STUDYING THE MODE OF ACTION OF NOVEL ANTI-INFLAMMATORY DRUGS

by

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ABSTRACT

Alzheimer's disease (AD) is ranked as the seventh leading cause of death in the US with over six million Americans currently diagnosed, and that number is projected to reach about 13 million by 2050. AD is currently believed to be caused by numerous factors ranging from genetics, lifestyle, and environmental conditions, but the exact pathogenesis of AD remains uncertain. What has been identified is the pathology associated with the disease includes the presence of amyloid beta (Aβ) plaques and neurofibrillary tangles composed of the protein tau in the brain. In cases of AD these proteins are misfolded and accumulate, causing disruptions in cell signaling and neuronal death, therefore worsening the disease. Aβ plaques also activate microglial cells, which produce cytokines and induce inflammation. Activation of an inflammasome protein, NLRP3, found in microglial cells, results in the production of the cytokine IL-1β which has been implicated in Alzheimer's due to its ability to induce and maintain this chronic cycle of inflammation, and possibly results in more amyloid-beta deposition. Studies have shown that the removal of NLRP3 results in decreased deposition of the proteins involved in AD. Our research examines novel anti-inflammatory drugs and their potential to reduce NLRP3-mediated inflammation-induced brain damage. We hypothesize that these drugs act to reduce inflammation by inhibiting a component of the NLRP3 inflammasome pathway. The levels of various proteins involved in the pathway were measured using Western Blot analysis, and preliminary results suggest that treatment with the drugs reduces LPS-induced NLRP3 inflammasome activation.

Alzheimer's Disease: Background

Alzheimer's disease (AD) is the leading cause of dementia, making up 60-70% of all cases and affecting over six and a half million Americans above the age of 65 [(1)]. Dementia is currently the seventh leading cause of death with over 55 million people worldwide diagnosed, yet there remains no cure [(2)]. Alzheimer's is a progressive neurodegenerative disease, that disrupts the normal function of neurons, resulting in symptoms such as forgetfulness, confusion, difficulty with decision-making, changes in mood, and much more [(2)]. The exact cause of the disease remains unknown, and while some genetic links have been made to mutations in proteins such as amyloid precursor protein, the vast majority of cases occur on what appears to be a sporadic basis [(3)]. Some medications are available to help ease the symptoms of the disease, but none tackle the pathology associated with the disease [(2)]. It has also been observed that in the presence of inflammation, the pathologies of AD are exacerbated, resulting in a worsening in the progression of the disease and in turn the symptoms. This has led to the development of drugs by P2D Biosciences aimed at reducing inflammation in order to slow the progression of the disease, but the exact mode by which they are acting on the cellular level is not well understood, thus leading to our hypothesis that the P2D drugs reduce inflammation by inhibiting a component of the NLRP3 inflammasome pathway.

AD Pathology

Key features of Alzheimer's pathology include the deposition of amyloid-beta (Aβ) plaques and neurofibrillary tangles (NFTs) [(3)]. Aβ originated from the Amyloid precursor protein (APP) which has been found to play a role in neuronal growth and repair [(4)]. In the brains of individuals with Alzheimer's however, there is an abundance of abnormally folded Aβ proteins that aggregate and form plaques. The enzyme gamma-secretase functions in cleaving the

transmembrane domain of the APP in the process of Aβ synthesis, so for cases of familial Alzheimer's disease (FAD) where mutations have been identified in this enzyme, researchers have begun developing therapeutic drugs with gamma-secretase as a potential target to treat this subset of individuals [5]. In addition to amyloid beta, dysregulation in the protein tau is seen as a secondary pathological signature associated with Alzheimer's. Tau is a microtubule-associated phosphoprotein (MAP) that functions in promoting and stabilizing the assembly of tubulin into microtubules in various tissues of the body [6]. In cases of dementia, a mutation can be seen in Tau in the frontotemporal region of an individual's brain resulting in abnormal hyperphosphorylation of the protein, causing the proteins to aggregate, and ultimately form the neurofibrillary tangles observed in patients.

