

Andrew Dennard

11/13/2023

BIO 610 Senior Comp

Dr. Mahita Kadmiel

Prednisone Mediated Apoptosis in Human Neuroblastoma Cells

Abstract

Glucocorticoids are commonly prescribed anti-inflammatory synthetic steroid hormones that are used to treat a wide variety of ailments. In many chemotherapy treatments they are included for their anti-inflammatory properties which are useful in regards to reducing the symptoms of other drugs included in chemotherapy treatments. Prednisone is one of these glucocorticoids that is often used for chemotherapy symptom reduction. Prednisone has also been found to have apoptotic inducing properties in some cancer cell lines. Human neuroblastoma (SH-SY5Y) is a type of cancer found in the nervous system that is often prevalent in children under the age of 5 years old. Other glucocorticoids such as dexamethasone have been shown to induce apoptosis in this cell line, but often come with unintended side effects such as the degeneration of neural cells. Prednisone having been already used in chemotherapy treatments is hypothesized to be a viable treatment for the induction of apoptosis in human neuroblastoma cells through the upregulation of pro-apoptotic genes and the down regulation of pro-survival genes Flow Cytometry will be used to test for apoptosis in the prednisone treated neuroblastoma cells in order to determine whether the treatment has resulted in the hypothesized outcome. qRT-PCR will also be performed to explore the pathway of apoptosis induction through a pro-survival gene Bcl-2 as well as a pro-apoptotic gene Bax. The expected results of both of these experiments are that a prednisone treatment of neuroblastoma cells will result in increased apoptosis among them as an upregulation of the chosen pro-survival gene and a downregulation of the chosen pro-apoptotic gene. With the expected results this research will be able to contribute to the growing data showing that the use of glucocorticoids as cancer-killing agents is

a viable route of treatment for patients who are already receiving glucocorticoids to mitigate chemotherapy symptoms.

Introduction

Neuroblastoma

Neuroblastoma is a specific type of neuronal cancer often found in relation to the spinal cord or brain stem [1]. This type of cancer is extremely common in children under the age of 5 [2]. Because of the age range of most neuroblastoma patients, it is of the utmost importance that treatment be as gentle as possible for them as their bodies often do not have the same defenses and stamina as older patients. The wide range of clinical presentations of neuroblastoma also dictates greater clinical research into the treatment options due to the varying and occasionally poor outcomes [18]. Symptoms vary with neuroblastoma, ranging from digestion and breathing problems to paralysis and other mobility issues [19]. Chemotherapy is extremely detrimental to a patient despite its ability to treat many forms of cancer, neuroblastoma included, and because of this the chemotherapeutic treatments used for this age group are under constant scrutiny in order to reduce their harmful side effects. Chemotherapy can cause decreased immune response, gastrointestinal problems, reproductive issues, and excessive fatigue as well as a multitude of other detrimental symptoms [21]. Neuroblastomas, just like the majority of other cancers, stems from a malfunction in the mechanism of programmed cell death resulting in excessive and uncontrollable cell growth causing tumors. These tumors continue to grow and infringe on other areas of the body which results in the impairment of natural bodily functions.

Chemotherapy

Chemotherapy is a common treatment for various types of cancers including neuroblastoma that uses multiple different chemicals based on the health of the patient and the severity of the cancer, often given intravenously [3]. These chemicals serve a variety of purposes regarding the treatment of cancer in patients. These active drugs often produce cytotoxic effects, such as apoptosis induction, that result in the death of cancer and tumor cells [3]. At this point in history this type of intravenous chemotherapy oftentimes coupled with radiation treatments has been shown to be the most effective if not only effective active treatment for cancer of any kind and cancer symptoms in patients (Figure 1). Despite its effectiveness in early to mid-stage cancer patients, chemotherapy significantly loses efficacy as the timeframe of the disease accelerates [3]. Even including the drawback of late-stage inefficiency, chemotherapy is used almost universally for cancer treatment with all types of cancer. Because of this widespread use, in depth research has been conducted on the side effects of chemotherapy treatments. This research has found that it is relatively impossible to provide effective tumor suppression and reduction without also causing the patient to incur dangerous levels of drug toxicity [3]. Because of these toxic side effects, a completely separate set of drugs is required to treat the dangerous levels of toxicity in a patient while undergoing chemotherapy. In regards to chemotherapy use in neuroblastoma patients, because of the explicitly young age of neuroblastoma patients their immune responses and overall stamina when undergoing chemotherapy can become an issue with all of the different chemicals, which vary between each patient, that are introduced into their system [21]. Due to this varying amount of chemicals, finding alternative drugs or being able to use already introduced drugs for multiple processes, such as the anti-inflammatory drug

prednisone which has been shown to have apoptotic effects in other human tissues, would be beneficial to these young patients and their overall health when undergoing chemotherapy.

