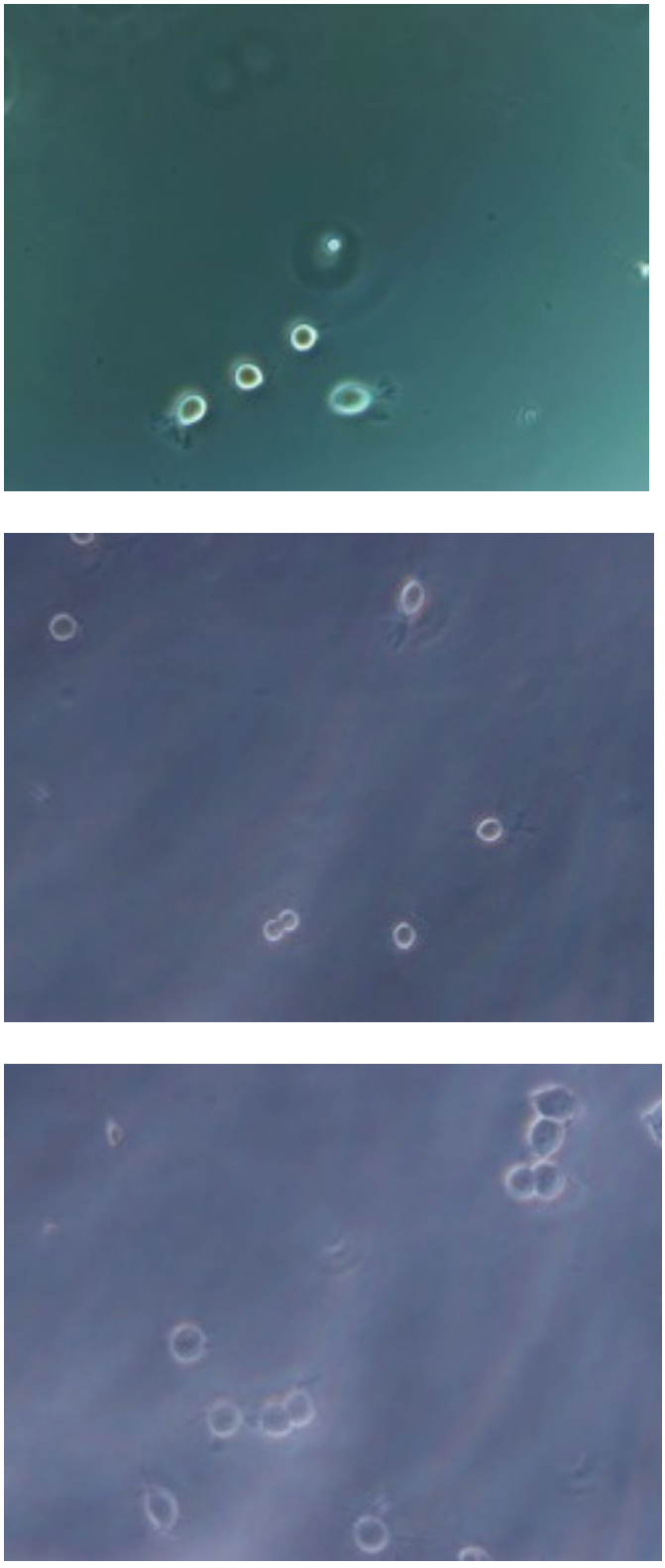

Figure 1. Control. Control group of N2A cells at 200x magnification various time points - 12hr (top), 24hr (middle), & 48hr (bottom).

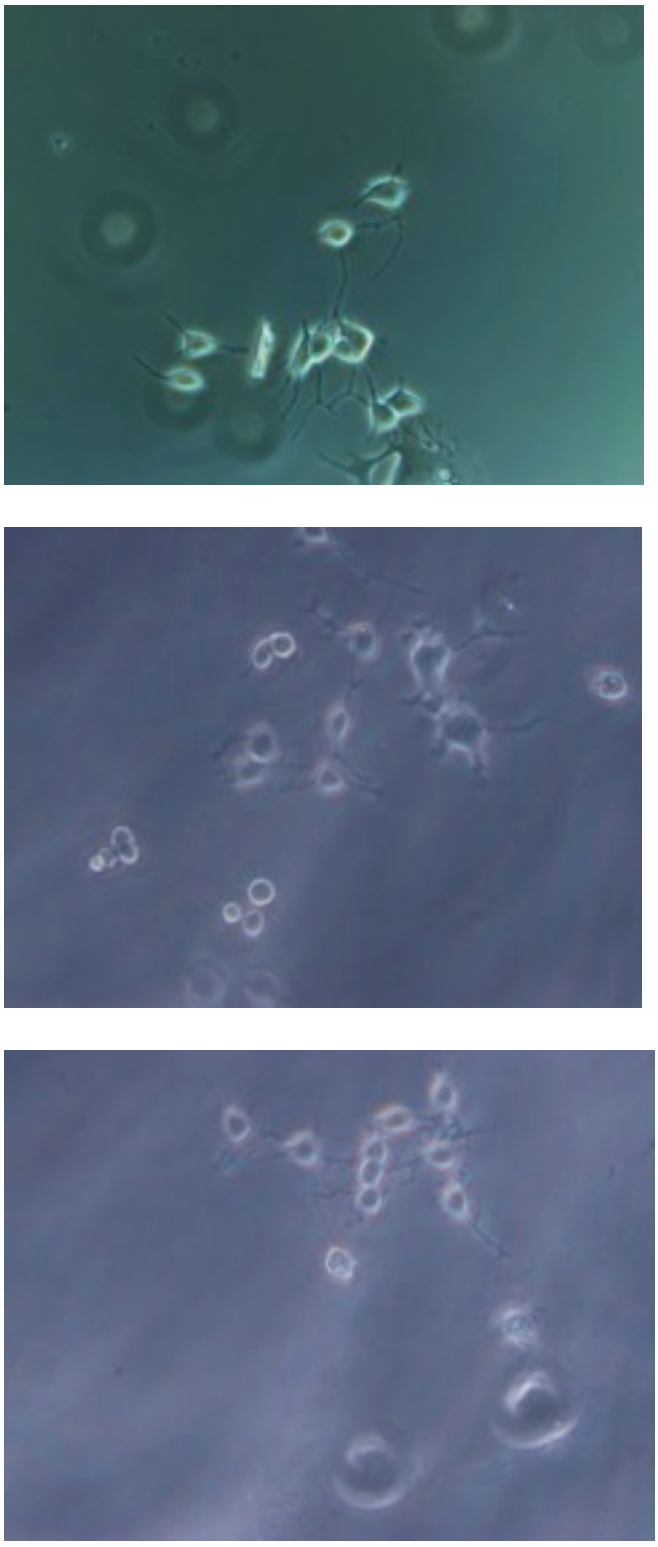

Figure 2. cAMP. 100mM dibutyryl treatment group of N2A cells at 200x magnification at various time points - 12hr (top), 24hr (middle), & 48hr (bottom).

Figure 3. Ceramide. 25μM ceramide treatment group of N2A cells at 200x magnification at various time points - 12hr (top), 24hr (middle), & 48hr (bottom).