AD & Chronic Inflammation

To date, numerous studies have identified links to chronic neuroinflammation playing a role in the progression of AD [7]. Microglia, immune cells of the central nervous system, are typically only active in the presence of some threat such as an injury or following the recognition of a pathogen. The presence of Aβ in the brains of those with Alzheimer's however has been hypothesized to result in the activation of microglia, thus resulting in the secretion of various pro-inflammatory cytokines (including interleukin-1 beta (IL-1β)), and toxic byproducts such as reactive oxygen species and nitric acid. Studies have also found that microglia function in phagocytosing Aβ, however, after a prolonged period of action, the microglia become enlarged and are no longer able to function in the clearance of Aβ. The microglia however remain active in response to the Aβ presence and continue secreting pro-inflammatory molecules that result in a self-feeding cycle of inflammation. The presence of inflammation has now also been linked to

exacerbating the pathologies associated with AD (Aβ plaques and NFTs), thus worsening the progression and symptoms of the disease.

NLRP3 Inflammasome

The NLRP3 inflammasome complex, found in microglial cells, typically plays a vital role in maintaining brain health [8]. Formation of the complex occurs as part of the innate immune response when damage-associated molecular patterns or pattern-associated molecular patterns interact with pattern-recognition receptors (PRRs), typically on the surface of cells. The NLRP3 pathway can be looked at in two stages: priming and activation. In the priming stage, activation of PRRs such as toll-like receptors, IL-1β receptors, and tumor necrosis factor (TNF) receptors leads to the activation of the transcription factor nuclear factor kB (NF-κB) that allow for synthesis of critical components of the NLRP3 inflammation pathway, specifically upregulation of NLRP3 as well as pro-IL-1β which is not constitutively expressed in the cell [9].

The activation stage can be initiated by various stimuli/ stressors from the presence of reactive oxygen species, extracellular ATP, RNA viruses, particulate matter, and more. Following exposure to the above-mentioned stimuli, the NLRP3 protein is activated and begins to oligomerize [10]. The NLRP3 protein contains three distinct domains: an amino-terminal pyrin domain, a central nucleotide-binding and oligomerization domain, and a C-terminal leucine-rich repeat domain. As part of the assembly process, NLRP3 interacts with the N-terminus of apoptosis-associated spec-like (ASC). The C-terminus of ASC contains a caspase recruiting domain (CARD) that interacts with the CARD domain of pro-caspase-1 [11]. This results in pro-caspase-1 being cleaved into its active form, caspase-1, via proximity-induced autolysis [11]. Once active, caspase-1 is released and converts pro-IL-1β into its active from IL-1β [8]. IL-1β is then

able to exit the cell via diffusion and go on to act as an inflammatory signal to neighboring cells or undergo autocrine signaling, thus amplifying the inflammatory process [(5)].

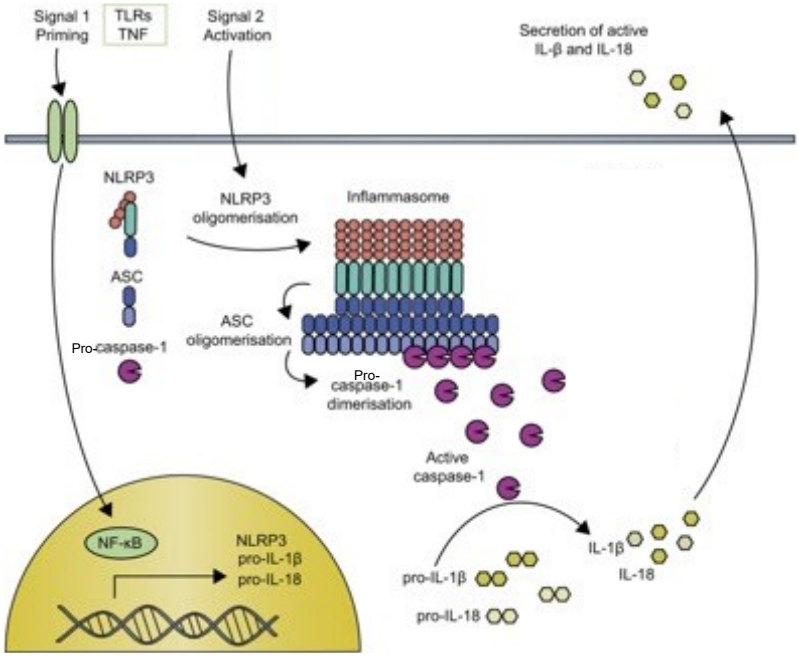


Figure 1: NLRP3 Inflammasome pathway [(8)].