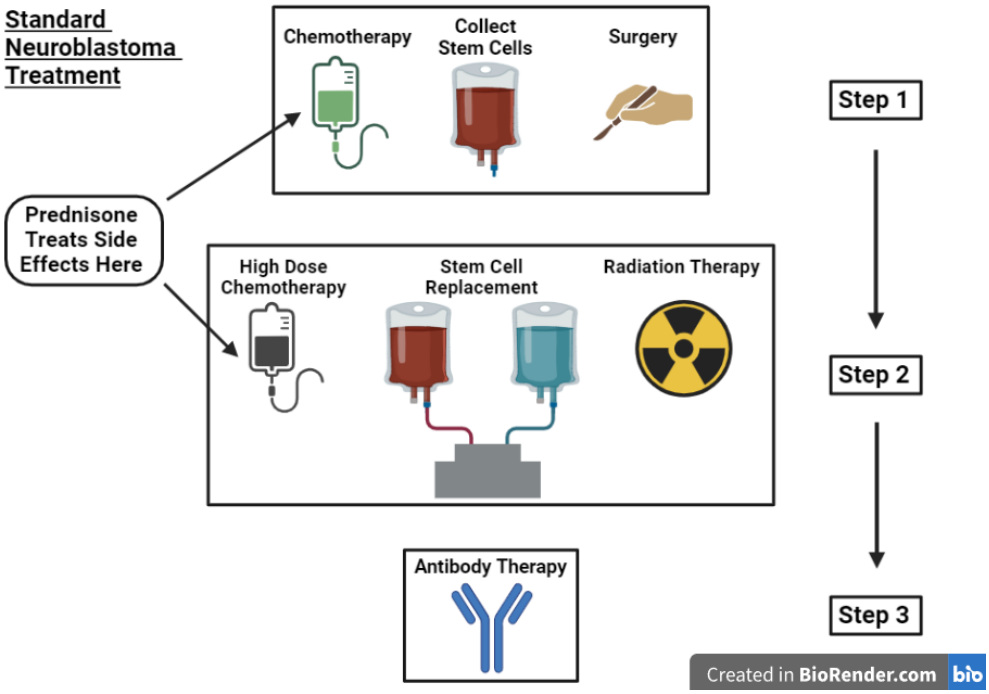


Figure 1: The pathway utilizing both chemotherapeutic and radiation treatments for patients with neuroblastoma. Specific points in which prednisone is used as a treatment are highlighted.

Glucocorticoids

Synthetic glucocorticoids mimic naturally occurring corticosteroids that are produced in the body. These naturally produced corticosteroids are synthesized in the body in response to inflammation or other bodily stress [4]. These corticosteroids then disperse throughout the bloodstream before being received by their target organs [4]. Once received, the corticosteroids enter the cell by binding to the glucocorticoid receptor and then translocating to the nucleus where they are able to regulate the transcription of certain genes through the glucocorticoid response element, many of which result in anti-inflammatory properties [5]. Synthetic glucocorticoids follow this same cellular pathway once they are distributed throughout the

bloodstream and produce these same anti-inflammatory effects as naturally occurring corticosteroids but can be more easily targeted to a specific organ or area of the body as synthetic glucocorticoids can be administered in a variety of fashions including orally, topically, and intravenously.

