Screening Novel Triazole Aurones for Anti-Inflammatory Properties

By
Ansley Morgan

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Thesis Committee:

Dr. Anthony Farone, Thesis Director

Dr. Paul Kline, Second Reader

Dr. Kim Sadler, Thesis Committee Chair

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APPROVED:

_____
Dr. Anthony Farone, Thesis Director
Professor, Biology Department

Dr. Paul Kline
Professor, Chemistry Department

_____
Dr. Kim Sadler, Thesis Committee Chair
Professor, Biology Department

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ABSTRACT

Inflammatory diseases affect millions of people and are typically treated with steroids or nonsteroidal anti-inflammatory drugs (NSAIDs) with serious side effects. A subgroup of flavonoids, called aurones, are potential alternatives. To test this hypothesis, 45 triazole aurones (ATs) were screened for anti-inflammatory characteristics in mouse macrophages. An inflammatory response was induced by the bacterial molecule, lipopolysaccharide (LPS), ATs were added, and the inflammatory response was determined. Nitric oxide (NO), and cell viability assays, indicated two ATs (AT 102 and AT 122) reduced NO without causing cytotoxicity. These were further analyzed for suppression of the pro-inflammatory cytokine tumor necrosis factor-alpha (TNF-α). Our analysis demonstrated that although AT 102 and AT 122 decreased NO production, TNF-α was not suppressed. This is possibly due to suppression of the signal transduction protein IκBζ, which is involved in normal inflammatory response regulation. Further studies are ongoing to elucidate this mechanism of action.

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Introduction

The innate immune system serves as a first line of defense against pathogens and responds to danger signals due to tissue damage or microbial invasion. This system provides a response to invading pathogens while triggering the adaptive immune response (Anaya *et al.,* 2013). It is comprised of many components including physical and anatomical barriers and phagocytic cells, such as macrophages that engulf and kill bacteria. These cells are responsible for stimulating the release of inflammatory mediators.

Inflammation is the result of the host's immune system recognizing, and removing, foreign stimuli. Chronic inflammation can be caused by autoimmune disorders, defects in the cells responsible for mediating inflammation, or the failure to eliminate an agent that causes acute inflammation (Pahwa *et al*., 2020). Crohn's disease, rheumatoid arthritis, and chronic obstructive pulmonary disease are common chronic inflammatory diseases. Non-steroidal anti-inflammatory drugs are commonly used medications utilized for chronic inflammation (Saad and Mathew, 2020.). This class of drugs consists of aspirin, ibuprofen, and naproxen that are known to suppress proinflammatory cytokines such as interleukin (IL)-1α, IL-1β, IL-6, TNF-α, and prostaglandins that cause the typical symptoms of inflammation such as fever, chills, and muscle stiffness (Maroon *et al*., 2010).

Inflammatory cytokines IL-1(α and β) are secreted by macrophages and large granular lymphocytes and can lead to cell death by apoptosis, macrophage stimulation, and fever (Justiz Vaillant and Qurie, 2020; Dinarello, 2018). IL-6 is produced by macrophages and fibroblasts and is involved in signaling that regulates inflammation

(Justiz Vaillant and Qurie 2020; Valle *et al.,* 2017). Another inflammatory cytokine produced by macrophages is TNF-α. It is produced during acute inflammation and is responsible for several signaling events that may lead to apoptosis (Idriss and Naismith, 2000). It is also rapidly released after trauma and plays a vital role in the pro-inflammatory cytokine amplification cascade (Parameswaran and Patial, 2010). Most inflammatory cytokines are able to be produced by macrophages, which are specialized immune cells involved in detection, phagocytosis, and destruction of harmful microorganisms. Macrophages also provide immediate defense against foreign pathogens before leukocyte migration and produce pro-inflammatory mediators, such as nitric oxide (NO) (Moncada *et al.,* 1991). NO, an intercellular mediator, can act as a defense molecule against infectious organisms, but when it is overproduced it can lead to tissue damage associated with chronic inflammation (Tripathi *et al.,* 2007).

