UNDERSTANDING THE EFFECT OF NOVEL ANTI-INFLAMMATORY COMPOUNDS ON NF-κB ACTIVATION

by

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ABSTRACT

Alzheimer's Disease (AD), the most common form of Dementia, is a brain disorder that affects memory, cognition, and behavior. It currently affects 6.7 million Americans in the United States and interferes with daily life. Neuroinflammation in the brain is thought to worsen symptoms and drive the progression of the disease. Inflammation is mediated by the transcription factor NF-κB, which typically leads to transcription of pro-inflammatory cytokines, including TNF-α and IL-1β. The transcription of these cytokines can lead to a cycle of chronic inflammation if left unregulated. In collaboration with P2D Biosciences and Dr. Kayla Green's Lab, we focused on testing compounds for their ability to reduce inflammation. Some of the compounds tested here have been shown to reduce cognitive defects in a mouse model of AD. In this study we are trying to understand the mechanism of action of these drugs. We are looking at the effect on the transcription factor NF-κB.

INTRODUCTION

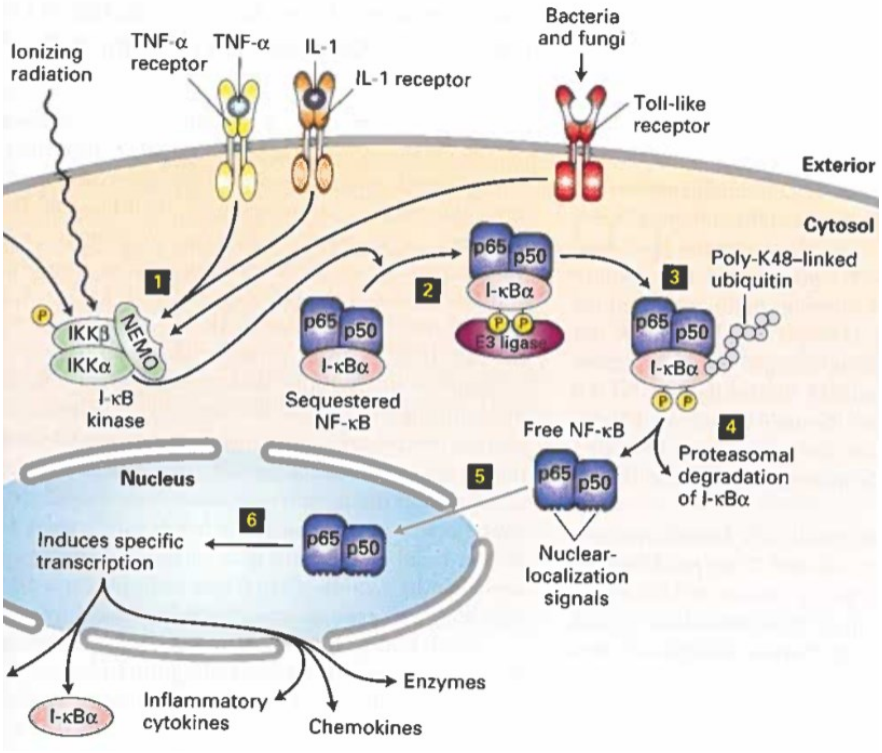

Figure 1: NF-κB Pathway[8]

Alzheimer's Disease (AD), the most common form of Dementia, is a brain disorder that affects memory, thinking, and behavior. As of 2024, there are nearly seven million Americans estimated to have AD.[10] It is currently ranked as the seventh leading cause of death in the United States and affects individuals aged sixty-five and older. As of current, the world population is aging, and the number of people aged sixty-five and older is expected to increase to nearly 1 billion by 2030.[1] AD is the most expensive disease in the United States.[3] According to the Alzheimer's Association, the earliest sign of AD is difficulty remembering newly learned information, but other common symptoms can include disorientation, mood and behavior changes, deepening confusion, more serious memory loss, and difficult swallowing, speaking, or even walking. As stated in many articles, due to both the clinical and financial burden that AD

places on the loved ones of affected patients and the government, it is becoming more and more pressing to find novel and therapeutic treatments to relieve this stress.

AD is driven by two main abnormal structures in the brain, namely beta-amyloid (Aβ) plaques and neurofibrillary tangles (NFTs) made up of the protein tau. Both of these structures damage neuronal synapses, that mediate memory and cognition, and kill nerve cells. Amyloid-beta peptides are the building blocks for plaques and consist of proteolytic fragments of the transmembrane amyloid precursor protein.[2] Meanwhile, tau is what comprises the tangles and is a brain-specific, axon-enriched microtubule-associated protein.[2]The combination of these is what drives normal, healthy neuronal cells into the diseased state that is seen in AD and results in the hallmark symptoms.