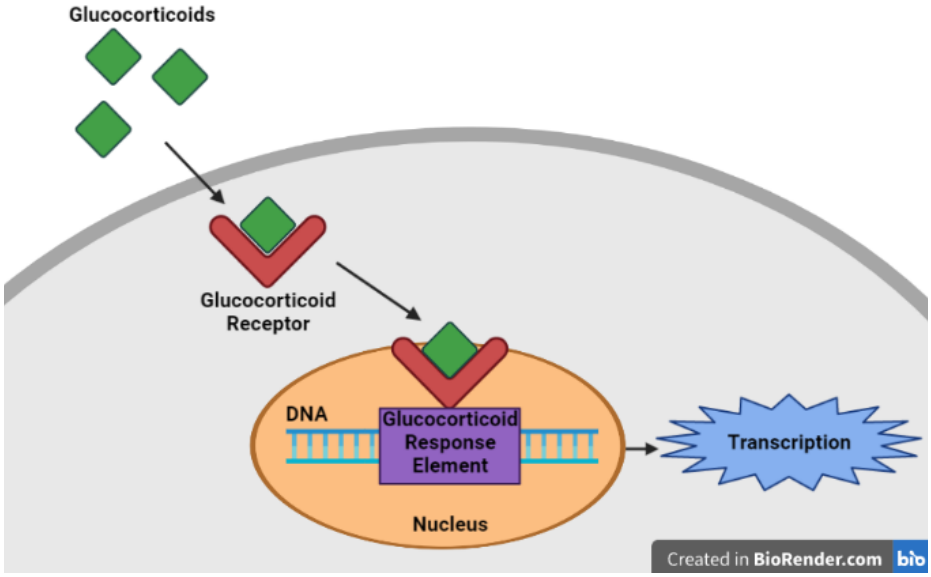


Figure 2: Diagram of the intracellular pathway depicting the route of both naturally occurring corticosteroids and synthetic glucocorticoids.

Along with their anti-inflammatory abilities synthetic glucocorticoids can also result in the induction of apoptosis in cells[6]. In regard to the induction of programmed cell death, also called apoptosis, these synthetic glucocorticoids follow the same pathway as they would when producing anti-inflammatory effects, but instead regulate the transcription of different genes linked to apoptosis [7]. These genes linked to apoptosis play a part in the intrinsic pathway of apoptosis. This pathway starts with the increase of mitochondrial reactive oxygen species in the cell along with the upregulation of pro-apoptotic genes through varying cellular processes including glucocorticoid regulation, either natural or artificial, through the glucocorticoid response element which promotes gene regulation in the nucleus [8]. These conditions then cause

the mitochondria to produce cytochrome c which binds to APAF-1, an integral member of apoptosomes which activate Caspase 9 [9,10]. APAF-1 then upregulates Caspase 9 which in turn activates Caspase 3, which acts as an enzyme, resulting in apoptosis of the cell through the cleavage of numerous cellular substrates that produces the morphological and chemical changes preceding and resulting in apoptosis. The extrinsic pathway of apoptosis is activated by external signals, including reactive oxygen species, that then bind to death receptors on the nucleus. These death receptors promote activator caspases such as pro-caspase 8 which in turn activate effector caspases like caspase 8. These effector caspases then activate caspase 3 which serves the same function in the extrinsic pathway as it does in the intrinsic pathway with the cleaving of cellular substrates and induction of apoptosis through numerous chemical and morphological changes within the cell. [11,12].