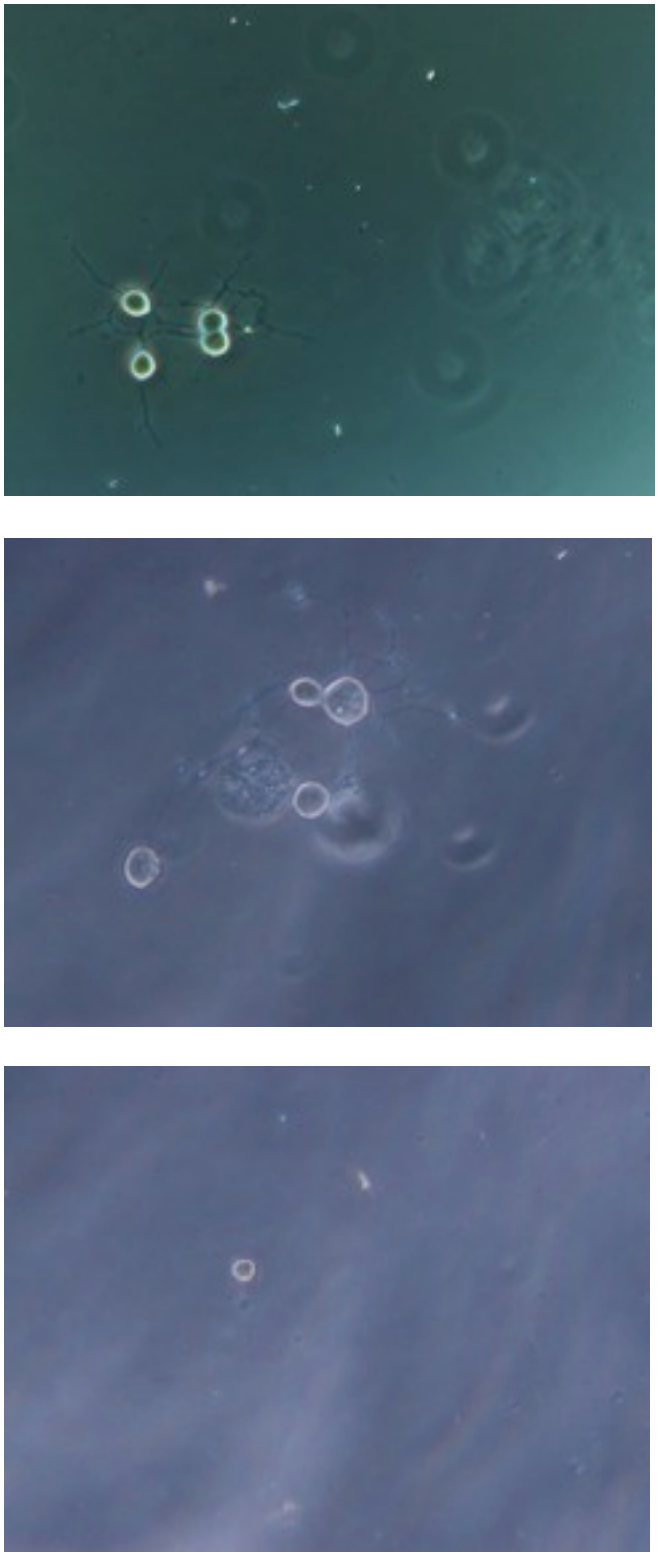

Figure 4. Mevinolin. 10μM mevinolin treatment group of N2A cells at 200x magnification at various time points - 12hr (top), 24hr (middle), & 48hr (bottom).

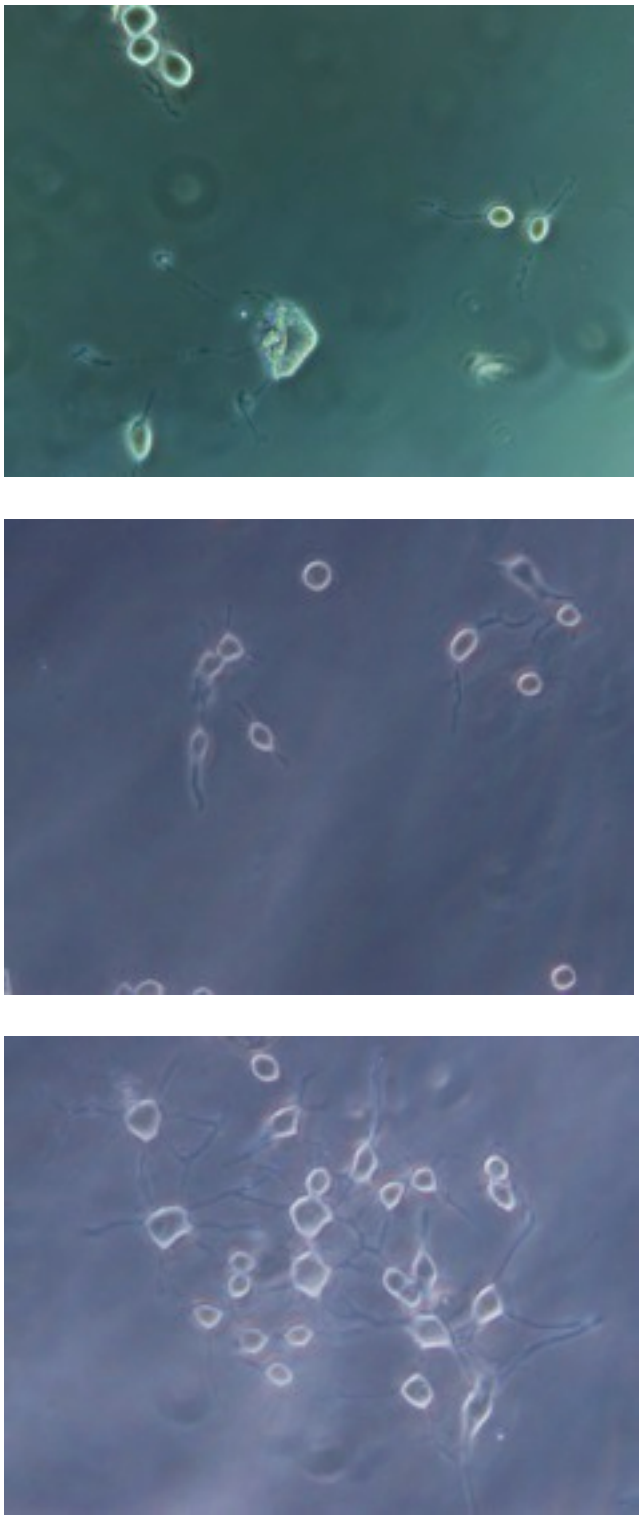

Figure 5. Perillyl alcohol. 2μM perillyl alcohol treatment group of N2A cells at 200x magnification at various time points - 12hr (top), 24hr (middle), & 48hr (bottom).

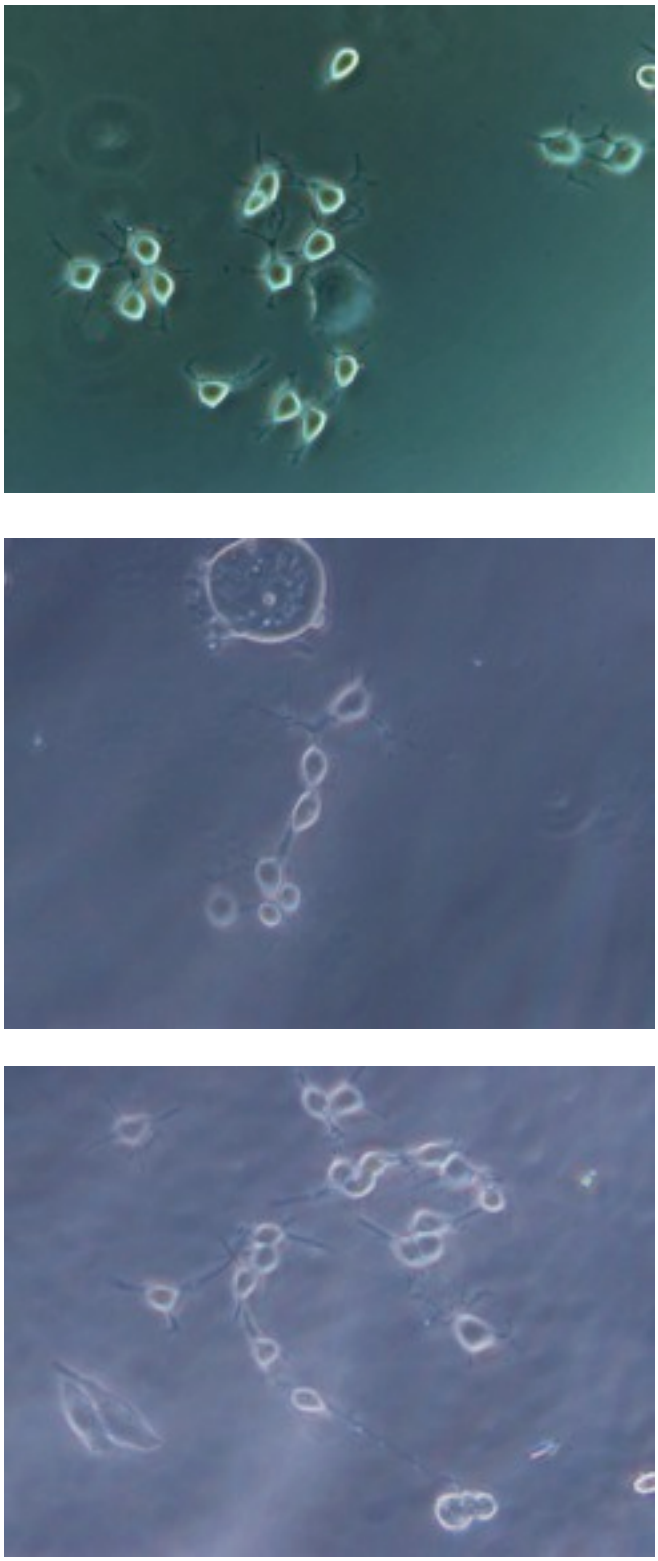

Figure 6. Retinoic acid. 20μM retinoic acid treatment group of N2A cells at 200x magnification at various time points - 12hr (top), 24hr (middle), & 48hr (bottom).