P2D Anti-Inflammatory Drugs

The NLRP3 inflammasome has also been found to be active in the in the presence of Aβ oligomers and tau aggregates in microglia, therefore contributing the cycle of chronic inflammation previously discussed, and contributing to the progression of AD [(12)]. This link between chronic inflammation and AD has resulted in researchers developing drugs aimed at inhibiting the cycle of inflammation to slow down the progression of the disease, one company being P2D biosciences. P2D244 and P2D340 are two drugs developed by P2D Biosciences that have been found to have positive effects on reducing inflammation in *in vivo* mice studies. The exact mode by which these two compounds may be acting to inhibit inflammation on a cellular

level however is not well understood, thus leading to our hypothesis that P2D2244 and P2D340 act to reduce inflammation by inhibiting a component of the NLRP3 inflammasome pathway.

a) **b)**

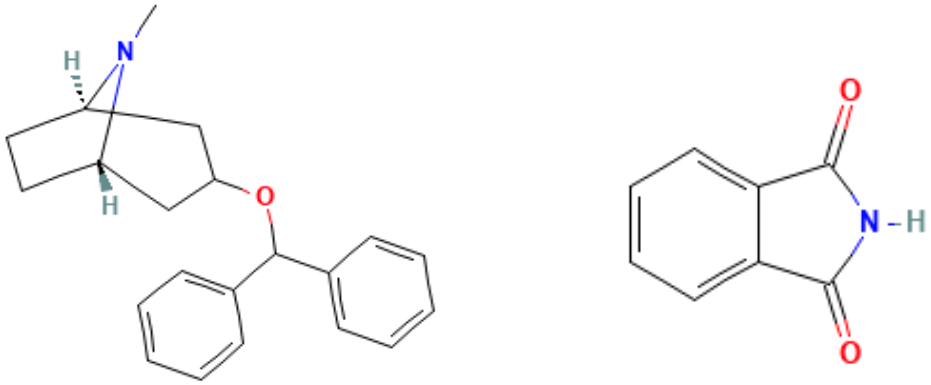


Figure 2: Parent compounds from which P2D drugs are derived. **(a)** P2D2244, tropane analog similar to benztropine. **(b)** P2D340, isoindoline analog.

Methods

Cell Culture

The mouse microglial (BV2) cell line was obtained from Dr. Michael Chumley (Department of Biology at Texas Christian University, Fort Worth, TX). The cells were cultured in high glucose Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), Penicillin (100 U)/ Streptomycin (0.1 mg/mL), and glutamine (1mM), incubated with 5% CO_2 at 37 C.

Western Blot Analysis

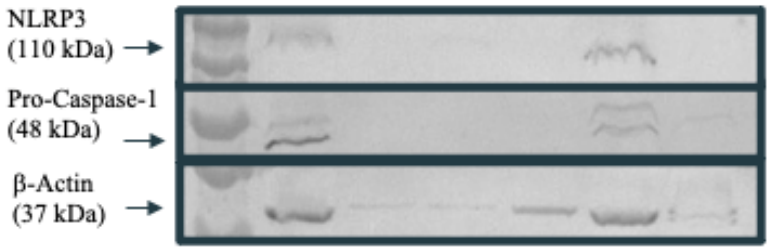
BV2 cells were plated on a 12-well plate (2.5 x 10^4 cells/well) before being treated with various concentrations of TNF-alpha for 16 hours. Following the incubation period, treated cells were extracted and harvested using RIPA buffer (from Thermo Fisher Scientific) and a protease inhibitor. The total protein concentration of each sample was then measured using a Bradford assay