A more recently discovered component of this disease is the presence of a sustained inflammatory response, which may provide the link between the two main abnormal structures. Typically, inflammation is a healthy process in the body that leads to healing injuries and fighting off foreign invaders. It is a natural defense mechanism against infection, toxins, and injury.[4] However, when inflammation is left unchecked and is allowed to propagate in a continuous cycle, this is when we see cell death in the body. One of the most important players and primary regulators of inflammation in the body is NF-κB, a transcription factor found free-floating in the cell. NF-κB has been found predominantly in the neurons and glial cells that surround Aβ plaques in patients with AD.[4] When there is a sustained immune response occurring in the brain, it exacerbates both the NFTs and the Aβ plaques.

The NF-κB pathway is activated by receptors on the cell surface which are primarily activated by pro-inflammatory cytokines such as IL-1β and TNF-α. The most well studied pathway involves the activation of the TNF-α receptor (TNFR) via TNF-α which results in a

cascade of signaling events. TNFR recruits IKK, an IκB kinase heterodimer composed of IKKα, IKKβ, and IKKγ also known as an NF-κB essential modulator (NEMO). IKK phosphorylates IκBα and triggers the degradation of IκBα via ubiquitination. IκBα is phosphorylated at two N-terminal serines and is degraded in the proteasome. This exposes the nuclear translocation signal on the NF-κB transcription factor and allows it to translocate into the nucleus where NF-κB promotes gene transcription. Among the genes expressed are IL-1β and TNF-α which are secreted from the cell and bind to receptors in autocrine and paracrine fashion, which then reinforces the inflammatory cycle.[8] NF-κB induced gene expression is responsible for many biological functions such as cell growth and proliferation and the immune response, specifically inflammation. Chronic inflammation when triggered, can lead to neurodegeneration, cell death in the brain, as seen in diseases such as AD and FTD.

Microglia are the resident immune cells within the central nervous system (CNS) and serve to survey the surrounding neurons and communicate with other glia cells. Microglia that become chronically activated release products such as proinflammatory cytokines, reactive oxygen species, and nitric oxide. When microglia find a threat to the CNS, it leads to activation and morphological changes that allow these cells to enlarge and migrate to the damaged area. It is thought that the presence of Aβ plaques in the brain is what drives the activation of these microglia in AD.[4] Eventually, as the microglia work to phagocytose the Aβ plaques, the sustained activation leads to a decrease in destructive efficiency. However, the microglial remain unchanged in their ability to produce pro-inflammatory cytokines which results in reactive microgliosis. The Aβ plaques are no longer being cleared from the brain and the immune system is perpetually activated causing the release of pro-inflammatory cytokines which results in

neuroinflammation. This turns into a vicious positive feedback loop and is what causes the exacerbation of neurodegeneration in AD.[4]

There is no cure for Alzheimer's Disease. Research on AD treatment has been mainly focused on two types of drugs, one type focused on treating the symptoms and the other type focused on changing the disease progression. Two of the most popular drugs offered that focus on disease progression of AD patients are Donanemab and Leqembi. Lecanemab, commercially known as Leqembi, is a monoclonal antibody intravenous (IV) infusion therapy that has US Food and Drug Administration (FDA) approval to treat early Alzheimer's.[6] It functions by targeting Aβ plaques and removing them from the brain by targeting them to be broken down. It has been shown to reduce cognitive and functional decline. Donanemab, created by Eli Lilly, has had very promising results in their phase 3 trial showing that the drug significantly slowed cognitive and functional decline again in individuals with early-stage AD.[7] Donanemab functions similarly to Lecanemab, also targeting amyloid as a monoclonal antibody. While it has not yet received FDA approval, it is on the forefront of treatments for this disease.

P2D biotech is a company that is focused on developing novel anti-inflammatory drugs for the treatment of CNS diseases, including Frontotemporal Dementia (FTD) and Alzheimer's disease. My project involves testing the effect of these drugs on their ability to modulate the NF-κB signaling pathway. This is a desirable approach to treating diseases such as AD and FTD because while inflammation is generally a positive mechanism used to fight infections and heal the body, it can become chronic and initiate or exacerbate a long list of disorders when it is left unchecked.