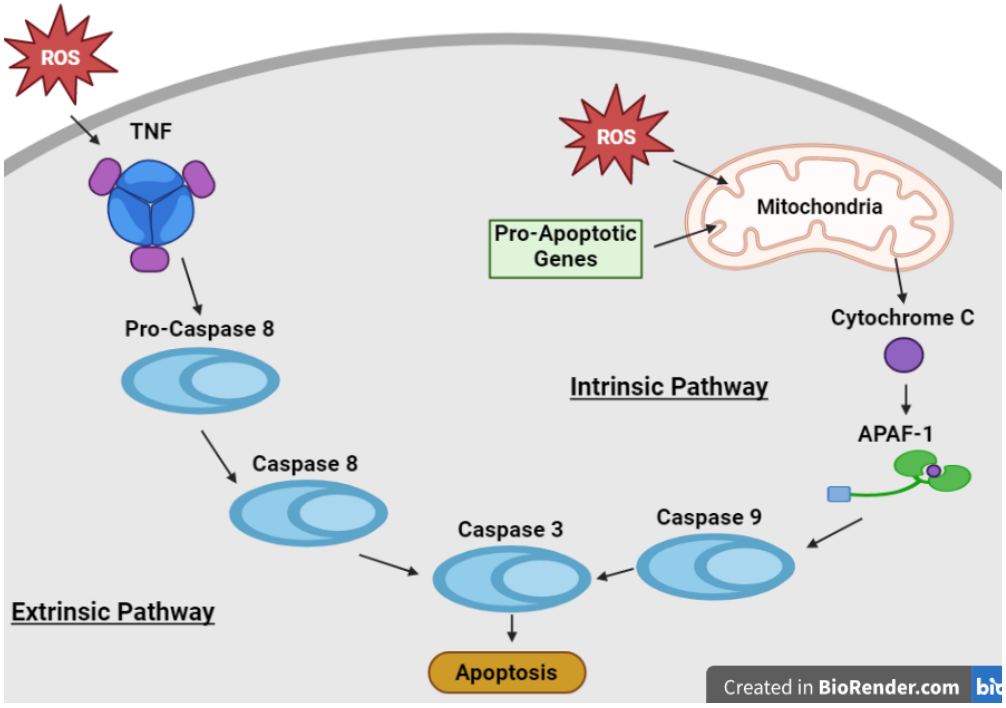


Figure 3: Depiction of the extrinsic and intrinsic pathways of cellular apoptosis in regard to treatment with glucocorticoids.

Pro-apoptotic and pro-survival genes which are regulated through the glucocorticoid signaling pathway if apoptosis is induced clinically can be used as clear indicators in regard to whether a cell is undergoing apoptosis. The upregulation and down-regulation respectively of these two categories of genes can be seen in cells that have gone through or are currently going through apoptosis and are generally absent in irregular levels otherwise [13,14]. Because of these easily quantified levels of gene expression, both pro-apoptotic and pro-survival genes are oftentimes chosen as measure of apoptotic activity at a cellular level.

Dexamethasone has been shown as an potent glucocorticoid that induces apoptosis in multiple different cell types including neuroblastoma cells [9,15]. Similarly, prednisone has shown apoptotic properties in several human cell lines [16]. Prednisone, a synthetic glucocorticoid that acts upon similar mechanisms as dexamethasone, is used as an anti-inflammatory treatment to help combat the side effects of chemotherapy [17]. Because of these apoptotic abilities and the pre-existing widespread application of prednisone as an agent to treat chemotherapeutic side effects for multiple cancers including neuroblastoma, it is hypothesized that prednisone can be used to induce apoptosis in human neuroblastoma cells in a dose dependent manner similar to the function of dexamethasone. Clinically this would imply that prednisone could be used for two purposes, both anti-inflammatory and pro-apoptotic, therefore gaining the ability to replace or reduce the number of active drugs used in a patient's chemotherapy treatment. This project aims to investigate the process by which glucocorticoids (prednisone and dexamethasone) are able to induce apoptosis in neuroblastoma by testing glucocorticoid function on neuroblastoma cells. Prednisone induced apoptosis mediated through pro-apoptotic and pro-survival genes provides clinical significance in its ability to add to the

literature regarding prednisone and its possible ability to replace or reduce some of the other apoptotic drugs used in the chemotherapy treatments for patients.

Methods

Cell Culturing

The human neuroblastoma cell line SH SH5Y (CRL-2266) was utilized throughout this research. This cell line is characterized by having both adherent and suspension live cells. The human neuroblastoma cells were grown in a Complete SH SY5Y Dulbecco's Modified Eagle Medium (DMEM) which includes 49% Dubecco's Modified Eagle Medium F12, 49% Eagle's Minimal Essential Medium (EMEM), and 2% Heat Inactivated Fetal Bovine Serum (FBS) [22]. Antibiotics and antimycotic drugs were also included in the SH SY5Y medium. These cells were cultured in a flask in this complete medium until 80% - 90% confluency was reached. After the cells reached the desired confluency, they were subjected to prednisone or control treatment for 6 hours at either 0 ng/μL (control), 1 ng/μL, 10 ng/μL, or 100 ng/μL concentrations.

Flow Cytometry

SH SY5Y cells were cultivated in 12 well plates until a concentration of 10^6 cells per well was achieved after 3 days of growth. At this point either prednisone (53-03-2), dexamethasone (50-02-2), or vehicle treatment was administered with prednisone and dexamethasone treatments being administered at 1 nM, 10 nM, or 100n nM concentrations [24]. These samples were created in triplicate in order to increase the credibility of the results. Cells were treated with either prednisone or dexamethasone for 6 hours at 1 ng/μL, 10 ng/μL, or ng/μL concentrations. After the selected timeframe the adherent cells were trypsinized while the nonadherent cells were centrifuged at 2500 rpm for 5 minutes in order to form a pellet. This