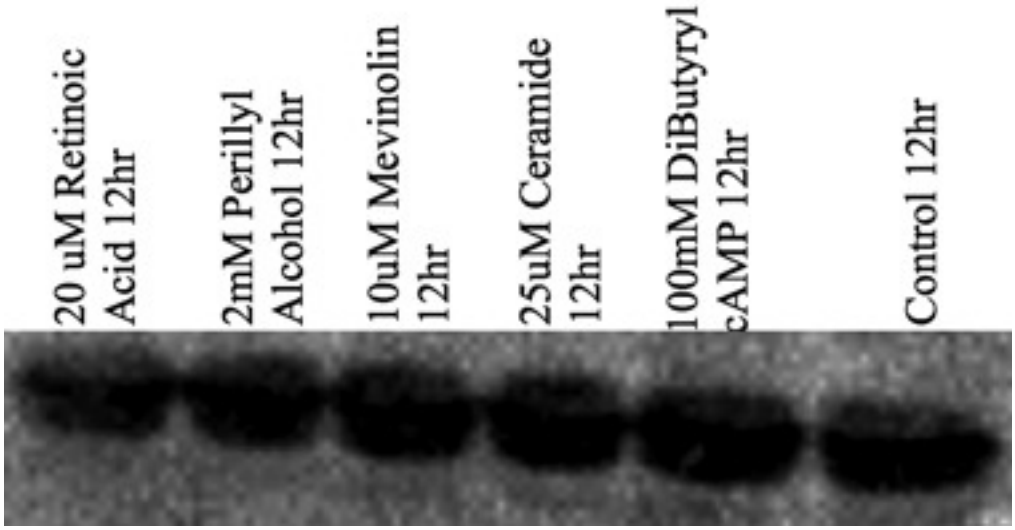

Figure 7. 12hr NeuN western blot. NeuN - 66kD, 48kD, and 46kD. The lanes are N2A samples with treatments as follows (left to right): 20µM Retinoic Acid 12hr, 2mM Perillyl Alcohol 12hr, 10µM Mevinolin 12hr, 25µM Ceramide 12hr, 100mM DiButyryl cAMP 12hr, and Control 12hr. All lanes showed the presence of the 66kD band of NeuN.

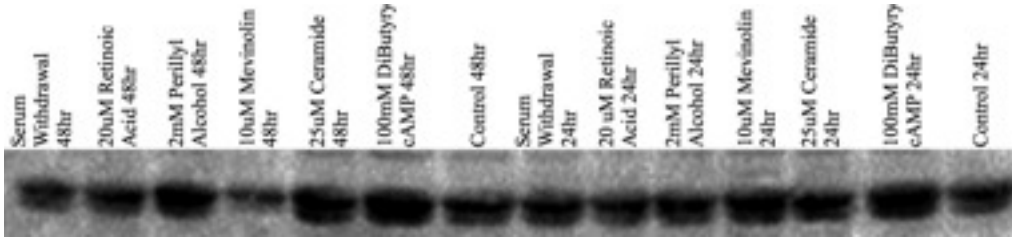

Figure 8. 24hr & 48hr NeuN western blot. NeuN - 66kD, 48kD, and 46kD. The lanes are N2A samples with treatments as follows (left to right): Serum Withdrawal 48hr, 20µM Retinoic Acid 48hr, 2mM Perillyl Alcohol 48hr, 10µM Mevinolin 48hr, 25µM Ceramide 48hr, 100mM DiButyryl cAMP 48hr, Control 48hr, Serum Withdrawal 24hr, 20µM Retinoic Acid 24hr, 2mM Perillyl Alcohol 24hr, 10µM Mevinolin 24hr, 25µM Ceramide 24hr, 100mM DiButyryl cAMP 24hr, and Control 24hr. All lanes are showing the 66kD band presence of NeuN.

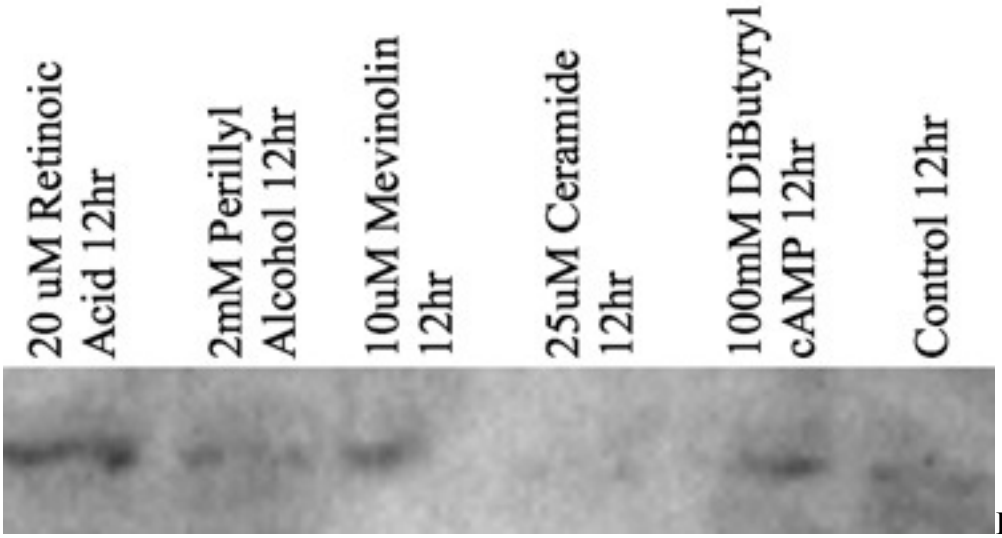

Figure 9. 12hr Acetylcholinesterase western blot. AcChE - 82kD. The lanes are N2A samples with treatments as follows (left to right): 20µM Retinoic Acid 12hr, 2mM Perillyl Alcohol 12hr, 10µM Mevinolin 12hr, 25µM Ceramide 12hr, 100mM DiButyryl cAMP 12hr, and Control 12hr. The 20µM Retinoic Acid 12hr lane is the only lane that definitively showed this band. The rest of the lanes showed some presence of this band with the exception of the 25µM Ceramide 12hr and Control 12hr lanes.

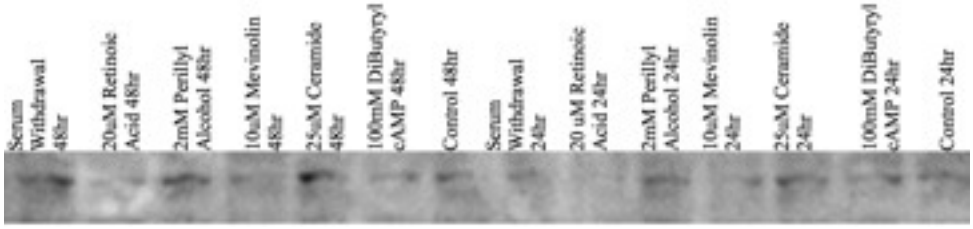

Figure 10. 24hr & 48hr Acetylcholinesterase western blot. AcChE - 82kD. The lanes are N2A samples with treatments as follows (left to right): Serum Withdrawal 48hr, 20 µM Retinoic Acid 48hr, 2mM Perillyl Alcohol 48hr, 10µM Mevinolin 48hr, 25µM Ceramide 48hr, 100mM DiButyryl cAMP 48hr, Control 48hr, Serum Withdrawal 24hr, 20µM Retinoic Acid 24hr, 2mM Perillyl Alcohol 24hr, 10µM Mevinolin 24hr, 25µM Ceramide 24hr, 100mM DiButyryl cAMP 24hr, and Control 24hr. Most lanes show a faded band with the exceptions of 20 µM Retinoic Acid 48hr, Serum Withdrawal 24hr, and 20 µM Retinoic Acid 24hr.

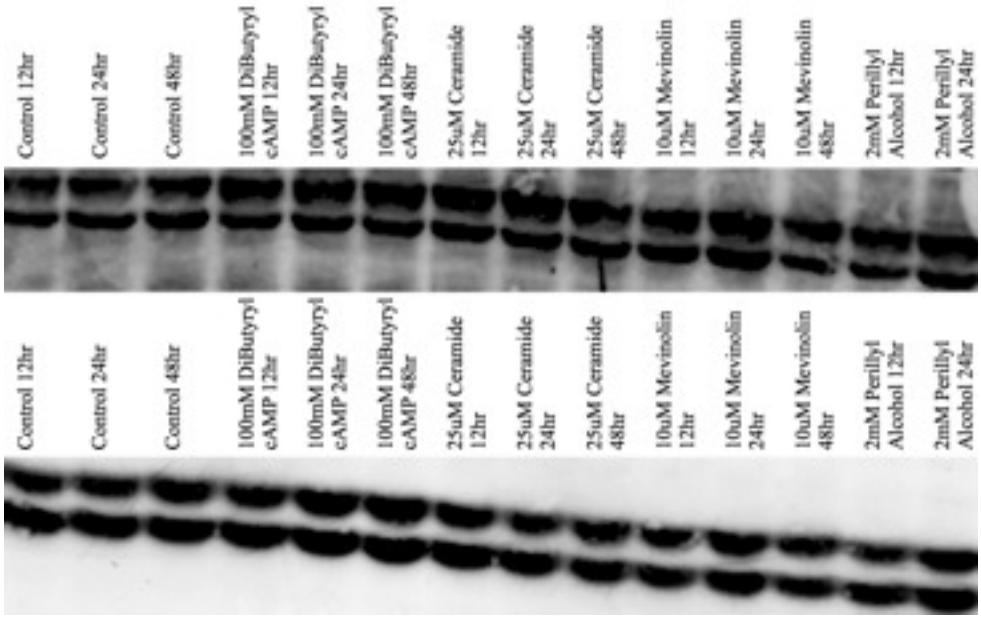

Figure 11. STAT5A & STAT5B western blot 1. STAT5A - 92kD. STAT5B-94kD. The lanes are N2A samples with treatments as follows (left to right): Control 12hr, Control 24hr, Control 48hr, 100mM DiButyryl cAMP 12hr, 100mM DiButyryl cAMP 24hr, 100mM DiButyryl cAMP 48hr, 25µM Ceramide 12hr, 25µM Ceramide 24hr, 25µM Ceramide 48hr, 10µM Mevinolin 12hr, 10µM Mevinolin 24hr, 10µM Mevinolin 48hr, 2mM Perillyl Alcohol 12hr, and 2mM Perillyl Alcohol 24hr. The twin dark bands above where the blot was cut are STAT5A & STAT5B expressed in every treatment group. The bottom bands below the cut are the loading control bands of ERK-1 at 44kD & 42kD.