to ensure equal amounts of protein were loaded per sample on a 9% sodium dodecyl sulfate-polyacrylamide (SDS) gel and separated by size. Gels were run for 75 minutes at 100 volts, following which the proteins were transferred to a polyvinylidene Difluoride (PVDF) membrane. The membrane was then blocked for one hour with BLOTTO (5% non-fat dried milk in TBST (Tris-Buffer saline with 1% Tween). Next, the membrane was incubated with various primary antibodies (1 part antibody for 500 parts BLOTTO) for half an hour on a rocker at room temperature, then overnight at 4º C. Next, the membrane was washed with BLOTTO on the shaker three cycles at 2x speed, for 10 minutes each cycle. The membrane was then incubated with the secondary antibody (1 part antibody for 1000 parts BLOTTO) for one hour at room temperature, and again washed with BLOTTO on the shaker for three cycles at 2x speed, 10 minutes each cycle. Both primary and secondary antibodies were obtained from Cell Signaling Technology. The membrane was then washed with Tris-Buffer Saline (TBS) for 5 minutes before being incubated with an Alkaline Phosphatase (AP) staining solution, NBT (from Promega), and BCIP (from Promega) following the manufacturer's protocol and left until the visualize bands. Lastly, images of the blots were analyzed using ImageJ software to quantify the data/ band thickness.

Results

a)

LPS (100 µg/mL)	-	+	+	-	-	-
TNF-α (5 mM)	-	-	-	+	+	+
ATP (5 mM)	-	+	-	+	-	-



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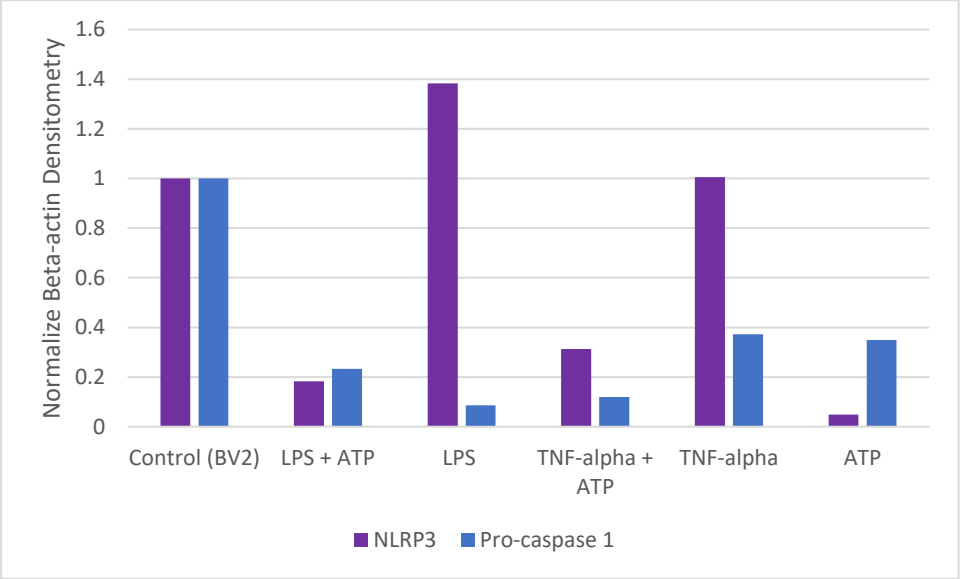
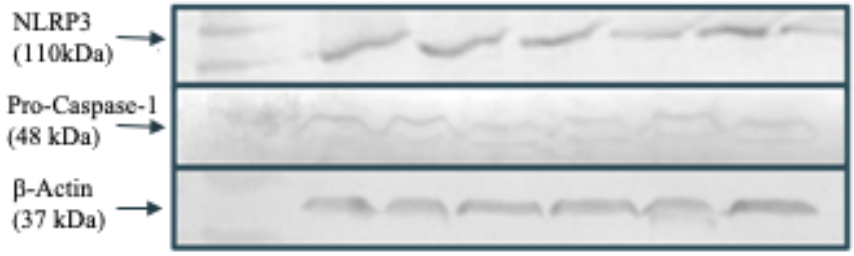


Figure 3: Effect of LPS, TNF-a, and ATP on the activation of the NLRP3 inflammasome. **(a)** Western Blot of BV2 treated with LPS and TNF-α. **(b)** Quantification of Western Blot using ImageJ analysis.