pellet was reintroduced to the trypsinized cells. The pellet was resuspended in the trypsin and cell mixture. This mixture was then moved to 1.5 mL centrifuge tubes and then centrifuged again at 2500 rpm for 5 minutes. The resulting supernatant was discarded, and the pellet was washed with PBS and centrifuged with the aforementioned conditions again. Annexin-binding buffer (HEPES, NaCl, & CaCl2) was used to resuspend the pellet. 100 µL of the mixtures was aliquoted into a new 1.5 mL centrifuge and then 5 µL of Annexin V conjugate was added and the mixture was allowed to incubate for 10-15 minutes protected from light. Propidium Iodide, to stain for dead cells, was also added at this point. The samples were then incubated for 15 minutes at room temperature before 400 µL of annexin-binding buffer was added and the sample was mixed gently. These samples were then analyzed by Flow Cytometry.

RT-qPCR

Cultivated SH SY5Y cells were treated with 1 nM, 10 nM, or 100 nM concentrations of prednisone or dexamethasone for 6 hours in 12 well plates. These treatments were performed in triplicate. To extract the RNA from these samples the medium was removed from the plate and centrifuged to form a pellet. This pellet was suspended in 1 mL of trizol. The wells were washed with PBS before the trizol, and resuspended cell mixture was added to the plate. The wells were then scratched, and the mixture was moved to a 1.5 mL centrifuge tube for each sample. 200 µL of chloroform was added and the tube was mixed by inverting 10 times before being allowed to incubate at room temperature for 10 minutes. These samples were then centrifuged at 12000 rpm for 15 minutes. The clear layer of liquid was removed and transferred to a new 1.5 mL centrifuge tube before adding 500 µL of isopropanol to this new tube. The samples were then incubated at room temperature for 10 minutes before being centrifuged at 12000 rpm for 10 minutes. The supernatant was discarded, and 1 mL of ethanol was added to the tubes before the samples were

centrifuged at 10000 rpm for 2 minutes. The resulting supernatant was removed, and the samples were centrifuged again at 12000 rpm for 2 minutes. The remaining liquid was removed, and the samples were incubated for 7 minutes in a fume hood before 30 µL of RNA free water was added to each sample. In order to quantify the RNA from each sample the following nanodrop procedure was performed. The loading arm and base were both wiped with water before loading RNA free water and running a blank sample in order to determine a baseline for testing. 5 µL of each individual sample was then added individually to the base and allowed to run while the 260/280 and the 260/230 ratios were recorded as well as the concentration. Between each sample water was used to wipe down the base and loading arm. To run RT-qPCR a master mix was made for each gene that was chosen for testing. *BAX* (qHsaCEP0040666) and *SURVIVIN* (qGgaCEP0015762) were observed as the genes of interest and *PPIB* (qHsaCIP0030902) was used as a housekeeping gene. 0.25 µL of TaqMan Gene Expression Assay 20X Primer Probe specific to each gene was added to the respective master mix in an amount that accounted for each individual sample being tested (i.e., 12 samples and a water constant requiring 2 replicates each resulting in a total volume of 6.5 µL of primer probe added) [23]. 2.5 µL of 2X qScript XLT One-Step RT-qPCR ToughMix (4444556) [20] was added to each master mix in an identical fashion as the primer probe. In a 96 well plate 2.25 µL of RNA at a concentration of 45 ng/µL from the RNA extraction samples was added to an individual well and referenced using a plate map. This was done in duplicate for each gene bringing the total to 13.5 µL of RNA taken from each RNA extraction sample. 2.75 µL of the master mix (Primer Probe and ToughMix) was added to each well for each gene being tested. Once completed the plates were loaded into the CFX Opus thermocycler by Bio-Rad for RT-qPCR. Data analysis for RT-qPCR was completed using the ΔΔCT method to show gene expression normalized to the housekeeping gene.

Results

RT-qPCR

RT-qPCR was performed following the aforementioned methods with a 6 hour treatment of 1 ng/µL, 10 ng/µL, and 100 ng/µL of both dexamethasone and prednisone separately performed in three separate experiments before RNA extraction and subsequent RT-qPCR. RT-qPCR was run for *GILZ* normalized to *PPIB* to check for the expected expression as *GILZ* expression has been shown in cell lines that utilize the GR receptor. RNA concentration for RT-qPCR was increased from 10 ng/µL to 45 ng/µL after preliminary RT-qPCR runs with each experiment resulted in a lack of expression for either gene. The second experiment RT-qPCR results had no expression in the FAM channel which is the expected channel expression for both *GILZ* and *PPIB*.