Having increased concentrations of TNF-α can trigger the nuclear factor-kappa light chain enhancer of activated B cells (NF- κB) signaling pathway in inflammatory cells (Liu, Y *et al.,* 2019). NF-κB is responsible for inducing expression of genes that code for IL-1β, IL-6, and cyclooxygenase (COX) 2 (Liu, T *et al.,* 2017). Cyclooxygenase 2 is an inducible enzyme that is responsible for producing prostaglandins, which induce inflammation (Ricciotti and Fitzgerald, 2011). Medications such as Celebrex and Bextra became popular to reduce inflammation due to selective inhibition of COX 2 (Fitzgerald, 2004). Though chronic inflammation can be mitigated by NSAIDs, long term use of these drugs has side effects, including gastrointestinal problems and increased risk of cardiovascular events (Maroon *et al.,* 2010). The commonality of these side effects creates a need for more natural, alternative therapies.

Flavonoids are a group of phenolic compounds that are responsible for the color of some plants and fruits, including lemons, grapefruits, and buckwheat (Kumar and Pandey, 2013). Flavonoids have been shown to have antiviral, anti-inflammatory, and anti-cancer properties, and can inhibit the expression of cyclooxygenase and lipoxygenase, inhibiting inflammation (Kumar and Pandey, 2013; Tunon *et al.,* 2009). The aurones tested in this study are a subgroup of modified flavonoids responsible for producing the yellow color of flowers, such as the yellow snapdragon, *Antirrhinum majus,* and have been shown to possess anti-inflammatory characteristics (Park *et al.,* 2016; Sato *et al.,* 2001). Recently Kafle *et al*. was able to synthesize aurones with a triazole structure (Figure 1) termed AT (Kafle *et al.,* 2020).

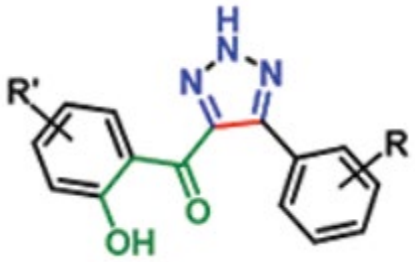

Figure 1: Triazole Aurone Structure This is the basic structure of a triazole aurone that was synthesized by Kafle *et al.* Each of the different triazole aurones would differ in their R groups.

The triazole structure is known to be immunomodulatory (Kharb *et al.,* 2011). Therefore, we hypothesized that newly synthesized triazole aurones may also have anti-inflammatory characteristics.

Using the murine Raw 264.7 macrophage-like cell-line, 45 novel ATs were screened. The process involved stimulating the Raw cells with LPS. LPS, also known as endotoxin, is found on the surface of gram-negative bacteria. It can stimulate an immune response through interacting with the membrane receptor CD14 found on macrophages (Meng and Lowell, 1997). When LPS binds to CD14, CD14 transfers LPS to the Toll Like Receptor 4 (TLR4)-MD2 complex (Gioannini *et al.,* 2004). This interaction initiates the signal transduction pathway and ultimately causes the expression of cytokines such as IL-1, IL-6 and TNF-α (Meng and Lowell, 1997). Once inflammation had been stimulated in the Raw cells, individual aurones were added to determine if the inflammatory response had been suppressed. Several inflammatory inhibitors, including Bay 11, dexamethasone, and dimethyl sulfoxide vehicle control (DMSO) were used as standards in order to validate the results.

Materials and Methods

2.1 Cell Culture

Raw 264.7 murine macrophages were cultured in Dulbecco's Modified Eagle's medium (DMEM), containing 4.5 g/L glucose, L- glutamine, sodium pyruvate, 10% fetal bovine serum (FBS), and penicillin-streptomycin**.** The cells were grown in an incubator at 37ºC supplemented with 5% CO_2 and passaged every 3-5 days. One tenth of the culture volume is passaged into a new flask containing fresh medium. Seeding a plate for screening involves following the same process as above except trypsin is used to dislodge the cells. The cells are then counted on a hemocytometer using trypan blue as a cell

viability stain. After counting, the cells are then diluted in medium until there are 5 x 10^4 cells/well. The cells are allowed to attach to the plastic surface of the wells for 16-18 hr.

2.2 Treatments

The stock solutions of 1 μg/mL LPS, 100 μM dexamethasone, 100 μM Bay 11, and 100% dimethyl sulfoxide (DMSO) were diluted using an Opentrons OT-2 pipetting robot. The DMSO treatment consisted of 100 ng/mL LPS and 1% DMSO. The dexamethasone treatment consisted of 100 ng/mL LPS and 10 μM dexamethasone. The Bay 11 treatment consisted of 100 ng/mL LPS and 1 μM Bay 11. The LPS treatment consisted of 100 ng/mL LPS. The AT treatments consisted of 100 ng/mL LPS and 100 μM of AT. The cell plates were washed with PBS, and the treatments were pipetted onto it in triplicate. The plate was then incubated for 24 hr.