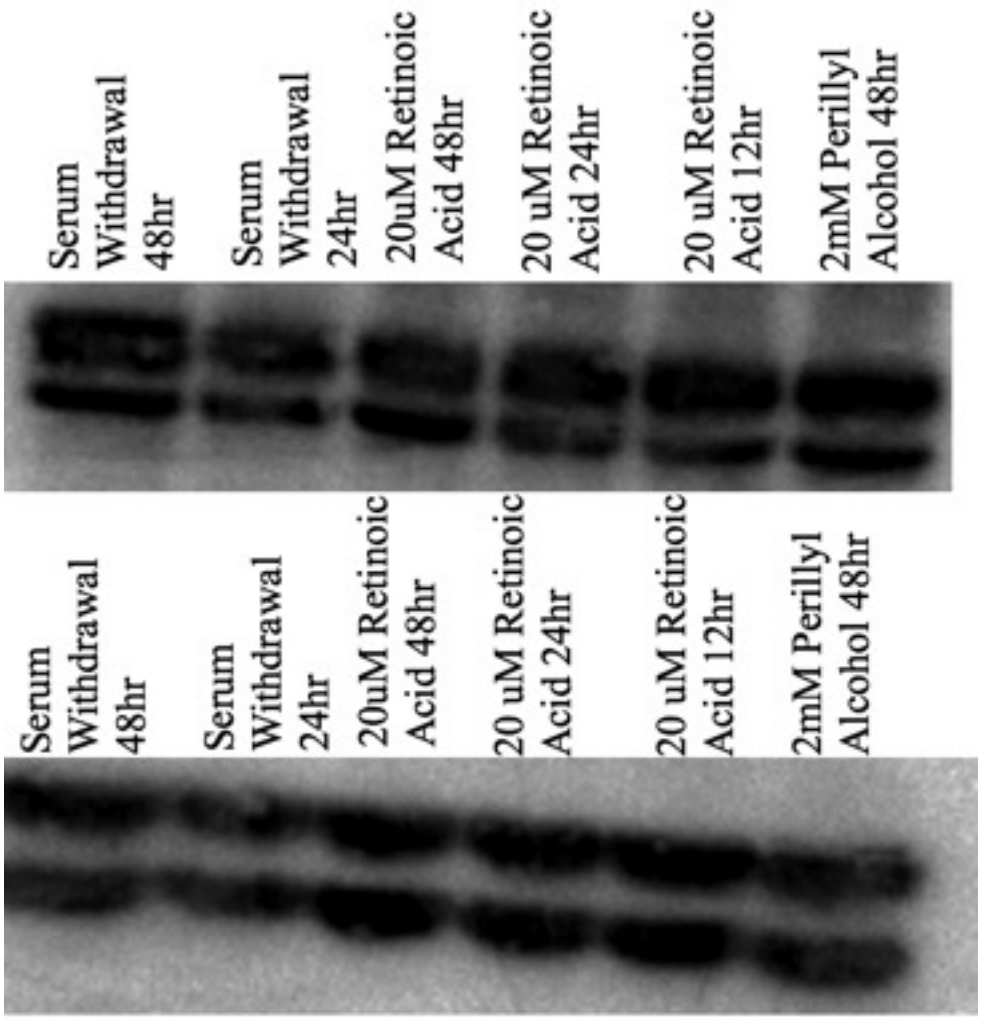

Figure 12. STAT 5A & STAT5B western blot 2. STAT5A - 92kD. STAT5B - 94kD. The lanes are N2A samples with treatments as follows (left to right): Serum Withdrawal 48hr, Serum Withdrawal 24hr, 20µM Retinoic Acid 48hr, 20µM Retinoic Acid 24hr, 20µM Retinoic Acid 12hr, and 2mM Perillyl Alcohol 48hr. The twin dark bands above where the blot was cut are STAT5A & STAT5B expressed in every treatment group. The bottom twin bands below the cut are the loading control bands of ERK-1 at 44kD & 42kD.

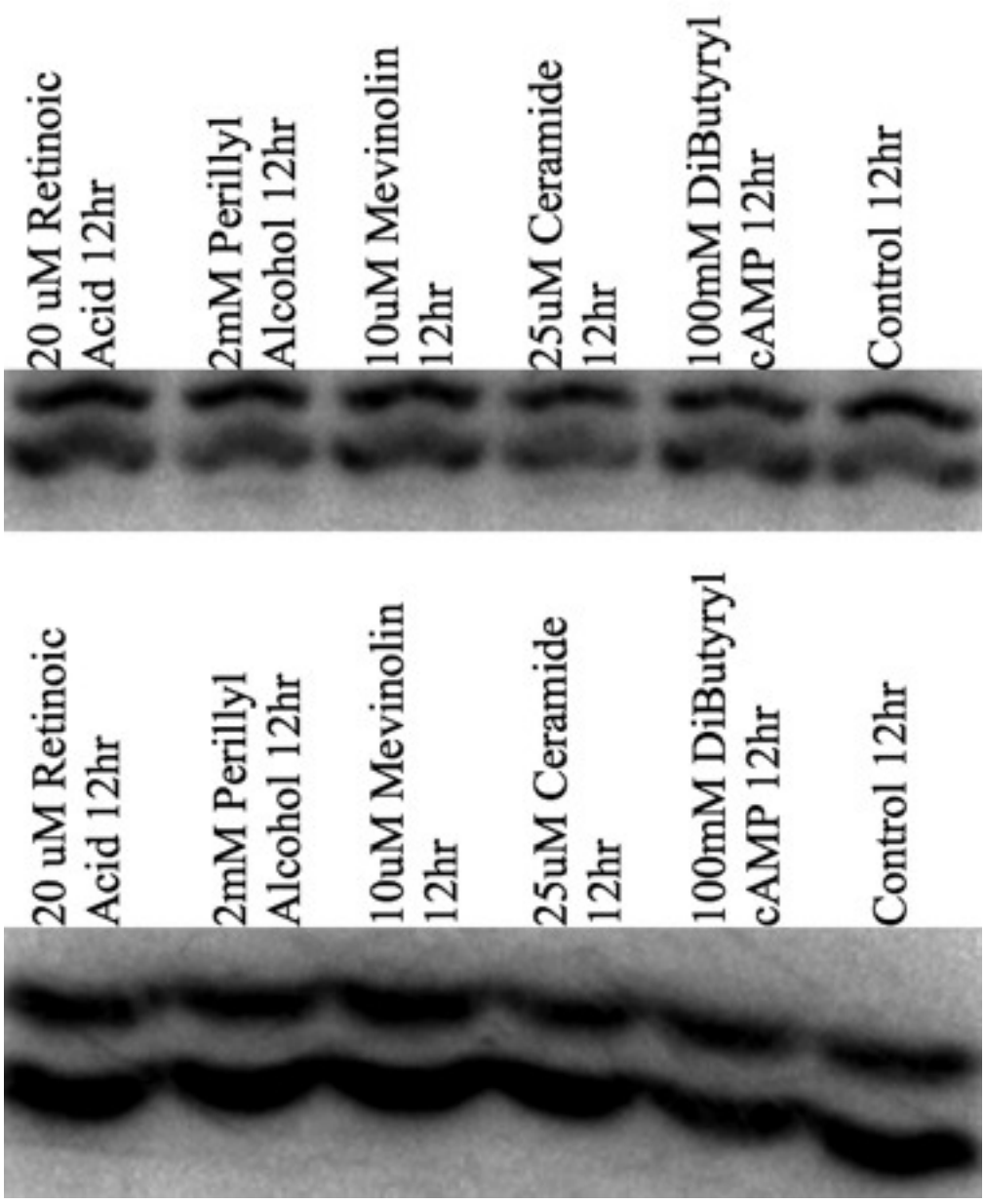

Figure 13. 12hr STAT 3 western blot. STAT3 Alpha- 91kD. STAT3 Beta - 86kD. The lanes are N2A samples with treatments as follows (left to right): Control 12hr, 100mM DiButyryl cAMP 12hr, 25µM Ceramide 12hr, 10µM Mevinolin 12hr, 2mM Perillyl Alcohol 12hr, and 20µM Retinoic Acid 12hr. The twin dark bands above where the blot was cut are STAT3alpha & STAT3beta expressed in every treatment group. The bottom twin bands below the cut are the loading control bands of ERK-1 at 44kD & 42kD.