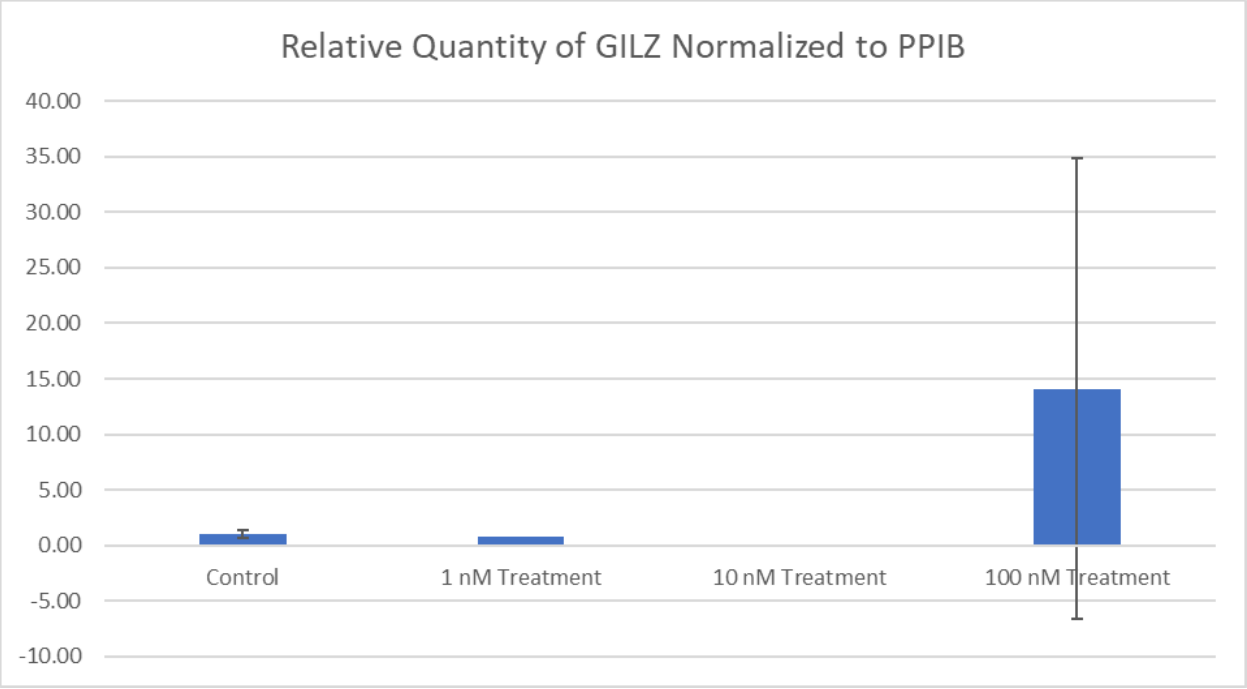


Figure 4: Graph demonstrating the relative quantity of GILZ normalized to PPIB for the first RT-qPCR run of dexamethasone treated cells. 3 biological replicates of dexamethasone treatment and their GILZ expression normalized to PPIB.

The RT-qPCR for the first run of dexamethasone treated cells resulted in extremely abnormal values for the expression of *GILZ* normalized to *PPIB* and it was advised not to waste primer probe aliquots of *BAX* and *SURVIVIN* for RNA that would produce results such as these. The data collected for expression is so far outside of the normal range of expression for RT-qPCR data that it was decided that there had to be an issue with the RNA used in the experiment either due to DNA remnant contamination or RNA extraction issues. The possibility of RNA degradation is also a likely factor in the quality of RNA experiments used.

