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Comparing the Anti-Inflammatory Properties of STATTIC to Indirubin-E804 in RAW264.7 Macrophages

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COMPARING THE ANTI-INFLAMMATORY PROPERTIES OF STATTIC TO INDIRUBIN-E804 IN RAW264.7 MACROPHAGES

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ABSTRACT

Natural products containing derivatives of the basic indole backbone have gained significant interest in the use against cancer cells, inflammation, and a multitude of disorders in the human body. The indole backbone is present in several endogenous hormones such as serotonin and melatonin. Additionally, the indole-containing amino acid tryptophan is the starting structure for many endogenous metabolites controlling natural physiological homeostasis such as circadian rhythms, healthy gut microbiota, and gut health. Natural indirubin is a deep red bis-indole isomer of indigo blue, both of which are biologically active ingredients used to treat neoplasia, chronic inflammation, and enhance xenobiotics' detoxification. Naturally derived indirubins and other indole-containing compounds have been shown to have anti-proliferative effects, mainly attributed to the inhibition of the cell cycle-related kinases, such as cyclin-dependent kinases (CDKs) and glycogen synthase kinase-3β (GSK-3β) with varying degrees of potency. Many indirubins are also aryl hydrocarbon receptor (AHR) agonists, with AHR-associated activities covering a wide range of potencies, depending on molecular structure. The AHR is a ligand-activated transcription factor that promotes drug-metabolizing enzymes leading to the degradation of these indirubins in an AHR-dependent manner. Our lab previously described the anti-inflammatory properties of indirubin-3'-(2,3 dihydroxypropyl)-oximether (E804), a novel indirubin derivative with potent STAT3 inhibitory properties, in murine RAW264.7 macrophages stimulated with lipopolysaccharide (LPS). This study compared the effects of a novel STAT3 inhibitor, 6-nitrobenzo[*b*]thiophene 1,1-dioxide (STATTIC), which is structurally designed as an

indole backbone to E804, on LPS-stimulated macrophage functions. I also determined if the effects of both STATTIC and E804 on these macrophage functions are modified by the AHR antagonist, 6,2',4'-trimethoxyflavone (TMF). Initial studies using AHR reporter transactivation assays show that E804, but not STATTIC or TMF, is an AHR agonist, which further corroborates early studies showing that E804 induces the drug-metabolizing monooxygenases (CYP1A1 and CYP1B1). Additionally, I demonstrate that STATTIC is more potent than E804 in suppressing LPS-induced IL-6 secretion, iNOS protein expression, and nitric oxide production. Macrophage intracellular reactive oxygen species (ROS) naturally generated during LPS-stimulation was suppressed by low levels of STATTIC but not by higher levels. In contrast, similar concentrations of E804 suppress ROS production. STATTIC completely inhibited phagocytosis, and less so by E804. When examined alone, TMF has anti-inflammatory properties as well, and when combined with E804 and STATTIC, further enhanced these compounds' effects. Collectively, these results indicate that E804 and STATTIC are potent modulators of pro-inflammatory profiles in LPS-treated macrophages. Additionally, these results suggest that AHR antagonism by TMF may antagonize the degradation of E804 and prolong its anti-inflammatory properties. Since STATTIC did not bind the AHR, any treatments using this novel small molecule may not require co-treatment such as TMF.

DEDICATION

I would like to dedicate this thesis to my family, who have helped me grow into the individual I am today. I would not have been able to get to where I am without the love and support I have received from them. My family always supported me in all aspects of life without asking for anything in return. They taught me how to work hard and to never give up in life, and for that, I am truly thankful. I would also like to dedicate this to all of my friends. Whenever I needed help with anything, they were there for me. I will always be grateful to them for all the memories we have made together.

ACKNOWLEDGMENTS

I want to thank my adviser, Dr. Charles D. Rice, for his help and advice throughout this entire process. I would also like to thank my lab mate Kursten Anderson for her assistance and friendship whenever I needed it.

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LITERATURE REVIEW

Macrophages in Inflammation

Inflammation is a complex response to protect the body from a broad range of insults ranging from toxic substances to invading pathogens. Acute inflammation is beneficial in destroying local pathogen invaders, but a sustained chronic inflammatory response can lead to pathology, such as autoimmunity that affects the gastrointestinal tract, the central nervous system, kidneys, skin, lungs, and joints (Ospelt, 2010). Upon tissue damage or pathogen recognition, the inflammatory response is coordinated by activating various signaling pathways that regulate both pro-and anti-inflammatory mediators in resident tissue cells and leukocytes recruited from the blood (Lawrence, 2009).