The goal of the experiment in **Figure 3** was to stimulate the inflammatory response in BV2 cells with LPS (an activator of the innate immune response) and TNF-α (a-strong inducer of the inflammatory response) to simulate a hallmark of Alzheimer's. A 1.4-fold increase in NLRP3 levels was observed in cells treated with LPS, compared to the control, while a 10-fold decrease was observed in levels of pro-caspase-1. No significant change was seen in NLRP3 levels in cells treated with TNF-α alone, but a roughly three-fold decrease was seen in pro-caspase-1 levels. A roughly five-fold decrease was seen in levels of both NLRP3 and pro-caspase-1 in cells creased with LPS and ATP. Similarly, a three-fold decrease in NLRP3 levels and a ten-fold decrease in pro-caspase-1 levels was observed in cells treated with TNF-α and ATP, potentially a result of cell death.

a)

LPS (100 mg/mL)	-	+	+	+	+	+
P2D2244 (3 μM)	-	-	+	+	-	-
P2D340 (3 μM)	-	-	-	-	+	+



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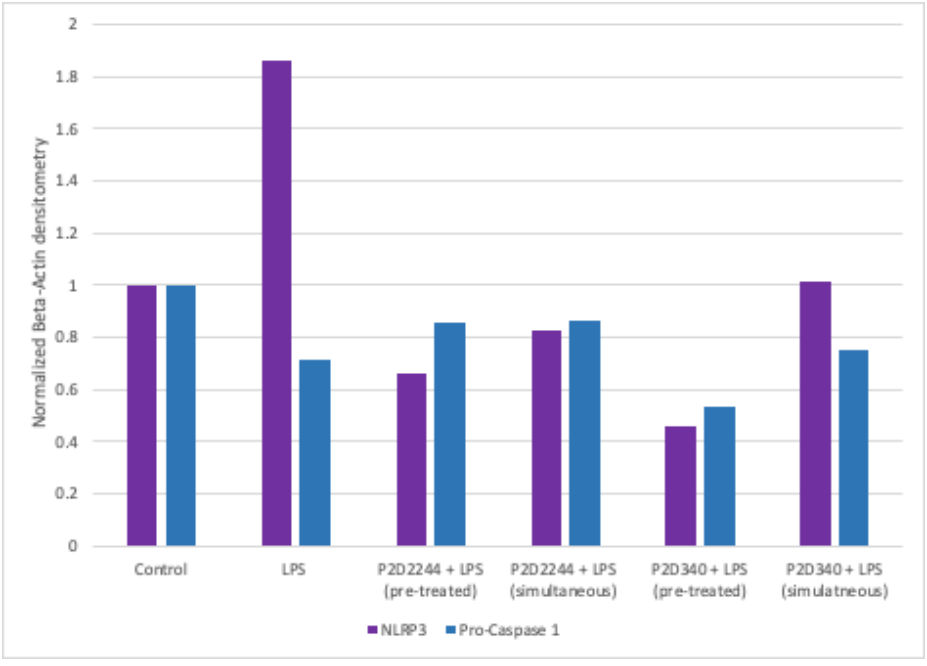

Figure 4: Effects of PD2244 and PD340 on Activation of the NLRP3 Inflammasome **(a)** Western Blot of BV2 treated with P2D2244 or P2D340 in the presence of LPS. Lanes 3 and 6 were pretreated with the drugs for 1 hour. Lanes 4 and 5 were treated with the drug and LPS simultaneously. **(b)** Quantification of Western Blot using ImageJ analysis.

Having shown that we were able to assess the activation state of the NLRP3 inflammasome using Western Blot, we next sought to determine whether the treatment of cells

with P2D2244 and P2D340 would result in the inhibition of the NLRP3 inflammasome pathway (**Figure 4**). We again did this by measuring various protein levels via Western Bot and analyzing imaging with ImageJ software. BV2 (mice microglial) cells were treated with both drugs (P2D340 and P2D2244) using two different protocols. In the first protocol, cells were pretreated with drug for 1 hour prior to treatment with LPS, in the second, cells were exposed to drug and LPS simultaneously. A 1.8-fold increase in NLRP3 was observed in the cells treated with LPS alone, along with a 30% decrease in pro-caspase-1 levels. In cells that were pre-treated with P2D2244 an hour before treatment with LPS, the level of NLRP3 was 35% of that seen in cells treated with LPS alone and about 66% of that seen in the control group. Levels of NLRP3 and pro-caspase-1 near that of the control were observed in BV2 treated with LPS and P2D2244 simultaneously.