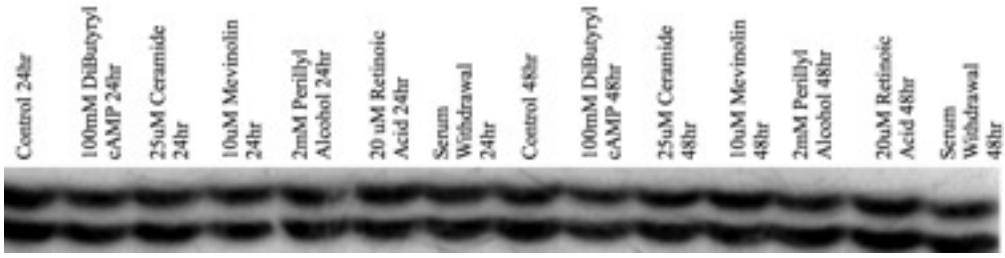

Figure 14. 24hr & 48hr STAT3 western blot. STAT3alpha - 91kD. STAT3beta - 86kD. The lanes are N2A samples with treatments as follows (left to right): Serum Withdrawal 48hr, 20µM Retinoic Acid 48hr, 2mM Perillyl Alcohol 48hr, 10µM Mevinolin 48hr, 25µM Ceramide 48hr, 100mM DiButyryl cAMP 48hr, Control 48hr, Serum Withdrawal 24hr, 20µM Retinoic Acid 24hr, 2mM Perillyl Alcohol 24hr, 10µM Mevinolin 24hr, 25µM Ceramide 24hr, 100mM DiButyryl cAMP 24hr, and Control 24hr. The twin dark bands above where the blot was cut are STAT3alpha & STAT3beta expressed in every treatment group. The bottom twin bands below the cut are the loading control bands of ERK-1 at 44kD & 42kD.

Discussion

In the morphometric study, the control cell level of differentiation seen in the experiment illustrates how neuroblastoma is one of the rare cancers with the ability to spontaneously regress into benign normal neuronal cells. The percentages of differentiated cells seen in the control groups increased for each of the time frames studied. This could be due to decreased amounts of cholesterol in the media over time due to cell utilization because in the experiment, it has been shown how cholesterol plays a role in cancer cell growth rather than growth arrest and terminal differentiation seen with the uses of the mevinolin and perillyl alcohol which disrupt the cholesterol biosynthesis pathway. The differentiation seen in the other treatment groups stayed nearly the same within their group between each of the time frames. The perillyl alcohol exhibited the highest percentage of differentiated cells. The mevinolin 48hr study group had zero percent differentiation due to the fact that the mevinolin is known to induce apoptosis and killed the majority of cells. The few cells in this treatment group that were still adhered were smaller than normal and rounded up as if they were already undergoing apoptosis as well. The 24hr retinoic acid group was over 10% lower than the 12hr and 48hr groups. This may have just been due to a bad series of photographs that were counted to establish the percentage.

In the western blots, the ERK-1 demonstrated even protein loading for gels showing that the BCA data used for loading 50μg of protein per lane was accurate. All of the STATs of interest were expressed in all treatments. However, it should be noted that some STAT bands were thicker than others when comparing between

STATs or between treatments looking at one particular STAT. STAT5B bands were thicker than STAT5A showing more expression of STAT5B, for example. This could mean that certain STATs are being activated more than others and thus the reason for their higher concentration. Mevinolin and retinoic acid treatments seem to cause differentiation based on morphometric analysis, but fail to express acetylcholinesterase at levels that one would expect. It was also interesting to see that NeuN was expressed strongly in all samples except for mevinolin at 48hr which was to be expected due to so much cell death compared to other treatments. NeuN like acetylcholinesterase is a definitive marker for terminal neural differentiation. Perhaps the antibody used was sensitive and was able to pick up even small traces of NeuN left behind from the small percentage of spontaneously differentiated cells in the control groups. As NeuN was the most scarce antibody for the project, different concentrations were not examined to try exclude the trace amounts of NeuN that were expressed in the control groups.

The literature has shown STAT5 activators have been found to induce 3T3-L1 cell differentiation into adipocytes (Stewart *et al.*, 2004). STAT5 activation has also been seen in A172 neural cells with granulocyte-macrophage colony-stimulating factor (Choi *et al.,* 2006). Future work may investigate STAT activation and cytoskeletal differences between the different treatments with these compounds. It would also be of interest to look at cyclooxygenase-1 in the treatments alongside NeuN and AcChE as a more definitive way of proving terminal neuronal differentiation. Also, it may be of interest to increase the concentration of ceramide

in future studies when looking at these proteins. It may not be worthwhile to look at mevinolin after 36 hours due to significant amounts apoptotic cellular death mevinolin induces. Something else that could be of interest with reference to mevinolin and cell death is that some of the literature has suggested that combining statins with cAMP gives great differences in protein expressions so perhaps using mevinolin and dibutyryl cAMP would give even a greater percentage of differentiation in terms of the morphometrics than any of the other treatments that were used in this study.

Conclusion

Studies such as this are needed for the development of a differentiation cancer therapy as an alternative treatment or an additional treatment to the methods already used in a clinical setting today. Using compounds such as the ones used in this study are a good start because these compounds can naturally be found in the body or even included as part of a nutritious diet in the case of perillyl alcohol. Future work looking at the activation of STAT proteins may prove a definitive mechanism for the differentiation induced therapy tested here through the usage of these compounds. From there, STAT activators could be used as a differentiation induced therapy.

References:

Agarwal, B., Bhendwal, S., Halmos, B., Moss, S.F., Ramey, W.G., Holt, P.R. 1999. Lovastatin augments apoptosis induced by chemotherapeutic agents in colon cancer cells. *Clin Cancer Res* 5: 2223-2229.

Allegretto, E.A., McClurg, M.R., Lazarchik, S.B., et al. 1993. Transactivation properties of retinoic acid and retinoid-X receptors in mammalian-cells and yeast-correlation with hormone-binding and effects of metabolism. *J Biol Chem* 268: 26625-26633.

Anzano, M.A., Byers, S.W., Smith, J.M., *et al.* 1994. Prevention of breast-cancer in the rat with 9-cis-retinoic acid as a single-agent and in combination with tamoxifen. *Cancer Res* 54: 4614-4617.

Anzano, M.A., Peer, C.W., Smith, J.M., *et al.* 1996. Chemoprevention of mammary carcinogenesis in the rat-combined use of raloxifene and 9-cis-retinoic acid. *J Natl Cancer Inst* 88:123-125.

Arnold, D.E. Gagne, C., Niknejad, N., McBurney, M.W., Dimitroulakos, J. 2010. Lovastatin induces neuronal differntiation and apoptosis of embryonal carcinoma and neuroblastoma cells: enhanced differentiation and apoptosis in combination with dbcAMP. *Mol Cell Biochem* 345:1-11.

Bardon, S., Foussard, V., Fournel, S., Loubat, A. 2002. Monoterpenes inhibit proliferation of human colon cancer cells by modulating cell cycle-related protein expression. *Cancer Letters* 2:187-194.

Blanco-Colio, L.M., Villa, A., Ortego, M., Hernandez-Presa, M.A., Pascual, A., Plaza, J.J., Egido, J. 2002. 3-Hydroxy-3-methyl-glutaryl coenzyme A reductase inhibitors, atorvastatin and simvastatin, induce apoptosis of vascular smooth muscle cells by downregulation of Bcl-2 expression and Rho A prenylation. *Atherosclerosis* 161: 17-26.

Blanco-Colio, L.M., Justo, P., Daehn, I. Lorz, C., Ortiz, A., Egido, J. 2003. Bcl-XL overexpression protects from apoptosis induced by HMG-CoA reductase inhibitors in murine tubular cells. *Kidney Int* 64:181-191.

Breder, C.D., Smith, W.L., Raz, A., Masferrer, J., Seibert, K., Needleman, P., Saper, C.B. 1992. The distribution and characterization of cycloxygenase-like immunoreactivity in the ovine brain. *J Comp Neurol* 337:543-567.

Brodeur, G.M. 2003. Neuroblastoma: Biological Insights Into A Clinical Enigma. *Nature Reviews Cancer* 3:203-216.

Burke, Y. Ayoubi, A.S., Werner, S.R., McFarland, B., Heilman, D.K., Ruggeri, B.A., Crowell, P.L. 2002. Effects of the isoprenoids perillyl alcohol and farnesol on apoptosis biomarkers in pancreatic cancer chemoprevention. *Anticancer Research* 22: 3127-3134.