METHODS

Cell Culture:

The immortalized human embryonic kidney cell line 293 (HEK293) is grown in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% fetal bovine serum (FBS), penicillin, streptomycin, and glutamine. HEK293 cells are kept in a humidified incubator at 37°C, supplemented with 5% CO2.

Luciferase Assay:

In order to determine the effect of various drugs on NF-κB mediated gene expression, a luciferase gene assay was implemented. HEK293 cells were plated at 25,000 cells per well in a 24 well plate. After being cultured for 24 hours, cells were then transfected with two plasmids, PRDII-Luc (100 ng/well) and CMV-Luc (50 ng/well). After being cultured for another 24 hours, cells were then pretreated with varying concentrations of drug for 1 hour. TNF-α (10 ng/uL) was added in order to stimulate the inflammation pathway, both in the absence and presence of the various drugs being tested. After 17 hours, cells are harvested using the manufacturers protocol and NF-κB-mediated gene expression was measured via a luciferase assay following the manufacturer's protocol (Promega Dual Luciferase Assay Kit).

MTT Cytotoxicity Assay:

In order to determine cell viability, an MTT assay was conducted for each drug. HEK293 cells were cultivated and plated 5,000 per well in a 96 well plate. Cells were incubated with increasing concentrations of the drug being tested. After dumping the original medium out, 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) solution **(**1 mg/mL**)** was then added to each well. The plate was incubated for 4 hours at 37° C, whereafter the medium was removed and DMSO solution was added to each well. After the formazan crystals dissolved in the DMSO solution, absorbance of the plate was read at 540 nm with a microplate reader.

RESULTS

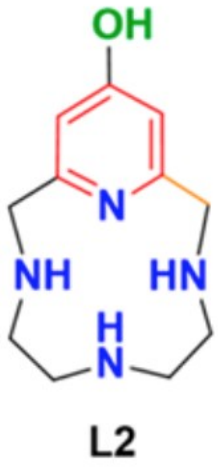

Figure 2: Structure of L2.

L2 is a compound that was developed in Dr. Kayla Green's Lab at Texas Christian University. The L2 molecule is made of multiple components that combat different causative agents of neurodegenerative diseases. The blue part of the molecule targets (Fig. 2) metal dysregulation and binds metal ions, the red has antioxidant capabilities, and the green part of the molecule has radical scavenging abilities.[5]

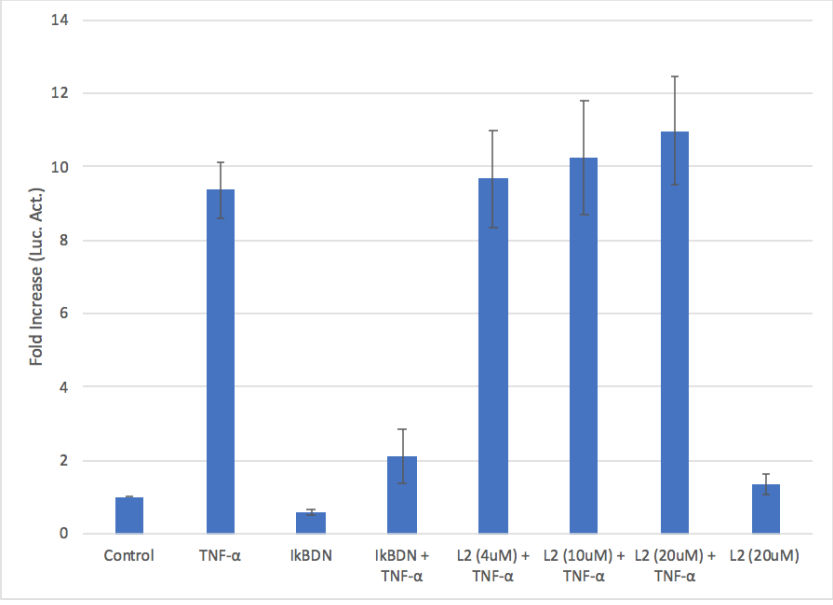

Figure 3: Effect of L2 on TNF-α-induced NF-κB Activation in HEK293 Cells

The drug L2 was designed to reduce Reactive Oxygen Species (ROS) and thus has been hypothesized to reduce inflammation but the mechanism by which it does so is unknown. Specifically, we wanted to test whether it inhibited cytokine-mediated activation of NF-κB. To test this hypothesis, we used an NF-κB reporter plasmid. PRDII-Luc contains the NF-κB regulatory region but instead of it transcribing the target genes, it now transcribes luciferase. Luciferase is a bioluminescent enzyme found in fireflies, so we can measure the luminescence of this pathway's product. CMV-Luc is used as a loading control and is compared to the levels of PRDII-Luc for transfection efficiency. The cells were pre-treated with the indicated concentrations of the drug for 1 hour. A dominant negative gene, IκBΔN, was used as a negative control. IκBΔN is a version of IkB that has the phosphorylation sites mutated. It therefore cannot be degraded, leaving it permanently bound to NFkB. Finally, the cytokine, TNF-α was added to the cells in order to stimulate the NF-κB pathway. After 17 hours of treatment with TNF-α, cells were harvested, and luciferase activity was measured.