Macrophages play an essential role in innate immunity as phagocytes, production of inflammatory cytokines, and initiation of the adaptive immune response by presenting antigens to T-lymphocytes (Janeway et al., 2001). Macrophages can be polarized into two different phenotypes, which have been historically described as classically activated (M1) and alternatively activated (M2) (Lee et al., 2020). M1 macrophages are typically induced by interferon-γ (IFN-γ) with a Toll-like receptor (TLR) agonist such as lipopolysaccharide (LPS) and are considered pro-inflammatory with the production of cytokines such as IL-1, IL-6, IL-12, and IL-23 (Lee et al., 2020). M2 macrophages are induced by T helper 2 (TH2) $CD4^+$ T cells which produce IL-4, IL-10, and IL-13, and are

considered anti-inflammatory through the production of cytokines such as IL-10, IL-22, and TGF-β (Murray 2017, Lee et al. 2020).

Different immune responses require M1 and M2 macrophages. Accordingly, M1 and M2 macrophages must regulate cellular metabolism to provide appropriate metabolites to meet energy demands for distinct immune responses. The key difference between M1 and M2 macrophages is the metabolism of arginine. M1 macrophages metabolize arginine to nitric oxide (NO) and citrulline via nitric oxide synthase 2 (NOS-2 or iNOS), these products are pro-inflammatory, cytotoxic, and increase the production of reactive oxygen species (ROS) and nitrogen species (Lee et al., 2020). M2 macrophages express ornithine decarboxylase and spermidine oxidase, which hydrolyzes arginine to produce ornithine and polyamine, suppressing pro-inflammatory responses (Lee et al., 2020). Understanding macrophage cellular metabolism in response to stimuli can potentially be exploited to identify anti-inflammatory compounds.

Indirubins role in inflammation

Severe and chronic inflammation can cause significant tissue damage and have previously been associated with numerous autoimmune diseases, cancer, and atopic disorders. Medicines that inhibit the signaling pathways of pattern recognition receptors (PRRs) such as TLRs can be considered potential anti-inflammatory agents. TLR-4, a key PPR, when activated by LPS, a principal component of Gram-negative bacteria's outer membrane, triggers the downstream nuclear transcription factor kappa-B (NF-κB) signaling pathway. NF-κB regulates the production of various pro-inflammatory

cytokines and the expression of many genes encoding inflammatory mediators (Lai et al., 2017).

NF-κB is one of the most important transcription factors in the inflammatory response and is commonly expressed by activated M1 macrophages. The activation of NF-κB involves the phosphorylation of IκBs at two serine residues (Ser32, Ser36) by the IκB kinase (IKK) complex (Kim et al., 2007). Once phosphorylated, IκBs are ubiquitinated and degraded by the 26S proteasome. The now free NF-κB translocates to the nucleus, binds to κB binding sites, and induces the transcription of pro-inflammatory mediators such as iNOS, Cox-2, TNF-α, IL-1β, IL-6, and IL-8 (Kim et al., 2007). Previous studies have shown that indirubin inhibits LPS-induced NF-κB P65 phosphorylation in a dose-dependent manner via the inhibition of IκBα degradation (Lai, 2017). Thus, indirubin potentially inhibits iNOS, Cox-2, TNF-α, IL-1β, IL-6, and IL-8 production. Indirubin and structurally related compounds containing indole backbones need to be further explored to determine the therapeutic anti-inflammatory potential of these compounds.

Aryl hydrocarbon receptor (AHR)

The AHR is a member of the basic helix-loop-helix family of ligand-activated transcription factors. AHR is activated by a variety of ligands, including environmental pollutants (e.g., polycyclic aromatic hydrocarbons), tryptophan metabolites (e.g., kynurenic acid, 6-formylindolo[3,2-b]carbazole (FICZ)), as well as endogenous compounds such as indirubins and indole-containing compounds (Dvořák et al., 2021).

Depending on the structure-activity relationships (SARs) of the AHR ligand, the ligand is defined as full or partial agonists, or as an antagonist leading to differential binding affinity (Blažević et al., 2015). The stronger binding a ligand has to AHR, the longer-lasting and toxic effects tend to be.

Inactivated AHR resides within the cytoplasm in a complex with several chaperone proteins such as 90-kDa heat shock protein (HSP90), AHR-interacting protein (AIP), cochaperone p23, and the c-Src protein kinase (Gutiérrez-Vázquez, 2018). Once activated by a ligand, AHR dissociates from the chaperone protein complex and undergoes translocation into the cell nucleus. Nuclear AHR forms a heterodimer with the AHR nuclear translocator (ARNT). The heterodimeric AHR/ARNT binds the target DNA sequences known as xenobiotic response elements (XREs) and triggers the expression of drug-metabolizing enzymes such as cytochrome P450 enzymes like CYP1A1 and CYP1B1 (Dvořák et al., 2021).

AHR activation by various indole-containing ligands has been linked to AHR recruitment to different target DNA sequences and ultimately triggering ligand-specific biological responses (Gutiérrez-Vázquez, 2018). Several different physiological processes have been linked to the activation of AHR, such as an inflammatory response (Gutiérrez-Vázquez, 2018), hematopoiesis (Boitano et al., 2010), as well as carcinogenesis (Kolluri, 2017).