Cho-Chung, Y.S., Nesterova, M., Becker, K.G., Srivastava, R., Park, Y.G., Lee, Y.N., Cho, Y.S., Kim, M.K., Neary, C., Cheadle, C. 2002. Dissecting the circuitry of protein kinase A and cAMP signaling in cancer genesis: antisense, microarray, gene overexpression, and transcription factor decoy. *Ann NY Acad Sci* 968: 22-36.

Choi, J.K., Byung, C.H., Yoon, H., Hyeonseon, P., Yoon, S.H., Park, H.C., and Park, S.R. 2007. Signal transduction pathways of GM-CSF in neural cell lines. *Neuroscience Letters* 420(3): 217-222.

Corsini, A., Maggi, F.M. Catapano, A.L. 1995. Pharmacology of competitive inhibitors of HMG-CoA reductase. *Pharmacol Res* 31: 9-27.

Cuende, J., Moreno, S., Bolaños, J.P., Almeida, A. 2008. Retinoic acid downregulates Rae1 leading to APC^{Cdh1} activation and neuroblastoma SH-SY5Y differentiation. *Oncogene* 27: 3339-3344.

de Saldanha da Gama Fischer, J., Liao, L., Carvalho, P.C., Barbosa, V.C., Domont, G.B., da Gloria da Costa Carvalho, M., Yates III, J.R. 2010. Dynamic proteomic overview of glioblastoma cells (A172) exposed to perillyl alcohol. *Journal of Proteomics* 73:1018-1027.

Dimitroulakos, J., Squire, J., Pawlin, G., Yeger, H. 1994. NUB-7: a stable I-type human neuroblastoma cell line inducible along N- and S-type cell lineages. *Cell Growth Differ* 5:373-384.

Dimitroulakos, J. Thai, S., Wasfy, G.H., Hedley, D.W., Minden, M.D., Penn, L.Z. 2000. Lovastatin induces a pronounced differentiation response in acute myeloid leukemias. *Leuk Lymphoma* 40: 167-178.

Dobrowsky, R. T., Werner, M.H., Castellino, A.M., Chao, M.V. and Hannum, Y.A. 1994. Activation of sphingomyelin cycle through low-affinity neurotrophin receptor. *Science* 265: 1596-1599.

Evangelopoulos, M.E., Weis, J., and Kruttgen, A. 2005. Signalling pathways leading to neuroblastoma differentiation after serum withdrawal: HDL blocks neuroblastoma differentiation by inhibition of EGFR. *Oncogene* 24: 3309-3318.

Evangelopoulos, M.E., Weis, J., and Krutten, A. 2009. Mevastatin-induced neurite outgrowth of neuroblastoma cells via activation of EGFR. *Journal of Neuroscience Research* 87: 2138-2144.

Garcia-Roman, N., Alvarez, A.M., Toro, M.J., Montes, A., Lorenzo, M.J. 2001. Lovastatin induces apoptosis of spontaneously immortalized rat brain neuroblasts: involvement of nonsterol isoprenoid biosynthesis inhibition. *Mol Cell Neurosci* 17: 329-341.

Garcia, D.G., Amorim, L.M.F., de Castro Faria, M.V., Freire, A.S., Santelli, R.E., Da Fonseca, C.O., Quirico-Santos, T., Burth, P. 2010. The anticancer drug perillyl alcohol is a Na/K-ATPase inhibitor. *Mol Cell Biochem* 345: 29-34.

Gottardis, M.M., Lamph, W.W., Shalinsky, D.R., Wellstein, A., and Heyman, R.A. 1996. The efficacy of 9-cis retinoic acid in experimental models of cancer. *Breast Cancer Research and Treatment* 38: 85-96.

Irving, H., Lovat, P.E., Campbell Hewson, Q., Malcolm, A.J., Pearson, A.D.J., and Redfern, C.P.F. 1998. Retinoid-induced Differentiation of Neuroblastoma: Comparison between LG69, an RXR-selective analogue and 9-cis Retinoic Acid. *European Journal of Cancer* 34(1):111-117.

Kizaki, M., Nakajima, H., Mori, S., *et al.* 1993. Effects of novel retinoic acid compounds, 9-cis-retinoic acid, on proliferation, differentiation, and expression of retinoic acid receptor-alpha and retinoid-X receptor-alpha RNA by H1-60 cells. *Blood* 82: 3592-3599.

Kizaki, M., Nakajima, H., Mori, S., *et al.* 1994. Novel retinoic acid, 9-cis-retinoic acid, in combination with all-trans-retinoic acid is an effective inducer of differentiation of retinoic acid-resistant H1-60 cells. *Blood* 83:3289-3297.

Lewis-Tuffin, L.J., Quinn, P.G., Chikaraishi, D.M. 2004. Tyrosine hydroxylase transcription depends primarily on cAMP response element activity, regardless of the type of inducing stimulus. *Mol Cell Neurosci* 25: 536-547.

Lipskaia, L., Grepin, C., Defer, N., Hanoune, J. 1998. Adenylyl cyclase activity and gene expression during mesodermal differentiation of the P19 embryonal carcinoma cells *J Cell Physiol* 176: 50-56.

Long, F., Schipani, E., Asahara, H., Kronenberg, H., Montminy, M. 2001. The CREB family of activators is required for endochondral bone development. Development 128: 541-550.

Lotan, R. 1980. Effects of vitamin A and its analogs (retinoids) on normal and neoplastic cells. *Biochem Biophys Acta* 605:31-91.

Loutari, H. Hatziapostolou, M., Skouridou, V., Papadimitriou, E., Roussos, C, Kolisis, F.N., Papapetropoulos, A. 2004. Perillyl alcohol is an angiogenesis inhibitor. *Journal of Pharmacol Exp. Ther* 311: 568-575.

Maltese, W.A. 1984. Induction of differentiation in murine neuroblastoma cells by mevinolin, a competitive inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase. *Biochemical and Biophysical Research Communications* 120: 454-460.

Maltese, W.A. and Sheridan, K.M. 1985. Differentiation of neuroblastoma cells by mevinolin, a competitive inhibitor of mevalonate synthesis: relation of neurite outgrowth and acetylcholinesterase activity to changes in cell proliferation and blocked isoprenoid synthesis. *Journal of Cellular Physiology* 125: 540-558.

Marcelli, M., Cunningham, G.R., Haidacher, S.J., Padayatty, S.J., Sturgis, L., Kagan, C., and Denner, L. 1998. Caspase-7 is activated during lovastatin-induced apoptosis of the prostate cancer cell line LNCaP. *Cancer Res* 58: 76-83.

Mo, H., Elson, C.E. 2006. Isoprenoids and novel inhibitors of mevalonate pathway activities. Edited by Heber, D., Blackburn, G.L., Go, V. L. G., and Milner, J. Nutritional Oncology. Waltham, MA: Academic Press. 629-644.

Park, W.H., Lee, Y.Y., Kim, E.S., Seol, J.G., Jung, C.W., Lee, C.C., Kim, B.K. 1999. Lovastatin-induced inhibition of HL-60 cell proliferation via cell cycle arrest and apoptosis. *Anticancer Res* 19: 3133-3140.

Ponzoni, M., Bocca, P., Chiesa, V., Decensi, A., Raffaghello, L, Rosso, C., Montaldo, P.G. 1995. Differential effects of N-(4-Hydroxyphenyl)retinamide and retinoic acid on neuroblastoma cells: appoptosis vs differentiation. *Cancer Research* 55: 853-861.

Prasad, K.N. 1991. Differentiation of neuroblastoma cells: a useful model for neurobiology and cancer. *Biological Reviews* 66: 431-451.

Prinetti, A., Bassi, R., Riboni, L., Tettamanti, G. 1997. Involvement of ceramide activated protein phosphatase in the differentiation of neuroblastoma Neuro2A cells. *FEBS Letters* 414: 475-479.

Rawlings, J.S., Rosler, K.M., Harrison, D.A. 2004. The JAK/STAT signaling pathway. *Journal of Cell Science* 117:1281-1293.

Ren, Z. and Gould, M.N. 1994. Inhibition of ubiquinone and cholesterol synthesis by the monoterpene perillyl alcohol. *Cancer Letters* 76: 185-190.

Ren, Z., Elson, C.E., Gould, M.N. 1997. Inhibition of Type I and Type II Geranylgeranyl-protein transferases by the monoterpene perillyl alcohol in NIH3T3 cells. *Biochemical Pharmacology* 54: 113-120.

Riboni, L., Prinetti, A., Bassi, R., Caminiti, A., Tettamanti, G. 1995. A mediator role of ceramide in the regulation of neuroblastoma neuro2a cell differentiation. J *Biochem Chemistry* 270 (45): 26868-26875.

Sakashita, A., Kizaki, M., Pakkala, S., *et al.* 1993. 9-cis-retinoic acid-effects on normal and leukemic hematopoiesis in vitro. *Blood* 81: 1009-1016.

Schneider, N., Lanz, S., Ramer, R., Schaefer, D., Goppelt-Struebe, M. 2001. Up-regulation of cycloxygenase-1 in neuroblastoma cell lines by retinoic acid and corticosteroids. *Journal of Neurochemistry* 77:416-424.