2.3 Cytotoxicity and NO Assays

The cell culture supernatants were collected and frozen for future analysis. The remaining cells were washed with PBS, and a Presto Blue viability stain, containing a 1:10 dilution of Presto Blue in medium, was pipetted onto the plate. This plate was then incubated for 1 hr. and was analyzed for the cell viability assay. Griess reagent for the NO assay and half of the previously harvested supernatant were pipetted onto a 96 well plate containing the nitrite standard curve, and the plate was incubated for 15 min. After the incubation periods, the plates were analyzed on a plate reader. The plate containing the nitrite standard curve was read first for the NO assay, and a linear regression fit was calculated. The r^2 value was then checked and must be at least 0.98 for the data to be accurate. The average and standard error were then calculated using the plate reader software. The values for the standards were also compared, and if in the range of the

known values then the experimental values for the AT's were assumed to be accurate. After incubation for 1 hr., the cell viability plate was complete, the plate was read and absorbance values for readings at wavelengths 570 nm and 600 nm were calculated. The difference in absorbance was then calculated, as well as the average and standard error. The experimental AT values were then compared to the values of the standards, and if an AT treatment was able to significantly reduce NO production compared to the vehicle control while having low cell toxicity it was considered for further investigation.

2.4 ELISA

An 800 ng/mL capture antibody in PBS was incubated overnight, on a hi-bind 96-well plate, at room temperature. After incubation, the plate was washed with 0.05% Tween-20 in PBS using a plate washer. The plate was blocked with Reagent Diluent for 1 hr. The plate was washed and a 1:100 dilution of the supernatants and a standard curve ranging from 2000 pg/mL to 31.2 pg/mL were pipetted onto the plate, incubated for 2 hr., then washed. 75 ng/mL of detection antibody was added to the plate. The plate was incubated for 2 hr., washed again, and a 1:40 dilution of Streptavidin-HRP was added for 20 min., then washed. A Substrate Solution containing equal parts of Color A and Color B was added for 20 min., then the reaction was halted using 50µL 2N Sulfuric Acid. Absorbance for each sample was determined using a plate reader for wavelengths 450 nm and 570 nm. A 4-parameter linear regression fit was calculated with r^2 value of at least 0.98.

2.5 Statistical Methods

An ordinary one-way ANOVA test was performed on the N=9 data set from initial screening cell viability assay, the dose-dependent NO assay and on the results of

the ELISA using GraphPad software. The mean results were compared to the vehicle control column for all three data sets.

Results

Initial screening of 45 novel triazole aurones are shown in Figure 2. Several samples caused an increase in NO production compared to the vehicle control (Figure 2). Though many samples had a high standard error, they were therefore inferred to not be anti-inflammatory, resulting in them being eliminated as the topics for further research. Many other ATs had a lower amount of NO production than the vehicle control but also had low cell viability. This meant that the low level of NO production was potentially due to the AT causing cell death. These cytotoxic samples were also ruled out of further research. AT 102 and AT 122 both showed extremely high cell viability and lowered NO production, indicating that these samples were able to reduce inflammation without killing the cells (Figure 2).