Indirubins have shown to interact with AHR to varying degrees; interactions tend to be brief due to indirubins' metabolism in an AHR-dependent manner by cytochrome P450 enzymes such as CYP1A1 and CYP1B1 (Knockaert et al., 2004). However,

indirubin derivatives with high AHR affinity and low molecular stability can be an attractive therapeutic alternative compared to the traditional, more stable, and potentially toxic AHR ligands studied, such as 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) (Knockaert et al., 2004).

The history of indirubins

Plants have been used for centuries to treat various ailments and diseases such as chronic inflammation. Natural products may also serve as a basis for the synthesis of derivatives to reduce toxic side effects and improve pharmacokinetic properties and efficacy. Until recently, the majority of active compounds within these medicinal herbs were unknown. After extensive research of one such medicinal herb traditionally used to treat chronic myelocytic leukemia, Danggui Longhui Wan, it was found that the active compound was a stable red isomer of blue indigo known as indirubin (Hoessel et al., 1999). Indirubin is chemically a 3,2'-bisindole commonly derived from tryptophan precursors (Blažević et al., 2015). Naturally derived indirubins, as well as other indole-containing compounds, have been shown to have anti-proliferative effects, mainly attributed to the inhibition of the cell cycle-related kinases, such as cyclin-dependent kinases (CDKs) and glycogen synthase kinase-3β (GSK-3β) (Blažević et al., 2015). Indirubin derivatives and structurally related compounds containing an indole core have also been shown to have anti-inflammatory properties via inhibition of NF-κB nuclear translocation (Kim et al., 2010).

Indirubin derivative E804

Naturally occurring indirubins generally have low bioavailability due to the structure's planarity, hydrogen bonds, and hydrophobic π-interactions. Accordingly, researchers have synthesized many artificial indirubin derivatives or structurally related compounds with improved chemical and pharmacological properties such as solubility and absorption (Busbee et al., 2013). One such indirubin derivative is indirubin-3'-(2,3 dihydroxypropyl)-oximether (E804). E804 possesses novel inhibitory activity of signal transducer and activator of transcription 3 (STAT3) in human breast and prostate cancer cells through the direct inhibition of c-Src kinase activity (Nam et al., 2005). The c-Src kinase has a vital role in cell proliferation, tumorigenesis, and metastasis resulting in the phosphorylation of tyrosyl residues of critical cellular substrates and leading to the activation of oncogenic signal transduction pathways such as STAT3 (Nam et al., 2005). In addition to upregulating numerous genes involved in the activation of oncogenic signal transduction pathways, STAT3 induces the expression of many cytokines, chemokines, and other immune mediators, such as IL-6 and COX-2, that are associated with cancer-promoting inflammation (Yu, 2009). Receptors for these pro-inflammatory mediators in turn further activate STAT3, forming autocrine and paracrine feed-forward loops that result in the promotion of cancer inflammation (Yu, 2009).

Interestingly, STAT3 also interacts with NF-κB on several levels. Many inflammatory factors encoded by NF-κB target genes, such as IL-6, are also critical STAT3 activators. In the context of pro-inflammatory tumor environments, STAT3 directly interacts with the NF-κB family member RELA by trapping it in the nucleus,

which results in constitutive activation of NF-κB (Yu, 2009). These reports suggest an indirect mechanism by which STAT3 maintains the expression of genes encoding pro-inflammatory mediators. Due to the pivotal role in which STAT3 influences the nature of pro-inflammatory environments, STAT3 inhibition by E804 and other structurally related compounds represent promising therapeutic compounds in alleviating inflammation.

STATTIC

As alluded to above, STAT3 is a crucial transcription factor regulating immunity and inflammatory pathways (Yu, 2009). Constitutive activation of the STAT3 pathway has also been shown to lead to aberrant growth and survival of human tumors (Mcmurray, 2006). STAT3 transduces signals from IL-6 family cytokines, epidermal growth factors, platelet-derived growth factor, as well as others. When activated, the receptor becomes phosphorylated on the tyrosine residues, and STAT3 is recruited through its SH2 domain. Tyr705 of STAT3 then becomes phosphorylated by JAK kinases, Src-family kinases. On dissociation, two STAT3 molecules dimerize through reciprocal pTyr705-SH2 domain interactions, and the dimer translocates to the nucleus, where it initiates the transcription of various genes (Mcmurray, 2006). Thus, treatment with STAT3 inhibitors has been proposed as a potential therapy for several diseases and pathologies associated with chronic inflammation (Liu et al., 2018).

6-Nitrobenzo[b]thiphene 1,1-dioxide (STATTIC) has since been identified as one of the top candidates for selectively inhibiting the function of the STAT3 SH2 domain regardless of the STAT3 activation state *in vitro* (Schus et al., 2006). STATTIC inhibits

the binding of a relevant tyrosine-phosphorylated peptide motif to the STAT3 SH2 domain. Consequently, STAT3 dimerization and DNA binding are inhibited by STATTIC, which has a minimal effect on other STAT family members such as STAT1, while inducing apoptosis of STAT3-dependent cancer cell lines (Schus et al., 2006). STATTIC has been shown to be an efficient radiosensitizing agent in normoxic and hypoxic esophageal squamous cell carcinoma cells by inhibiting STAT3 activation, and downregulating HIF-1α and VEGF expression (Zhang et al., 2015). Additionally, STATTIC inhibits RANKL-induced expression of osteoclast-related transcription factors c-Fos and NFATc1 induced by STAT3 and NF-κB, preventing bone loss caused by ovariectomy (Li et al., 2018). Taken together, the novel small nonpeptidic molecule STATTIC provides a potential avenue in the therapeutic application for the treatment of inflammatory diseases caused by STAT3 signaling.