Samaila, D., Toy, B.J., Wang, R.C., Elegbede, J.A. 2004. Monoterpenes enhanced the sensitivity of head and neck cancer cells to radiation treatment in vitro. *Anticancer Res* 24: 3089-3095.

Shi, W. and Gould, M.N. 1995. Induction of differentiation in neuro-2A cells by the monoterpene perillyl alcohol. *Cancer Letters* 95: 1-6.

Sidell, N. 1982. Retinoic acid-induced growth inhibition and morphologic differentiation of human neuroblastoma cells in vitro. *Journal of the National Cancer Institute* 68(4):589-596.

Skou, J.C. 1998. The identification of the sodium pump. *Biosci Rep* 18: 155-169.

Stewart, W.C., Baugh, Jr., J.E., Floyd, Z.E., and Stephens, J.M. 2004. STAT 5 activators can replace the requirement of FBS in the adipogenesis of 3T3-L1 cells. *Biochemical and Biophysical Research Communications* 324: 355-359.

Stewart, W.C., Pearcy, L., Floyd, Z. E., and Stephens, J.M. 2011. STAT5A Expression in Swiss 3T3 Cells Promote Adipogenesis in Vivo in Athymic Mice Model System. *Obesity* 19 (9): 1731-1734.

Tettamanti, G. and Riboni, L. 1994. Gangliosides turnover and neural cell function: a new perspective. *Prog Brain Res* 101: 77-100

Tettamanti, G., Prinetti, A., Bassi, R., Viani, P., Giussani, P., and Riboni, L. 1996. Sphingoid bioregulators in the differentiation of cells of neural origin. *J Lipid Mediators Cell Signal* 14: 263-275.

Tong Cao, Boon Chin Heng. 2005. Differentiation therapy of cancer. Potential advantages over conventional therapeutic approaches targeting death of cancer/tumor cells. *Medical Hypotheses* 65(6):1202-1203.

White, U.A., Stewart, W.C., and Stephens. J.M. 2011. Gp130 Cytokines Exert Differential Patterns of Crosstalk in Adipocytes Both In Vitro and In Vivo. *Obesity* 19(5): 903-910.

Wiseman, D.A., Werner, S.R., Crowell, P.L. 2007. Cell cycle arrest by the isoprenoids perillyl alcohol, geraniol, and farnesol is mediated by $p21^{Cip1}$ and $p27^{Kip1}$ in human pancreatic adenocarcinoma cells. *The Journal of Pharmacology and Experimental Therapeutics* 320: 1163-1170.

Yermakova, A. V. Rollins, J., Callahan, L.M., Rogers, J., O'Banion, M.K. 1999. Cyclooxygenase-1 in human Alzheimer and control brain: quantitative analysis of expression by microglia and CA3 hippocampal neurons. *J Neuropathol Exp Neurol* 58: 1135-1146.

Appendix A:

Solutions, Buffers, and Media Used

Dulbecco's Modified Eagle's Medium/Ham's F-12 50/50 (10% FBS media)

500mL Dulbecco's modified Eagle's medium/Ham's F-12 50/50

5mL MEM vitamins

5mL Penicillin-Streptomycin antibiotics

55mL Heat inactivated fetal bovine serum (10% FBS by volume)

Dulbecco's Modified Eagle's Medium/Ham's F-12 50/50 (0% FBS media)

500mL Dulbecco's modified Eagle's medium/Ham's F-12 50/50

5mL MEM vitamins

5mL Penicillin-Streptomycin antibiotics

Freeze-down media cells were frozen in to begin with

80% Dulbecco's modified Eagle's medium/Ham's F-12 50/50 (10% FBS)

10% DMSO

Lysis Buffer Cocktail (per microcentrifuge tube of cells)

500uL Cell Lytic

5uL 100mM EDTA

5uL Protease Inhibitor Cocktail

1uL 0.2M Sodium Vanadate (3.7g in 100mL Deionized water)

10X Electrode Buffer (1 Liter)

30g Tris Base

144g Glycine

50mL 20% SDS solution (or 10 grams dry SDS)

Fill to 1000mL with deionized water

Store 2-8 degrees C.

1X Electrode Buffer (1 Liter)

100mL 10X Electrode Buffer

900mL Deionized water

Store 2-8 degrees C.

7.5% Polyacrylamide Resolving Gel (1 Gel)

7.5ml Protogel (Acrylamide Solution)

7.5ml 1.5M Tris@pH 8.8 solution

15mL Deionized water

36uL Temed

112.5uL Ammonium Persulfate

Stacking Gel (Tops 1 Gel)

1mL Protogel

2.5mL 0.5M Tris@pH 6.8

6.4mL Deionized water

50uL 20% SDS Solution

12uL Temed

37.5uL Ammonium Persulfate

2X Loading Buffer (25mL)

1.5mL 20% SDS

3.3mL 0.5M Tris (pH 6.8)

6.5mL 100% Glycerol

13.7mL Deionized Water

Beta Mercaptoethanol added to make up 2% of the final volume of aliquot to be used just before usage.

4X Loading Buffer (25mL)

2.25mL 20% SDS

5mL 0.5M Tris (pH 6.8)

10mL 100% Glycerol

7.75mL Deionized Water

Beta Mercaptoethanol added to make up 2% of the final volume of aliquot to be used just before usage.

10X TBS-T (1 Liter)

0.25M Tris pH 8.6 (30.3g Tris)

1.25M NaCl (78.05g NaCl)

pH solution to 8.0 before adding Tween

1% Tween (10mL Tween - using 1% due to being a 10X stock)

Store 2-8 degrees C

1X TBS-T (1 Liter)

100mL 10X TBS-T

900mL Deionized water

Store 2-8 degrees C

10X Transfer Buffer (1 Liter)

500mL Deionized water

29g Glycine

58.05g Tris Base

3.7g SDS powder (or 18.5mL 20% SDS solution)

Fill to 1,000mL with deionized water

Store 2-8 degrees C

1X Transfer Buffer (2 Liters as 20% methanol by volume solution)

200mL 10X Transfer Buffer

400mL Methanol

1,400mL Deionized water

Mix up the night before needed so make sure it is cold for usage

Store 2-8 degrees C

Dibutyryl cAMP (100mM stock)

50mg dibutyryl cAMP

1.2mL 0% FBS Media

Good for 4 weeks at 2-8 degrees C

Ceramide (2mM stock)

5mg ceramide

7.3mL DMSO

Good for 2 months at 2-8 degrees C

Mevinolin (24.7mM stock)

25mg

2.5mL DMSO

Good for 3 months at 2-8 degrees C

Perillyl Alcohol (6.3M stock)

Used as bottled, good for 4 weeks once opened at 2-8 degrees C

Retinoic Acid (20mM stock)

50mg

8.3mL DMSO

ZERO light exposure and good for 2 weeks at 2-8 degrees C

Coomassie Blue Stain (1 Liter)

1g Coomassie blue

500mL Methanol (50% by volume)

400mL Deionized water (40% by volume)

100mL Glacial acetic acid (10% by volume)

Stir 3-4hrs then filter

Store at room temperature

0.1M Sodium Hydroxide Solution For Blot Stripping Protocol

0.5g Sodium hydroxide

125ml Deionized water

Appendix B:

Additional Morphometric Graphs

Due to the compounds having a labile nature and short half life, it was hard to coordinate with the suppliers to be able to replicate the entire study to completion three times. Cell passage numbers were also different for each of the attempted replicates. As such, only one study was properly completed in its entirety and is included in the earlier text. The following pages graphically illustrates the data collected from all of the morphometric studies performed. The number of replicates per treatment will be listed as "*n*" in the figure legend below each graph.