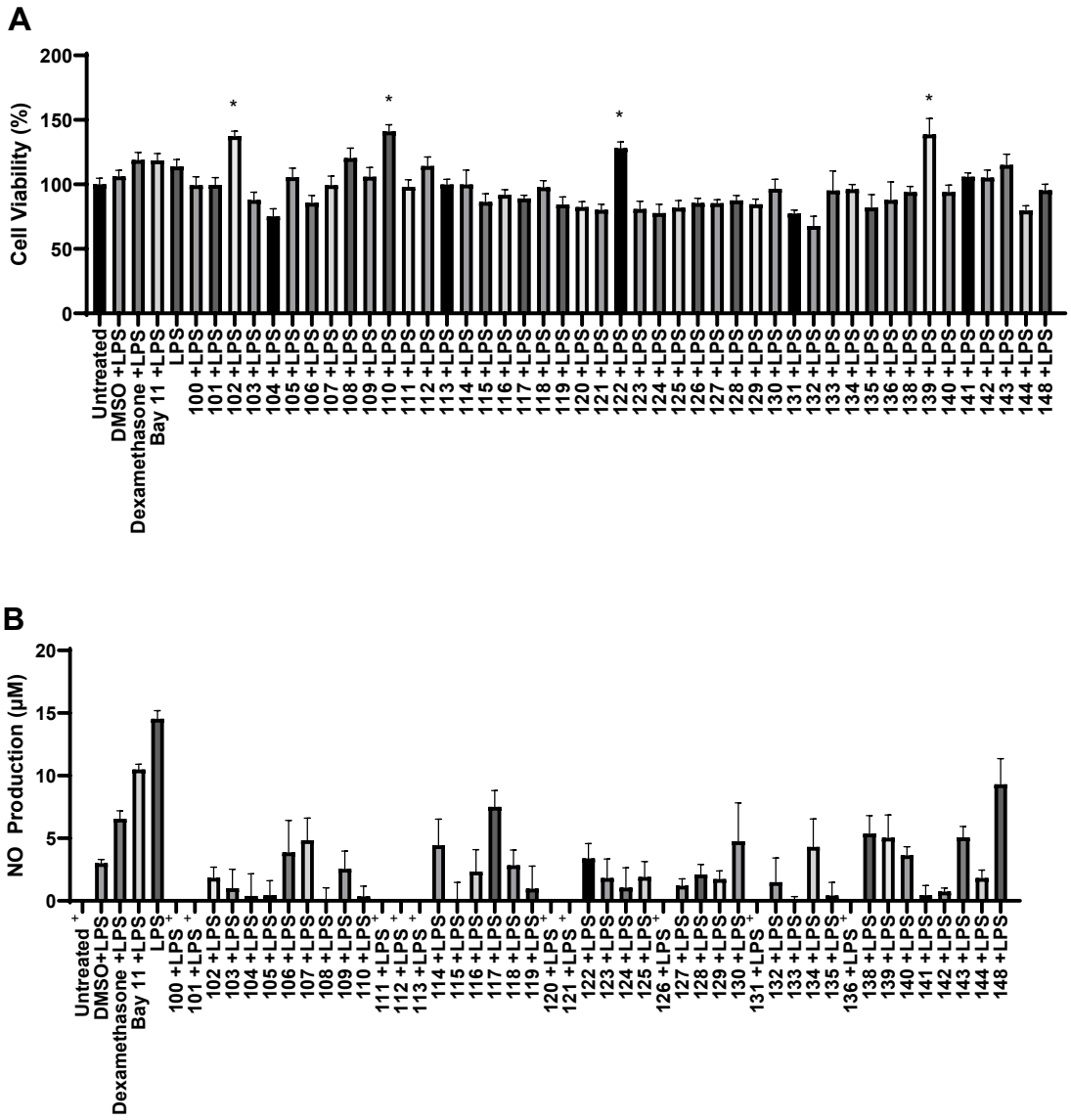

Figure 2: Cell Viability and NO Production. A shows the results of an ordinary one-way ANOVA on the *N* =9 initial screening cell viability data set. The asterisks represent samples that had significantly higher cell viability than the DMSO vehicle control. **B** shows the average NO production of each sample *(N*=9). Samples with a $^{+}$ showed values that were lower than the limit of detection.

To further investigate these effects, AT 102 and 122 (Figure 3) were examined at increasing concentrations to determine if there was a dose-dependent effect.

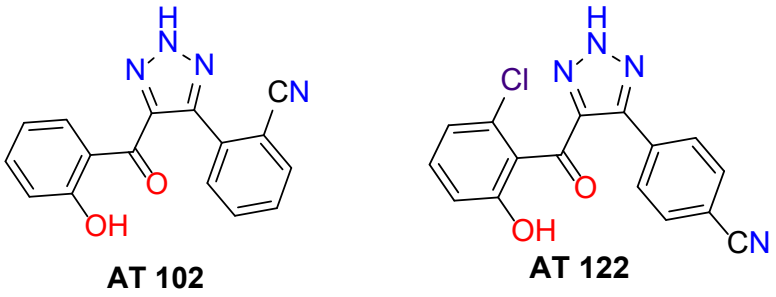

Figure 3: AT 102 and AT 122 Structures. This figure shows the structures of the two ATs chosen for further investigation. These structures were synthesized, and images provided by Kafle, A., Bhattarai, S. and Handy, S. T.