Hypothesis and aims

In the study described herein, I investigated the potential role of AHR antagonism in regulating the response of RAW 264.7 macrophages to a pro-inflammatory stimulus such as LPS. This study tested the hypothesis that AHR antagonism plays a critical role in mediating the therapeutic properties of indole-derived compounds E804 and STATTIC. Additionally, I investigated the potential of STATTIC as an anti-inflammatory molecule and as an AHR ligand.

INTRODUCTION

The immune system protects the body from a broad range of insults via induction of an inflammatory response. Acute inflammation for instance, is critical for protection against microbial pathogens. However, unresolved or chronic inflammation can result in various types of pathology, including tissue damage, autoimmunity, atherosclerosis, inflammatory bowel diseases, and cancer progression (Ospelt, 2010; Doherty et al., 2003; Bouma and Strober, 2003; Multhoff et al., 2012). Macrophages play an essential role in coordinating a balance between driving inflammation by producing pro-inflammatory mediators, including cytokines, and nitric oxide (NO) (Mantovani, 2006; Dall'Asta et al., 2012; Janeway et al., 2001; Babcock et al., 2013), and resolution of inflammation via secretion of anti-inflammatory cytokines and mediators.

Macrophage activation by an inflammatory stimulus, such as lipopolysaccharide (LPS) from Gram-negative bacteria, is primarily recognized through pattern recognition receptors (PPRs) such as the Toll-like receptor 4 (TLR-4) (Lee et al., 2020). Following TLR-4 activation, several intracellular signaling events initiate through the adaptor molecules myeloid differentiation primary response gene 88 (MyD88), TIR-domain-containing adapter-inducing interferon-β (TRIF), transforming growth factor-β (TGF-β)-activated kinase (TAK1), and tumor-necrosis factor(TNF)-receptor-associated factor 6 (TRAF6) (Akira and Takeda, 2004). In turn, these adaptor molecules activate several transcription factors including, nuclear transcription factor kappa-B (NF-κB) and signal transducer and activator of transcription 3 (STAT3) signaling pathways resulting in increased production of pro-inflammatory enzymes and cytokines, such as iNOS, COX-2,

TNF-α, IL-1β, IL-6, and IL-8. These pro-inflammatory mediators further promote the recruitment and activation of additional immune effector cells (Grivennikov and Karin, 2010; Kim et al., 2007; Fu et al., 2020). In view of NF-κB and STAT3's central role in driving inflammation, efforts have focused on inhibiting these pathways as a therapeutic approach to treat immune and inflammatory-mediated pathologies (Yu and Kone, 2004; Grivennikov and Karin, 2010; Li et al., 2018).

A promising avenue for discovering new anti-inflammatory therapeutics involves the naturally occurring compound indole, its metabolites, and synthetic derivatives. Indole is a planar bicyclic molecule in which the benzene ring is fused through 2 and 3 positions of N-containing pyrrole ring (Sravanthi and Manju, 2016). The indole ring is widely distributed in biological systems as an essential constituent of biomolecules and natural products such as the essential amino acid tryptophan, plant hormones, alkaloids, and the neurotransmitter serotonin (Kumari and Singh, 2019). Due to its wide distribution, indole-based compounds have numerous biological activities and can function as anti-microbial, anti-convulsant, anti-cancer, and anti-inflammatory mediators (Sravanthi and Manju, 2016). A naturally-derived indole ring containing compound that has shown promising anti-inflammatory applications is indirubin and its derivatives (Chan et al., 2012; Miyoshi et al., 2012). Indirubin is chemically a 3,2'-bisindole commonly derived from tryptophan precursors (Blažević et al., 2015), and has shown to exhibit anti-leukemic, anti-proliferative, hepatoprotective, and strong anti-inflammatory properties (Babcock et al., 2013; Lai et al., 2017; Blažević et al., 2015). Despite indirubin's natural therapeutic potential, poor solubility and absorption have led

investigators to design new indirubin derivatives to combat this problem (Busbee et al., 2013).

Many indole ring-containing compounds, such as indirubin and several indirubin derivatives, have shown to have a high affinity for the aryl hydrocarbon receptor (AHR). The AHR is a member of the basic helix-loop-helix family of ligand-activated transcription factors and becomes activated by various xenobiotic and endogenous ligands (Nebert, 2017; Dvořák et al., 2021). Depending on structure-activity relationships (SARs), AHR ligands are defined as full agonists, partial agonists, and antagonists leading to differential binding affinity (Blažević et al., 2015). Upon activation, the AHR is recruited to different target DNA sequences and ultimately triggering ligand-specific biological responses (Gutiérrez-Vázquez, 2018). The most well-known of these genes in the fields of toxicology and pharmacology are the expression of drug-metabolizing enzymes such as cytochrome P450 enzymes, namely CYP1A1 and CYP1B1 (Guengerich et al., 2004; Dvořák et al., 2021; Köhle and Bock, 2007).