In figure 3, cells treated with TNF-α alone showed approximately a nine-fold increase in luciferase activity showing that it stimulated inflammation in the cell. The negative control, IκBΔN, showed a significant reduction in luciferase activity as compared to TNF-α. No significant reduction in luciferase activity was observed in cells treated with increasing concentrations of L2. Some cytotoxicity was observed at the higher concentrations of L2.

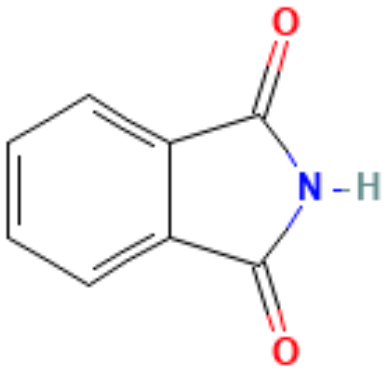

Figure 4: Structure of Isoindolinedione

Due to the nature of the research at P2D Biosciences, we are unable to know the true structure of the compounds we are looking at, we only know what they are derivatives of. Therefore, three of the compounds tested in this study are derivatives of isoindolinedione. These compounds are PD340, PD2254, and PD2024.

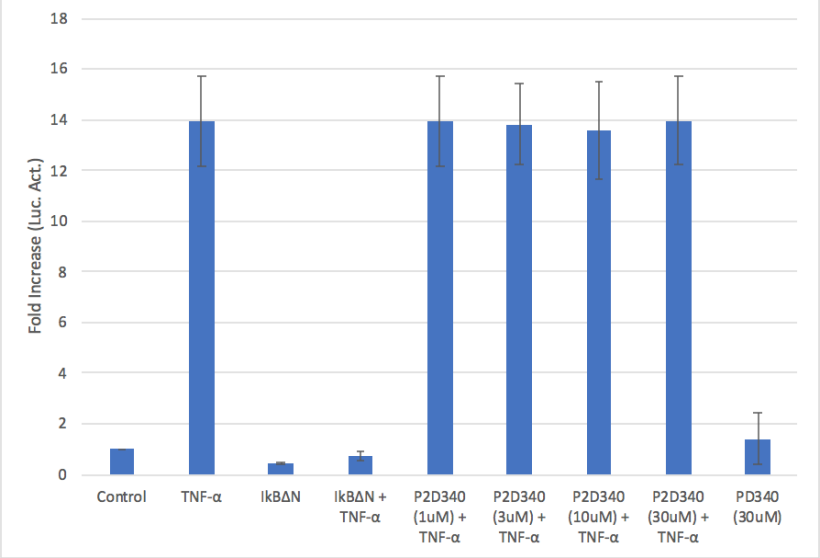

Figure 5: Effect of PD340 on TNF-α-induced NF-κB Activation in HEK293 Cells

In figure 5, cells treated with TNF-α alone showed a fourteen-fold increase in luciferase activity showing that it stimulated inflammation. The negative control, IκBΔN, showed a significant

reduction in luciferase activity as compared to TNF-α. No significant reduction in luciferase activity was observed in cells treated with increasing concentrations of PD340.