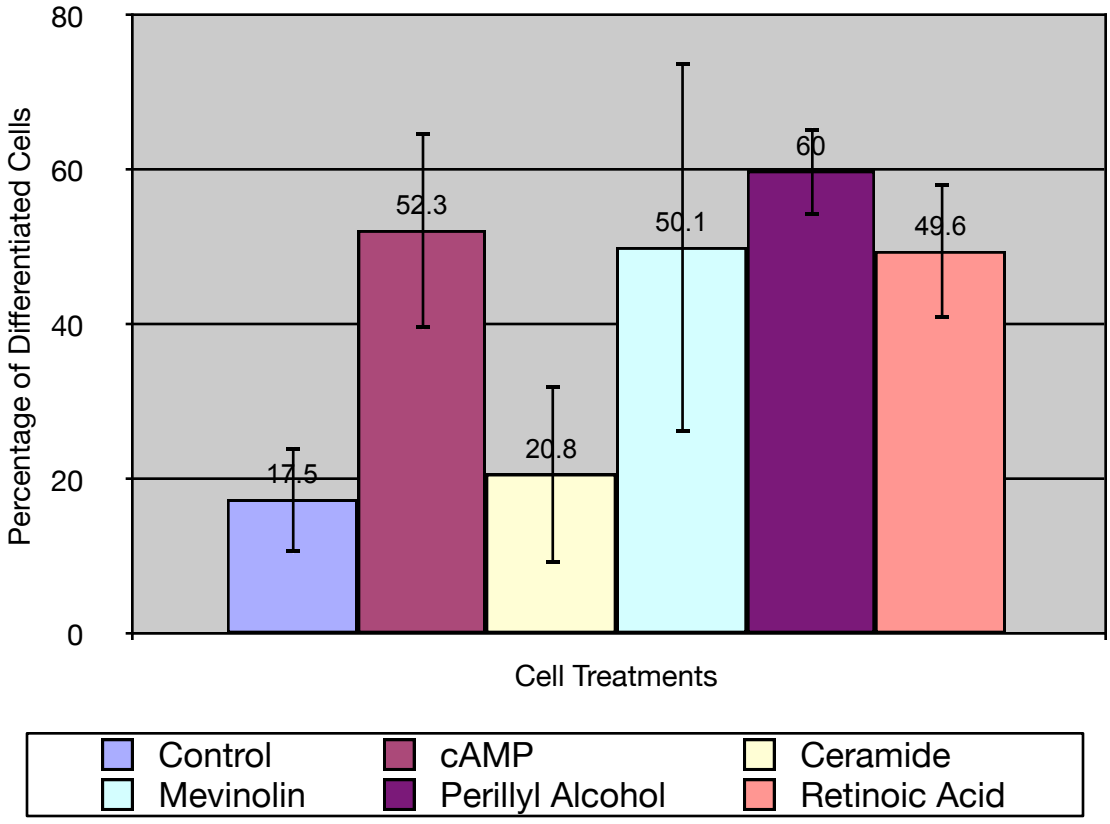

Chart 1. 12 hour differentiation graph. N2A cells were control (*n*=3), treated with 100mM dibutyryl cAMP (*n*=3), 25μM ceramide (*n*=3), 10μM mevinolin (*n*=3), 2mM perillyl alcohol (*n*=3), and 20μM retinoic acid (*n*=3). The cells were counted at 12 hours post treatment and a differentiation percentage was calculated for each. This color coded bar graph shows the differences in differentiation between each of the treatment groups during this time frame.

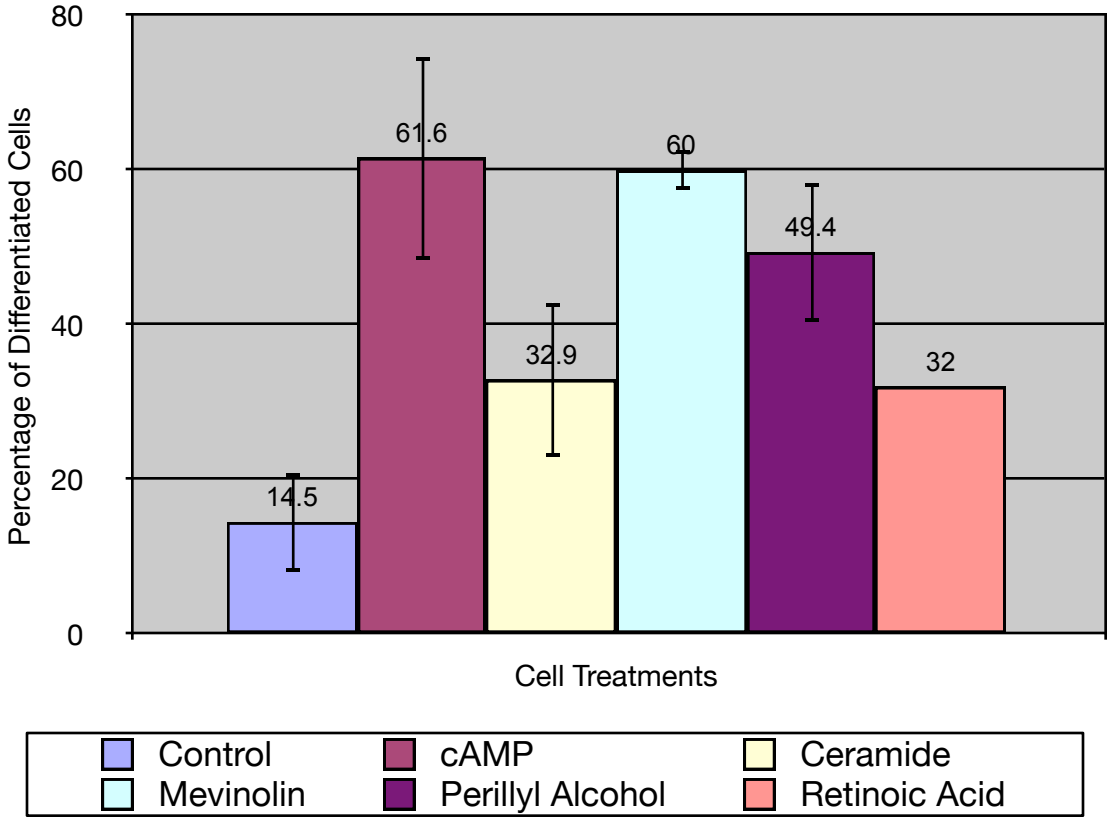

Chart 2. 24 hour differentiation graph. N2A cells were control (*n*=5), treated with 100mM dibutyryl cAMP (*n*=4), 25µM ceramide (*n*=3), 10µM mevinolin (*n*=4), 2mM perillyl alcohol (*n*=5), and 20µM retinoic acid (*n*=1). The cells were counted at 24 hours post treatment and a differentiation percentage was calculated for each. This color coded bar graph shows the differences in differentiation between each of the treatment groups during this time frame.

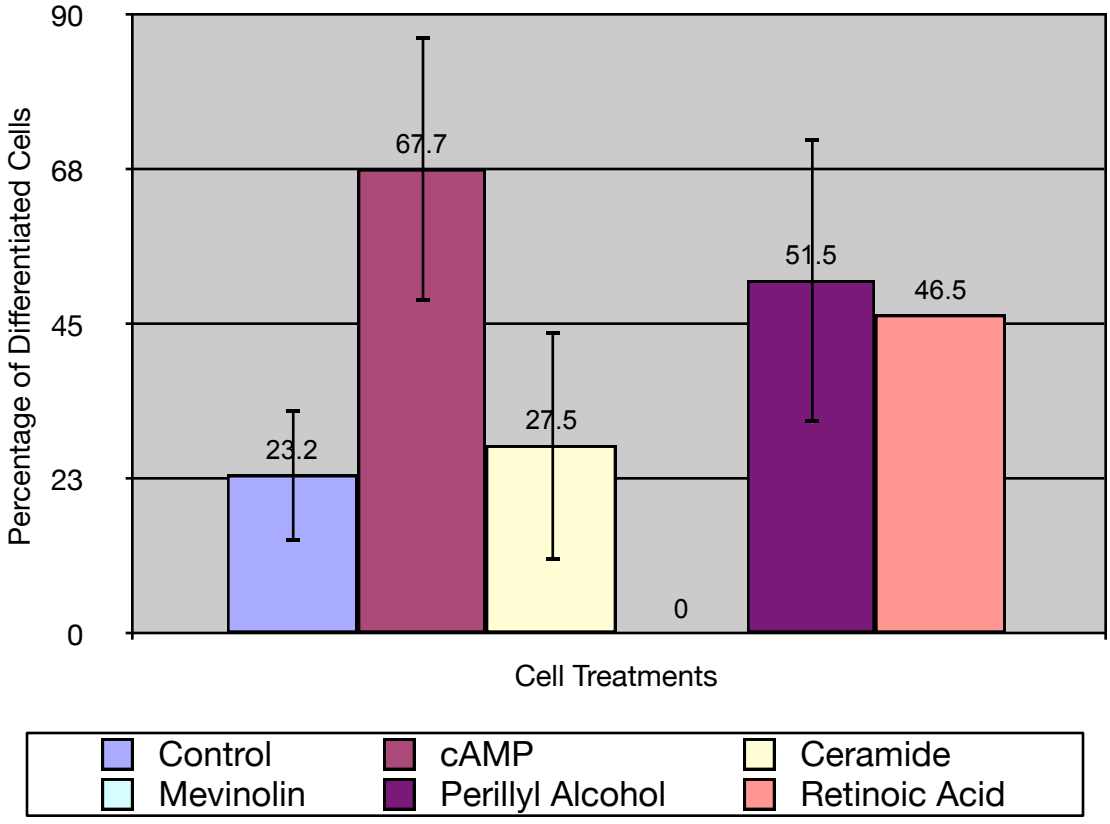

Chart 3. 48 hour differentiation graph. N2A cells were control (*n*=3), treated with 100mM dibutyryl cAMP (*n*=3), 25µM ceramide (*n*=3), 10µM mevinolin (*n*=3), 2mM perillyl alcohol (*n*=3), and 20µM retinoic acid (*n*=1). The cells were counted at 48 hours post treatment and a differentiation percentage was calculated for each. This color coded bar graph shows the differences in differentiation between each of the treatment groups during this time frame. ***10µM mevinolin at 48hrs is "0" due to no cell viability.