The indirubin derivative, indirubin-3'-(2,3 dihydroxypropyl)-oximether (E804), possesses novel activity against STAT3 signaling through the direct inhibition of c-Src kinase activity and tyrosyl phosphorylation (Nam et al., 2005). E804 has also been shown to be a potent anti-inflammatory agent by inhibiting the expression of iNOS, IL-6, and COX-2 (Babcock et al., 2013). In addition to E804's therapeutic potential via the STAT3 pathway, E804 has been implicated in the activation of AHR in the human glioblastoma cell line T98-G (Babcock et al. 2013, Scobie 2019). Due to structural instability, E804 is quickly metabolized by enzymes induced through AHR activation, such as CYP1A1 and

CYP1B1 (Scobie, 2019; Spink et al., 2003). However, with the application of AHR antagonists such as 6,2',4'-trimethoxyflavone (TMF), the metabolism of E804 can potentially be inhibited to enhance the anti-inflammatory properties of E804 as well as other indole containing compounds such as STATTIC.

In the study described herein, I investigated the potential role of AHR antagonism in regulating the response of RAW 264.7 macrophages to a pro-inflammatory stimulus such as LPS. This study tested the hypothesis that AHR antagonism plays a critical role in mediating the therapeutic properties of indole-derived compounds E804 and STATTIC. Additionally, I investigated the potential of STATTIC as an anti-inflammatory molecule and as an AHR ligand.

MATERIALS AND METHODS

Cells and cell culturing

The murine macrophage cell line RAW 264.7 was obtained from ATCC ((# TIB-71) Manassas, VA, USA) and cultured phenol red-free minimum essential media (MEM-alpha; Gibco) supplemented with 10% fetal bovine serum (FBS; HyClone Thermo), 20 mM HEPES (pH 7.5), 4 mM L-glutamine, 100 U/ penicillin, 100 μg/ streptomycin, 1 mM sodium pyruvate, 44 mM $NaHCO_3$ (each from Sigma Aldrich), and 1% non-essential amino acid solution (100X, HyClone Thermo). Cells were typically grown to near confluence in T-75 flasks until the time of assays.

Chemicals

E804 (Figure 1A) was obtained from Alexis Biochemical (CA, USA) and solubilized in dimethyl sulfoxide (DMSO) (Sigma Aldrich) to a stock solution of 10^{-2} M and stored at -20°C. The AHR antagonist TMF (Figure 1B) was purchased from Sigma-Aldrich (MO, USA) and solubilized in DMSO to a stock solution of 10^{-2} M and stored at -20°C. STATTIC (Figure 1C) was obtained from Tocris (UK) and was solubilized in DMSO to a stock solution of 4.12 X 10^{-3} and stored at -20°C. Working dilutions were made with DMEM.

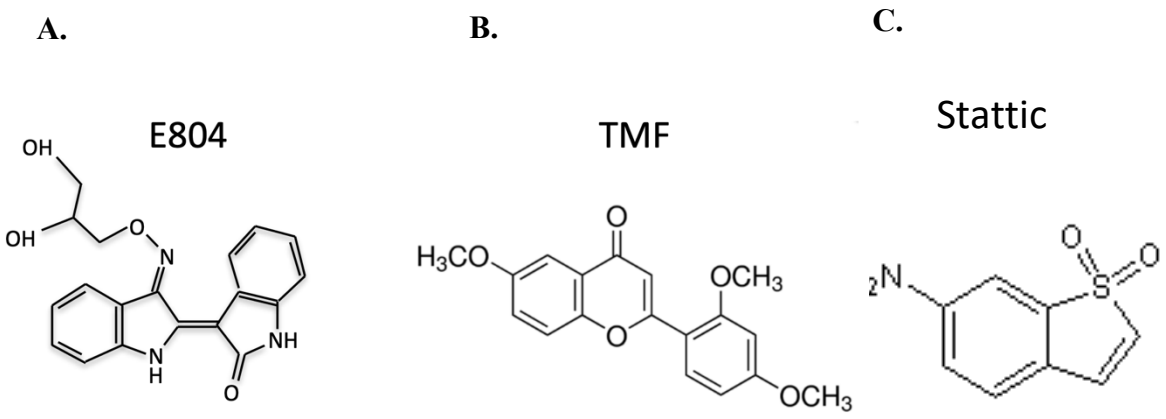

Figure 1: Chemical structures for (A) indirubin-3'-(2,3 dihydroxypropyl)-oximether (E804), (B) 6,2',4'-trimethoxyflavone (TMF), (C) 6-nitrobenzo[b]thiophene 1,1-dioxide (STATTIC).