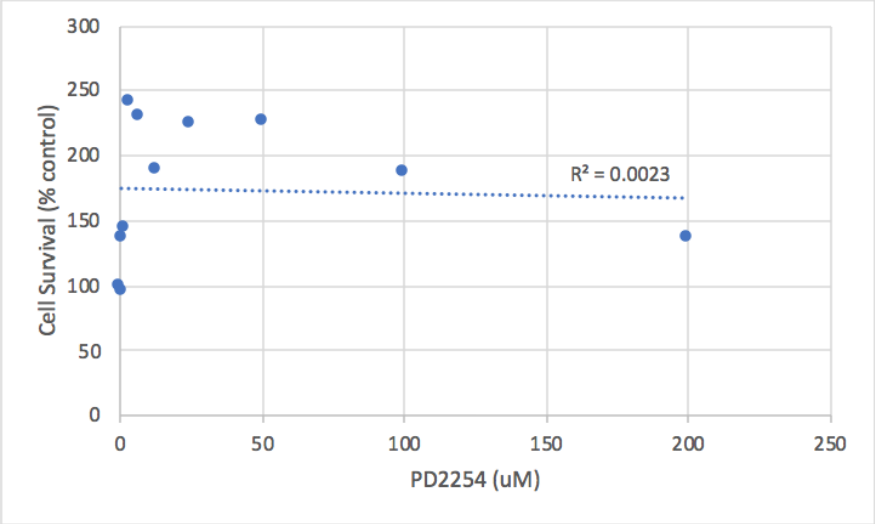

Figure 6: Cytotoxicity of PD2254 in HEK293 cells

Before testing any novel compound, it is important to determine its cytotoxicity so as to identify suitable concentrations to use in the luciferase assay. An MTT assay was performed in order to assess the cytotoxicity of PD2254. Our data suggests that the compound is not cytotoxic at all concentrations of PD2254 tested, since cell survival remains above one hundred percent.

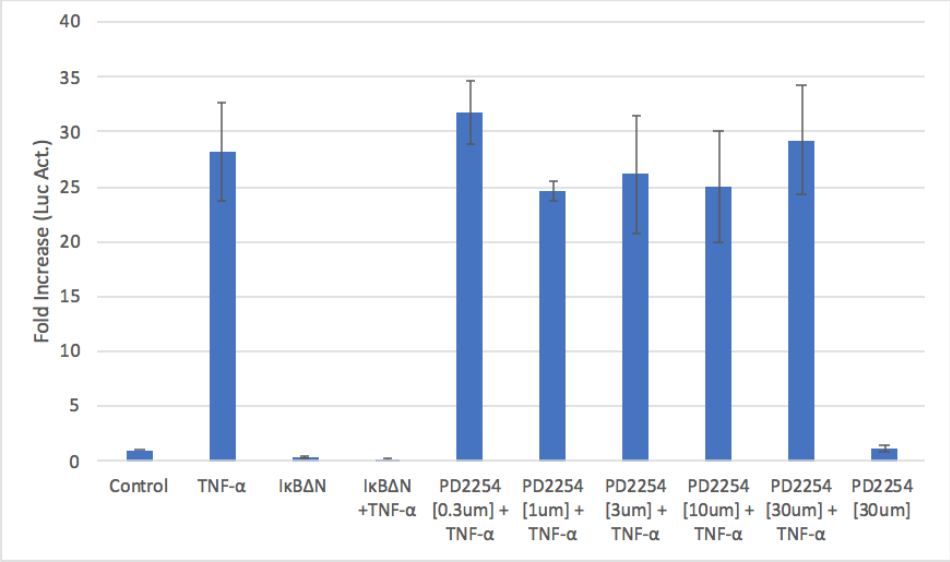

Figure 7: Effect of PD2254 on TNF-α-induced NF-κB Activation in HEK293 Cells

Next we examined the effect of PD2254 on TNF-a-induced NF-kB activation. In figure 7, cells treated with TNF-α alone showed a twenty-six-fold increase in luciferase activity showing that it stimulated inflammation. The negative control, IκBΔN, showed a significant reduction in luciferase activity as compared to TNF-α. No significant reduction in luciferase activity was observed in cells treated with increasing concentrations of PD2254.