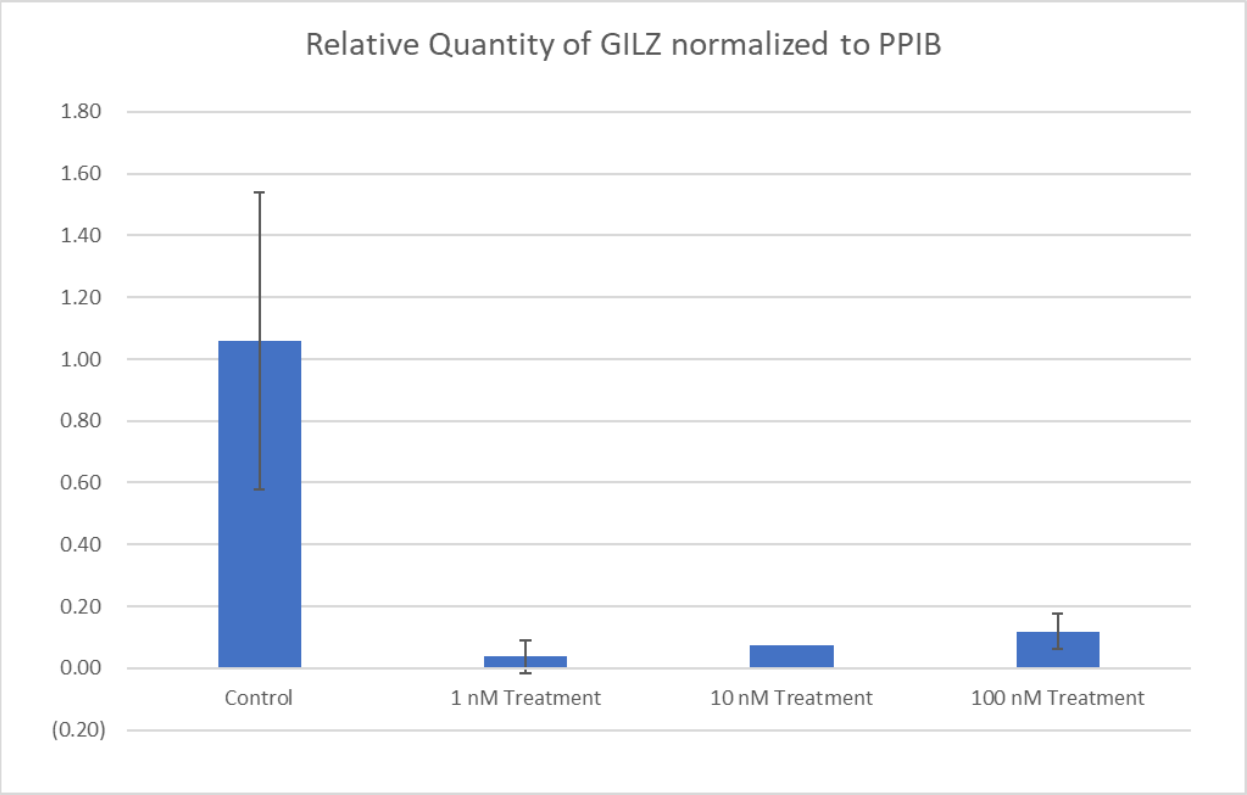


Figure 6: Relative quantity of *GILZ* normalized to *PPIB* for the third RT-qPCR run of dexamethasone treated cells. 3 biological replicates of dexamethasone treatment and their GILZ expression normalized to PPIB.

The RT-qPCR for Experiment 3 followed a similar trend as the results of Experiment 1 resulting in values that fall far outside of the normal values expected for RT-qPCR data. It was also

advised that this experiment not be continued in regard to the utilization of *BAX* and *SURVIVIN* primers in order to avoid the waste of resources.

Flow Cytometry

Flow Cytometry was unable to be performed during this project due to numerous mechanical errors with the flow cytometer. There was no point within the timeline of this research that the flow cytometer was able to be run without encountering issues that resulted in the termination of this portion of the project.

Discussion

The data gathered from this research holds no significance or clinical relevance as all data values recorded were far outside of the normal expected values for their respected experiments. The RNA for RT-qPCR was unable to go through a general quality control process due to time constraints and other events during the RNA extraction portion of these experiments which could have resulted in its unreliability during RT-qPCR. These RNA quality issues could stem from the RNA extraction methodology, specifically the cell count prior to RNA extraction or the DNAse treatment preceding RNA extraction. RNA quality may have been poor due to one or a combination of multiple factors including organic contamination, DNA contaminations, or RNA degradation. The flow cytometry experiments failed due to mechanical error and not researcher error as the flow cytometer was not able to run samples without a malfunction at any point during the time of this research. The methodology of this research could be repeated and result in data with relevance if all machinery was functional and ample time was given for quality control of the RNA from the SH-SY5Y cell line. This research could continue in the future as either a repetition of the same methodology or expand to encompass other cancerous cell lines that are

treated with chemotherapy as prednisone is commonly used in conjunction with many chemotherapy treatments and if shown to have apoptotic effects could be useful for the treatment of other cancers.