Cytotoxicity assays

Cell viability was quantified as a function of succinate dehydrogenase activity, a measure of cellular respiration, by performing an MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. RAW264.7 macrophages were plated into 96 well

Costar plates at 10^6 cells per well in 100 µl of DMEM media. After 4 h of incubation at 5% CO_2 and 37°C for adherence, compounds and the carrier control DMSO were added in quadruplicates over serial dilutions beginning with 100 µM per well in a total volume of 200 µl. Plates were incubated for 24 h. Four hours before the end of the assay, 20 µl of 5mg/mL MTT solution in DMEM was added to each well. The supernatant for each well was discarded, and cells containing reduced MTT solubilized with 100 µl of acidified isopropanol (4 mM HCl, 0.1% NP-40 in isopropanol). After a shaking period of 5 min, the optical density (O.D.) for each well was measured at 550 nm Synergy H1 Hybrid plate reader (BioTek). The experiment was repeated three times. For statistical purposes, data were compared between treatment groups using ANOVA and Bonferroni's multiple contrast post-hoc tests using GraphPad 9 statistical package. Prior to experiments, a α value of 0.05 was established as statistically significant for cytotoxicity determinants and throughout this study. Based on these assays it was determined that 0.1 and 1 µM E804 and STATTIC, and 10 µM TMF were suitable for further investigation.

Phagocytosis assay

The phagocytic capability of RAW264.7 macrophages following 24 h of compound treatment was determined by measuring phagocytosis of 1 µM fluorebrite carboxylate YG microspheres (Polyscience) diluted in 50 mM Na_2HPO_4. Washed beads were added to 10^5 treated cells in opaque flat-bottom 96 well plates at a ratio of 100:1 in a total of 200 µl of media, and plates incubated for 90 min at 37°C with 5% CO_2.

Phagocytosis was stopped by performing 3 washes with 100 µl of ice-cold 0.01 M phosphate-buffered saline (PBS) containing 1 mM $CaCl_2$ and $MgCl_2$. Extracellular fluorescence was quenched with trypan blue (2 mg/ml) dissolved in 20 mM citrate and 150 mM NaCl with a pH of 4.5. Plates were then read with a Synergy H1 Hybrid plate reader (BioTek) at an excitation wavelength of 441 nm and an emission wavelength of 486. Data were compared between treatment groups using ANOVA, followed by Bonferroni's post-test using GraphPad 9 statistical package. The experiment was repeated three times.

AHR Reporter Assay

AHR-reporter assay was performed using a Human AHR Reporter Assay system (Indigo Biosciences). The AHR Reporter cells express luciferase under the AHR promoter. The AHR reporter cells were treated in triplicate with serial dilutions of STATTIC, E804, and MeBIO, a known AHR agonist and positive control included in the kit, in the presence or absence of TMF. Following a 24 h incubation period at 37°C with 5% CO_2, relative luminescence units (RLU) were measured using Synergy H1 Hybrid plate reader (BioTek). RLU data were subjected to a nonlinear regression (curve fit) model using the [agonist] versus response (three parameters) equation for each treatment and plotted on a log10 scale using GraphPad 9 statistical package. Additionally, TMF and E804 antagonist RLU data were normalized individually and presented as a maximum activity percentage. This data was then subjected to a nonlinear regression (curve fit)

model using the [inhibitor] versus response (three parameters) equation and plotted on a log10 scale using GraphPad 9 statistical package.

Measuring NO production

Nitric oxide (NO) production by LPS-stimulated RAW264.7 macrophages is a reliable screening assay for the anti-inflammatory properties of new pharmacological compounds of interest (Silva et al., 2021). Therefore, this assay was used to demonstrate the anti-inflammatory activity of E804, STATTIC, and TMF. RAW264.7 macrophages were plated in 96 well Costar plates at 10^6 cells per well in 100 µl of phenol-free DMEM-alpha media (Gibco). Following a 3 h incubation period at 37°C with 5% CO_2 for adherence, phenol-free DMEM media was replaced, and cells treated with E804 (0.1, 1 µM), STATTIC (0.1, 1 µM), in combinations with both TMF (10 µM), LPS (0.1 µg/ml), or neither in a final volume of 200 µl in triplicates. After 24 h, supernatants were harvested and 100 µl transferred to a 96-well plate for determination of NO_2 production, a stable non-volatile product of NO production, measured using an equal volume of Griess reagent (0.1% N-(1-naphthyl)-ethylenediamine, and 1% sulfanilamide in 5% H_3PO_4 solution) at room temperature for 10 min. The O.D. was measured with a Synergy H1 Hybrid plate reader (BioTek) at 550 nm and compared to a $NaNO_2$ standard curve. Values per replicate were averaged and then plotted. Nitrite concentrations were compared between treatment groups using ANOVA, followed by Bonferroni's post-test using GraphPad 9 statistical package.