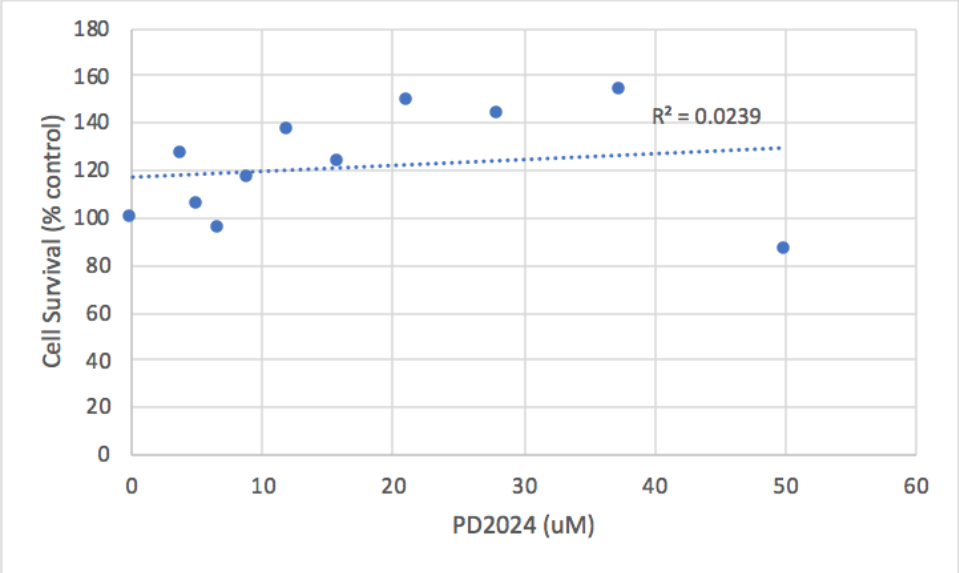

Figure 8: Cytotoxicity of PD2024 in HEK293 Cells

An MTT assay was also performed in order to assess the cytotoxicity of PD2024. Our data suggests that the compound is not cytotoxic at all concentrations of PD2024 tested, since cell survival remains above one hundred percent.

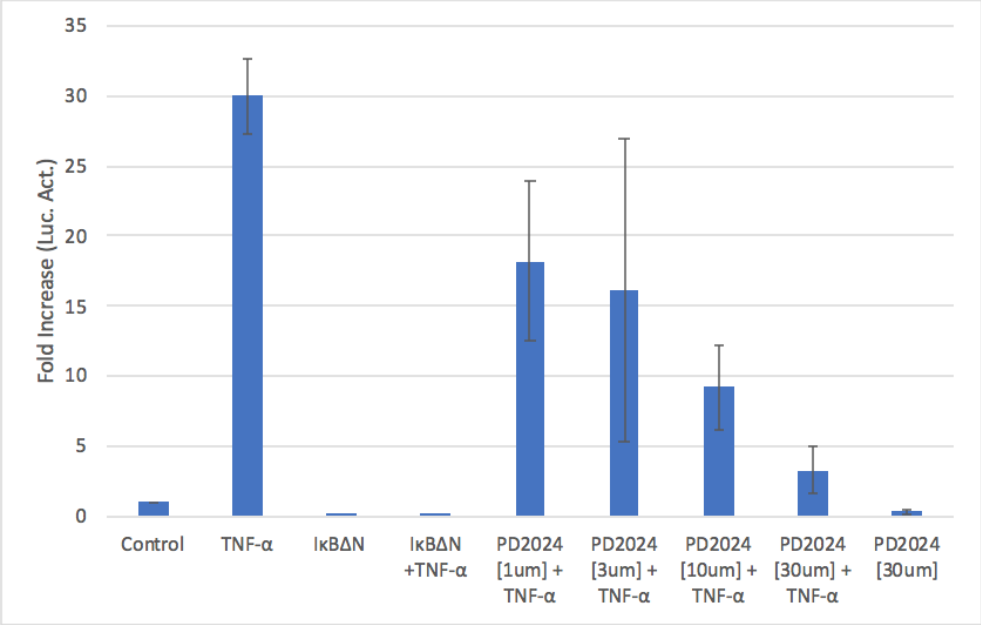

Figure 9: Effect of PD2024 on TNF-α-induced NF-κB Activation in HEK293 Cells

In figure 9, cells treated with TNF-α alone showed a twenty-four-fold increase in luciferase activity showing that it stimulated inflammation. The negative control, IκBΔN, showed a significant reduction in luciferase activity as compared to TNF-α. A significant reduction in luciferase activity was observed in cells treated with increasing concentrations of PD2024.

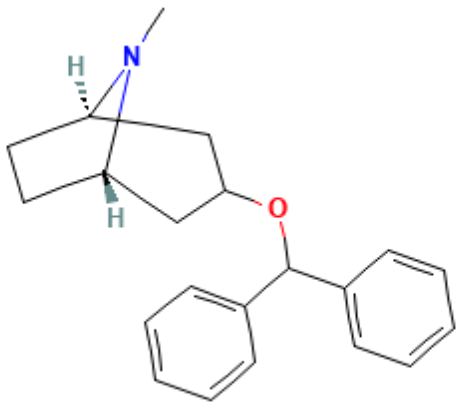

Figure 10: Benztropine Structure

Due to the nature of the research at P2D Biosciences, we are unable to reveal the true structure of the compounds we are looking at as this information is proprietary. We do know what they are derivatives of. Therefore, one of the compounds tested in this study is a derivative of benztropine. This compound is PD2244.