The same method for NO and cell viability assays were used. The highest concentration was 25 μM and a serial dilution was performed. The expected results were that the amount of NO produced would increase as the concentration of the AT decreased because the weaker concentrations of AT would not be able to inhibit NO production caused by the addition of LPS. The results of the ANOVA, shown below in Figure 4, showed this trend, but did not show that there were significant differences between the differing concentrations and the vehicle control.

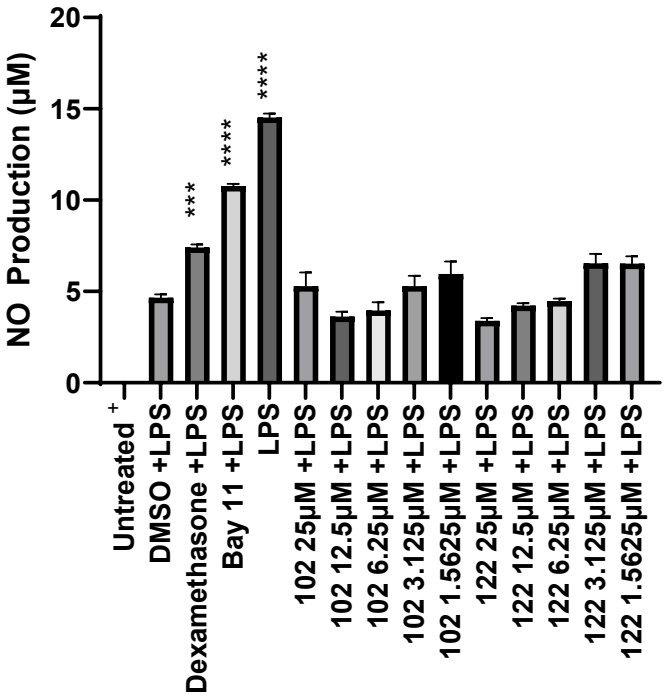

Figure 4: Dose-Dependent NO Production. This graph shows the amount of NO that was produced by differing concentrations of AT 102 and AT 122. The asterisks represent how significant the difference was between the different samples compared to the vehicle control according to the results of the ANOVA. Samples with a $^{+}$ showed values that were below the limit of detection.

The ELISA for TNF-α results were unexpected. In many studies, TNF-α levels follow the NO trends. However, Figure 5 illustrates that cell supernatants containing a higher concentration of AT that produced low levels of NO had high levels of TNF-α present. Almost all samples also showed a significantly higher amount of TNF-α present than the vehicle control.

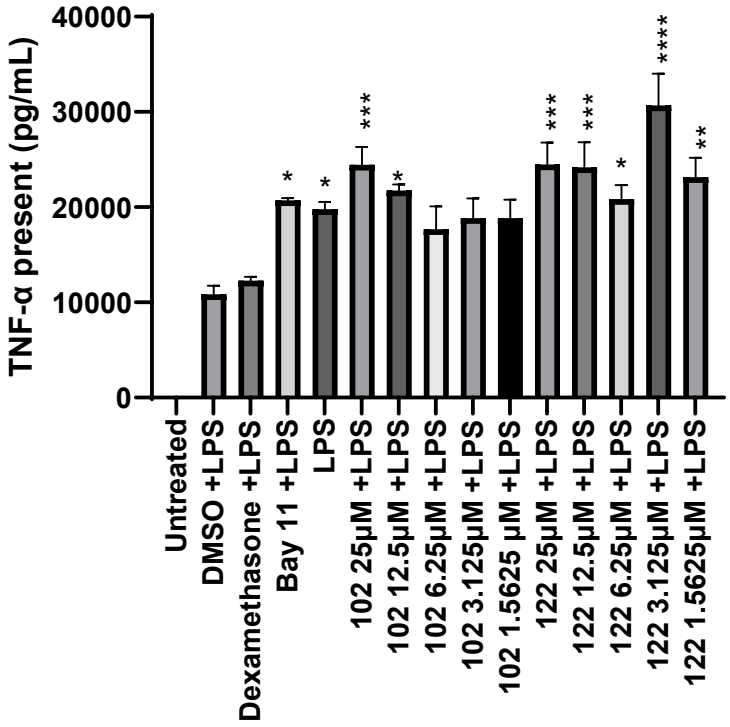

Figure 5: TNF-α Production. This graph shows the differing levels of TNF-α present in each sample. The asterisks represent how significant the difference between the samples and the vehicle control was according to a one-way ordinary ANOVA.