Discussion

Alzheimer's disease is ranked as the seventh leading cause of death in the US with over 6 million Americans currently diagnosed, and that number is projected to reach about 13 million by 2050. AD is currently believed to be caused by numerous factors ranging from genetics, lifestyle, and environmental conditions. The pathogenesis of Alzheimer's however remains even less certain as scientists continue looking into theories based on the factors related to the disease such as the formation of amyloid beta peptide (Aβ) plaques and neurofibrillary tangles in the brain. The Aβ plaques seen in AD disrupt normal function and induce inflammation, activating microglial cells, which produce cytokines and induce chronic inflammation. Studies have shown that the inhibition of NLRP3 results in decreased deposition of the proteins involved in AD [(10)]. Our study aims to understand the mode of action of novel anti-Alzheimer drugs. Cytokines are signaling molecules produced by immune cells that mediate inflammatory signaling. Activation

of the inflammasome protein NLRP3, found in microglial cells, results in the production of the cytokine IL-1β which has been implicated in Alzheimer's due to its ability to induce and maintain the cycle of inflammation. The goal of our research was to investigate novel anti-inflammatory drugs and their potential to reduce NLRP3-mediated inflammation-induced brain damage which could ultimately slow down the progression of the disease.

There are currently no approved drugs used to treat AD that work via the inhibition of the NLRP3 inflammasome, however several are in the trial stage and have been proposed for approval by the US Food and Drug Administration (FDA). One such drug being Entrectinib (ENB) which acts to inhibit the NLRP3 inflammasome by targeting the singling intermediate protein NEK7[(14)]. NEK7 is a mammalian NIMA-related kinase (NEK protein) that acts upstream of the NLRP3 protein, effecting oligomerization and activation of NLRP3 by interacting with NLRP3 following potassium efflux [(15)]. The anti-inflammatory components of these drugs have the potential to ease a wide variety of inflammasome-related diseases beyond Alzheimer's, including arthritis, inflammatory bowel diseases, and much more [(16)].

In collaboration with the company P2D Biosciences, we tested two drugs, P2D2244 and P2D340 for their ability to inhibit the NLRP3 pathway. These drugs were shown to have a positive effect in reducing LPS-induced IL-1β and TNF-α secretion in mouse brain (P2D Biosciences Unpublished results). However, the mechanism by which they act on a cellular level is not known. The NLRP3 inflammasome is believed to play a role in the progression of AD as its activity is upregulated in response to detecting protein aggregates in the brains of those with Alzheimer's, resulting in further progression of the disease [(13)]. We therefore hypothesized that the positive effect these drugs have on Alzheimer's pathology is by reducing inflammation in the brain, specifically by inhibiting activation of the NLRP3 inflammasome. If the hypothesis is true,

treatment of mouse microglial cells with our anti-inflammatory drugs should result in the inhibition of LPS-induced activation of the NLRP3 inflammasome.

Following treatments with LPS, levels of NLRP3 increased while levels of pro-caspase-1 decreased relative to their controls as was expected. Next, as hypothesized, a decline of NLRP3 levels was observed in groups treated with PD2244, and the levels of pro-caspase-1 were closer to that of the control compared to the group treated with LPS alone, suggesting that our drug may inhibit the cleavage of caspases by inhibiting the activation of NLRP3. It is worth noting that the group pre-treated with P2D2244 for an hour showed a greater decline in levels of NLRP3, but virtually identical levels of pro-caspase-1 compared to cells treated with LPS alone. Similarly, the groups treated with PD340 saw a decrease in levels of NLRP3 expression, however, levels of pro-caspase-1 did not see a significant rebound.

In conclusion, our preliminary results suggest that these novel drugs decrease activation of the NRLP3 inflammasome, as assessed by Western Blot. These results are preliminary in nature and need to be replicated to be confirmed. Other methods of assessment may also be used, such as ELISA assays, to measure the secretion of IL-1β. The observed inhibition in the first component of the pathway, the NLRP3 protein itself, may also indicate that the drug is acting on an upstream component of inflammation such as NF-κB. NF-κB is a transcription factor responsible for the synthesis of various proteins involved in the immune response, including NLRP3 and pro-IL-1β, therefore similar results could be observed (e.g. an increase in NLRP3) if the drug act to inhibit NF-kB [(9)].

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