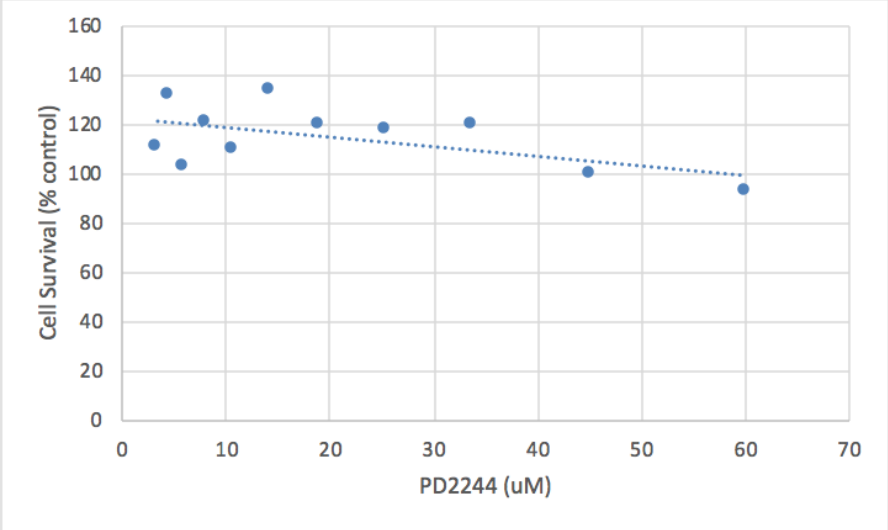

Figure 11: Cytotoxicity of PD2244 in HEK293 Cells

An MTT assay was performed in order to assess the cytotoxicity of PD2244. Identifying the range of cytotoxicity of the compound helps us select suitable concentrations to test its efficacy as an anti-inflammatory agent. Our data suggests that the compound is not cytotoxic at all concentrations of PD2244 tested, since cell survival remains above one hundred percent.

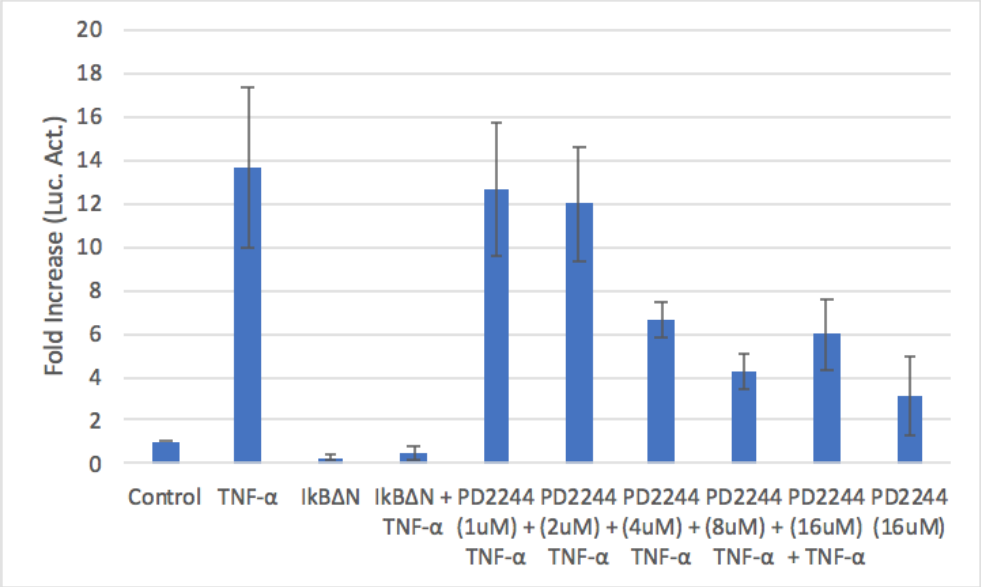

Figure 12: Effect of PD2244 on TNF-α-induced NF-κB Activation in HEK293 Cells

In figure 12, cells treated with TNF-α alone showed a thirteen-fold increase in luciferase activity showing that it stimulated inflammation. The negative control, IκBΔN, showed a significant reduction in luciferase activity as compared to TNF-α. A significant reduction in luciferase activity was observed in cells treated with increasing concentrations of PD2244. Some cytotoxicity was visually observed in higher concentrations of PD2244.