Discussion

Interestingly, the triazole aurones, AT 102 and AT 122, were able to reduce the amount of LPS induced NO production in the initial screenings but during the dose-dependent assays no sample produced a result that was significantly different than the DMSO vehicle control (Figure 3). The expected trend was present, but the differences between samples and the vehicle control were not statistically significant. Though the concentration of each AT was reduced by two-fold for each dilution, only small differences in NO production were observed. DMSO, an anti-inflammatory agent, is able to reduce inflammatory cytokine production in LPS stimulated macrophages (Elisia *et al.,*

2016). The lack of significant differences could be due to the vehicle control containing four times more DMSO than the highest concentration did during the dose-dependent assays. The vehicle control contained 1% DMSO and the sample containing the highest concentration in the dose-dependent assays, 25 μM, only contained 0.25% DMSO. The concentrations of the ATs also could have been too low for them to produce statistically different effects. Nevertheless, the initial screening containing 100 μM ATs did show a decrease in NO production, indicating a slight anti-inflammatory effect (Figure 2).

High levels of inflammatory cytokine TNF-α should correlate with high levels of NO production. Surprisingly, the TNF-α did not demonstrate the typical effect. It seems to suggest that although AT 102 and AT 122 were able to decrease NO production, TNF-α expression actually increased. This may be due to a different mechanism of action. There has not been any published work on the mechanism of action of ATs, but we are able to speculate that a possible mechanism of action may be to suppress NF-κB activity. This is due to LPS being able to activate NF-κB pathways and the role of inhibitor of kappa B zeta (IκBζ) in LPS-stimulated macrophages (Kitamura *et al.,* 2003). IκBζ is a member of the inhibitor of kappa B proteins and shows an increased amount of expression after LPS-stimulation (Kitamura *et al.,* 2003). In this pathway IκBζ interacts with the p50 subunit of NF-κB in the nucleus and causes an increase in pro-inflammatory cytokines. Although IκBζ is able to promote production of certain pro-inflammatory cytokines, it suppresses TNF-α production (Hildebrand *et al.,* 2013). This suggests that AT 102 and AT 122 may act by suppressing IκBζ in their mechanism of action. This could potentially explain the decrease in NO production and the increase in TNF-α levels shown by the ELISA.

Many chronic inflammatory diseases can lead to patients living a lifetime of pain. Although, there are many treatments currently available the most commonly used treatments involve taking NSAIDs, which are beneficial for treating acute inflammation, but when taken for long periods of time can have detrimental side effects. Long term use of NSAIDs can cause peptic ulcer disease, acute renal failure, strokes, myocardial infarctions, and can worsen other chronic conditions (Marcum and Hanlon, 2010). Exploring more natural options could be the solution due to their ability to be used for long periods of time without detrimental effects (Maroon *et al.,* 2010). Flavonoids have already been a topic of research and some such as Quercetin and Apigenin are already available commercially. Quercetin is a yellow pigment present in plants that is commonly taken as a dietary supplement and has been used in treatments for diabetes, irregular blood pressure, and other cardiovascular issues (Patel *et al.,* 2018; Eid and Haddad, 2017). Apigenin is another yellow pigment found in plants but possess anticancer, antidiabetic, and anti-inflammatory properties (Salehi *et al.,* 2019). ATs are a subgroup of flavonoids that have already been shown to possess anti-inflammatory characteristics, so the combination of all of those characteristics suggests the potential to be anti-inflammatory therapeutics. This project demonstrated that two novel ATs were able to decrease NO production without harming the cell, meaning that they could potentially serve as a treatment for inflammation.

Conclusion

The purpose of this study was to determine if 47 novel triazole aurones possessed anti-inflammatory properties. NO and cell viability assays showed that there were a few

compounds that were able to reduce NO production, compared to the vehicle control of 1% DMSO, while also maintaining high cell viability. AT 102 and AT 122 were chosen for further investigation that examined their dose-dependent effect on LPS induced NO and TNF-α production, because those compounds were able to reduce NO production with no apparent cytotoxicity. In particular, these studies demonstrated an unexpected increase in the amount of TNF-α present in all doses. Further investigation of the mechanism of action of these triazole aurones could better explain the details of what was observed.

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