iNOS protein expression was also quantified by immunocytochemistry that determined the relative amount of iNOS protein expressed in cells. RAW264.7 macrophages were plated into 96 well Costar plates at 10^5 cells per well in 100 µl of DMEM media. After 4 h of incubation at 37°C with 5% CO_2 for adherence, macrophages were treated for 24 hr with compounds of interest. The overlying media was then removed and wells were washed 3 times with PBS containing 0.1% tween-20 (PBS-TW20). Each well then received 200 µl ice-cold methanol for 15 min to fix cells attached to the wells followed by another round of washing 3 times with PBS-TW20. Each well was then blocked with 10% FBS in PBS overnight. Blocking buffer was then removed and washed once with PBS-TW20. Cells were then incubated with rabbit anti-mouse iNOS antibody (BD Transduction Labs, 1:500 dilution) for 24 h. Following three washes with PBS-TW20, macrophages were incubated for an additional hour with a secondary goat anti-mouse IgG (H+L) antibody conjugated with Alex Fluor Plus 488 (ThermoFisher) at room temperature in the dark. The relative fluorescence units (RFU) for each well was determined at ex/em of 495-515 nm. Data were compared between treatment groups using ANOVA, followed by Bonferroni's post-test using GraphPad 9 statistical package.

Measuring intracellular reactive oxygen species (ROS) production

RAW264.7 macrophages were plated into 96 well Costar plates at 10^5 cells/well in 100 µl of DMEM media for 3 hours and then treated with E804 (1 or 0.1µM), STATTIC (0.1, 1µM), in the presence or absence of TMF (10 µM) and LPS (0.1 µg/ml).

After 24 h of incubation at 5% CO_2 and 37°C, dichlorofluorescin diacetate (DCFDA) (molecular Probes, Eugene OR) was added at 10^{-5} M to all wells and incubated for an additional 30 min at 5% CO_2 and 37°C to detect total superoxide. The plates were washed three times with PBS-TW20. The plates were read at ex/em 485-530 nm. RFU's were subject to a comparison between treatment groups using ANOVA, followed by Bonferroni's post-test using GraphPad 9 statistical package.

IL-6 enzyme-linked immunosorbent assay (ELISA)

An ELISA MAX™ Deluxe Sets for Interleukin-6 (IL-6) secreted by RAW264.7 macrophages following treatment at 6 and 24 h. 100 µl of supernatants from macrophages treated with E804 (1, 0.1µM), STATTIC (0.1, 1µM), and in combination with or without TMF (10 µM) or LPS (0.1 µg/ml) were used as the source. A commercially available mouse IL-6 ELISA MAX Deluxe Set (Biolegend) was used for IL-6 cytokine assays. Supernatants were added to a pre-coated capture antibody Costar maxi-sorb plate provided by the kit and incubated overnight at 4°C. The plates were washed and blocked using reagents provided by the vendor. Detection antibody was added for 1 hour, followed by treatment with avidin-HRP and a colorimetric substrate solution. A phosphoric acid stop solution was added, and the plates were read at 450 nm. Concentrations of cytokines were plotted against the standard curve of known concentrations supplied by the manufacturer and expressed as relative concentrations compared to the positive control.

RESULTS

Defining the cytotoxicity of E804, STATTIC, and TMF

Initially, I defined a concentration range of E804, STATTIC, and TMF that did not affect RAW264.7 macrophage viability. For this purpose, an MTT assay, which measures succinate dehydrogenase activity and cellular respiration level (Tim, 1983) was employed. RAW264.7 macrophages were cultured for 24 h with varying concentrations of E804, STATTIC, and TMF and cellular respiration measured. Up to 3 μM of E804 and up to 1 μM STATTIC was seen to have no effect on RAW264.7 macrophage cellular respiration (Figure 2A,B). Similarly, up to 25 μM of TMF (Figure 3C) had no effect on cellular respiration. Therefore, a maximum of 1 μM E804, 0.1 μM STATTIC, and 25 μM TMF was used for subsequent assays.

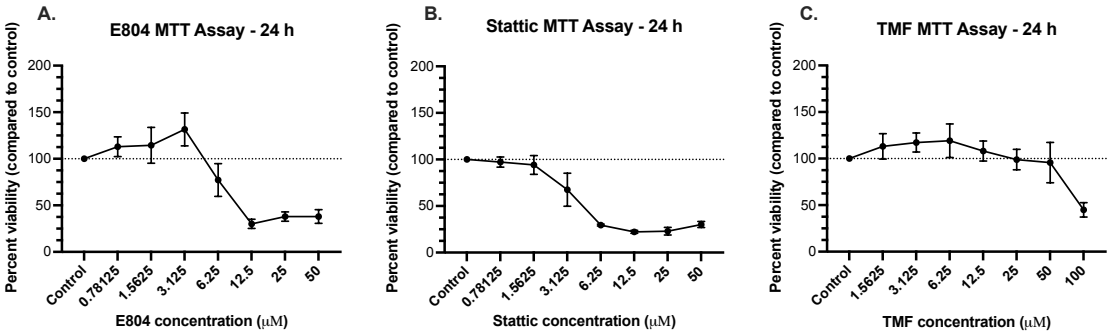

Figure 2: Effects of (A) indirubin-3'-(2,3 dihydroxypropyl)-oximether (E804), (B) 6,2',4'-trimethoxyflavone (TMF), (C) 6-nitrobenzo[b]thiophene 1,1-dioxide (STATTIC) on cellular respiration in RAW264.7 macrophages, as determined by the MTT assay. Data represent the percent change in O.D. compared to control, shown as mean ± standard error (n = 3 individual experiments).