References

1. Neuroblastoma - Symptoms and causes - Mayo Clinic[Online]. Available: https://www.mayoclinic.org/diseases-conditions/neuroblastoma/symptoms-causes/syc-20351017. [Accessed: 28-Nov-2022]
2. Neuroblastoma - Childhood: Types of Treatment | Cancer.Net[Online]. Available: https://www.cancer.net/cancer-types/neuroblastoma-childhood/types-treatment. [Accessed: 28-Nov-2022]
3. Nygren, P. (2001) What is cancer chemotherapy? *Acta Oncol (Madr)* 40, 166–174
4. Newton, R. Molecular mechanisms of glucocorticoid action: what is important? DOI: 10.1136/thorax.55.7.603
5. Kadmiel, M. *et al.* (2016) Glucocorticoid action in human corneal epithelial cells establishes roles for corticosteroids in wound healing and barrier function of the eye. *Exp Eye Res* 152, 10–33
6. Reed, J.C. (2000) Mechanisms of apoptosis. *Am J Pathol* 157, 1415–1430
7. Schmidt, M. *et al.* (1999) Glucocorticoids Induce Apoptosis in Human Monocytes: Potential Role of IL-1β. *The Journal of Immunology* 163, 3484–3490
8. Mechanisms of Glucocorticoid-mediated Apoptosis in Hematological Malignancies | Clinical Cancer Research | American Association for Cancer Research[Online]. Available: https://aacrjournals.org/clincancerres/article/8/6/1681/199853/Mechanisms-of-Glucocorticoid-mediated-Apoptosis-in. [Accessed: 29-Nov-2022]
9. Xing, K. *et al.* (2015) Dexamethasone enhances programmed cell death 1 (PD-1) expression during T cell activation: an insight into the optimum application of glucocorticoids in anti-cancer therapy. *BMC Immunol.* 16, 39
10. Ryu, J.S. *et al.* (2017) Prednisolone induces apoptosis in corneal epithelial cells through the intrinsic pathway. *Scientific Reports 2017 7:1* 7, 1–10
11. Smith, L.K. and Cidlowski, J.A. (2010) Glucocorticoid-Induced Apoptosis of Healthy and Malignant Lymphocytespp. 1–30
12. Cohen, G.M. (1997) Caspases: the executioners of apoptosis. *Biochemical Journal* 326, 1–16
13. Lalier, L. *et al.* (2007) Bax activation and mitochondrial insertion during apoptosis. *Apoptosis* 12, 887–896
14. Dohi, T. *et al.* (2004) Mitochondrial survivin inhibits apoptosis and promotes tumorigenesis. *J Clin Invest* 114, 1117–1127
15. He, J. *et al.* (2017) Dexamethasone affects cell growth/apoptosis/chemosensitivity of colon cancer via glucocorticoid receptor α/NF-κB. *Oncotarget* 8, 67670

16. Ryu, J.S. *et al.* (2017) Prednisolone induces apoptosis in corneal epithelial cells through the intrinsic pathway. *Scientific Reports 2017 7:1* 7, 1–10

17. Prednisone - NCI[Online]. Available: https://www.cancer.gov/about-cancer/treatment/drugs/prednisone. [Accessed: 28-Nov-2022]

18. Weinstein, J et al. (2003) Advances in the Diagnosis and Treatment of Neuroblastoma. The Oncologist 2003 8:278-29

19. United Therapeutics Corporation. (2023) What is neuroblastoma - causes and symptoms. Neuroblastoma info 2023

20. *TaqMan™ Fast Advanced Master Mix for qPCR*. (n.d.). Retrieved December 16, 2023, from https://www.thermofisher.com/order/catalog/product/4444556?SID=srch-srp-4444556

21. *Chemotherapy Side Effects | American Cancer Society*. (n.d.). Retrieved December 16, 2023, from https://www.cancer.org/cancer/managing-cancer/treatment-types/chemotherapy/chemotherapy-side-effects.html

22. SH-SY5Y - CRL-2266 | ATCC. (n.d.). Retrieved December 16, 2023, from https://www.atcc.org/products/crl-2266

24. Cellular Culture and Analysis (n.d.) Retrieved December 16, 2023 from Cell Culture & Analysis (sigmaaldrich.com)