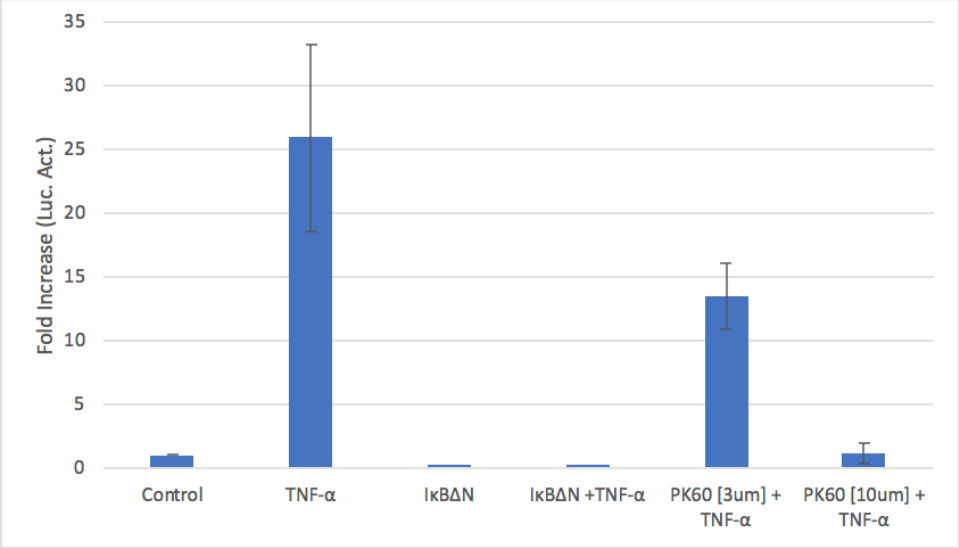

Figure 13: Effect of PK60 on TNF-α-induced NF-κB Activation in HEK293 Cells

In figure 13, cells treated with TNF-α alone showed a twenty-five-fold increase in luciferase activity showing that it stimulated inflammation. The negative control, IκBΔN, showed a significant reduction in luciferase activity as compared to TNF-α. A significant reduction in luciferase activity was observed in cells treated with increasing concentrations of PK60. Some cytotoxicity was observed at [10uM] of PK60.

DISCUSSION

NF-κB is an important determinant of inflammation because it is one of the main pathways activated in response to infection and in chronic degenerative disorders. Therefore, inhibition of NF-κB activation is a logical approach for inflammatory conditions such as AD, traumatic brain injuries, and FTD because it will prevent long term damage associated with these diseases. In this study, we were testing the capabilities of various compounds to affect cytokine induced activation of NF-κB.

To test the effect of our novel compounds on inflammation, a luciferase assay was utilized to measure TNF-alpha-induced NF-κB activation. Using the luminescence properties of luciferase, levels of activation can be measured and quantified to determine if these compounds are acting at the level of nuclear activation and transcription.

Results for the compound L2 suggest that it is not acting at the transcriptional level of the inflammation pathway. If L2 was functioning at the transcriptional level, we would expect to see a stepwise decrease in NF-κB activation with increasing concentration of L2. As shown in figure 3, regardless of concentration of drug used, we did not see a significant reduction in NF-κB activation, and thus inflammation. This suggests that the drug is reducing inflammation in the cell via a downstream part of the pathway, perhaps in regulating the function of the genes activated by NF-κB.

Results for the compounds PD340 and PD2254, the isoindolinedione derivatives, suggest that they are also not acting at the transcriptional level of the inflammation pathway. If these compounds were functioning at the transcriptional level, we would expect to see a stepwise decrease in NF-κB activation with increasing concentration of these compounds As shown in figure 5 and figure 7, regardless of concentration of drug used, we did not see a significant

reduction in NF-κB activation, and thus inflammation. This suggests that the drug is reducing inflammation in the cell via a downstream part of the pathway, perhaps in regulating the function of the genes activated by NF-κB.

Results for the compound PD2024, one of the isoindolinedione derivatives, and PD2244, the benztropine derivative, suggest that they are acting at the transcriptional level of the inflammation pathway. If these compounds were functioning at the transcriptional level, we would expect to see a stepwise decrease in NF-κB activation with increasing concentrations of these compounds. As shown in figure 9 and 12, we observed that as concentrations of each drug increased, there was a reduction in NF-κB activation and thus inflammation in the cell. This suggests that the both of these drugs' mechanisms likely occur via blocking the nuclear translocation of NF-κB and transcription of the cytokines following this translocation.

In summary, the compounds that showed a reduction in inflammation need to be explored in future studies to determine where exactly the compounds are targeting this pathway. These compounds show promise as potential anti-inflammatory drugs, and treatments for diseases such as AD.

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