Defining the AHR activity of E804, STATTIC, and TMF

To determine if E804, STATTIC, and TMF were AHR ligands, a human AHR cell-based genetic reporter assay was used. As expected, the AHR agonist MeBio induced a significant dose-dependent increase in AHR activity (Figure 3A). Even though reduced relative to MeBIO stimulation, E804 induced significant AHR activation (Figure 3B), which peaked at approximately 80 nM but diminished with an increasing concentration (Figure 3B). On the other hand, TMF elicited only weak AHR activity at high concentrations (e.g. 2 μM) (Figure 3C), whereas STATTIC failed to induce any AHR activation (Figure 3D). These findings indicate that: i) E804 is an AHR agonist, ii) TMF despite being a known AHR antagonist, can stimulate weak AHR activity at high concentrations, and iii) STATTIC is not an AHR ligand, as measured in this reporter assay.

To determine the antagonistic properties of TMF, TMF was serially diluted and used in combination with E804 (EC85 value of 76 nM). The results indicate that TMF inhibited AHR activation at 80 nM and below. At 400 nM and above, TMF showed partial AHR activity (Figure 4). These results suggest that TMF is an AHR antagonist, blocking E804-dependent AHR activation by competitively binding to AHR at lower concentrations. At higher concentrations, TMF still competitively binds to AHR but is a partial agonist, slightly activating AHR, which has been previously reported.

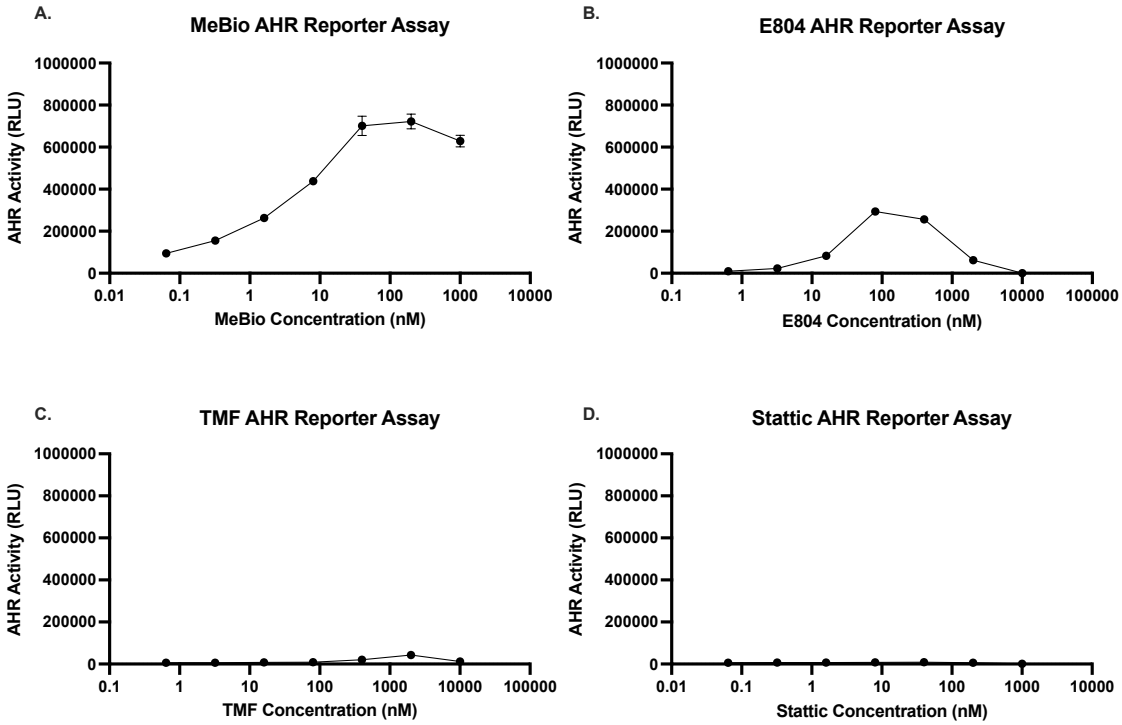

Figure 3: Effect of 24-h treatment with indirubin-3'-(2,3 dihydroxypropyl)-oximether (E804), 6-nitrobenzo[b]thiophene 1,1-dioxide (STATTIC), 6,2',4'-trimethoxyflavone (TMF), or MeBIO on AHR activity. (A) MeBIO showed a normal dose-response curve. (B) E804 showed AHR agonist activity. (C) TMF showed partial agonist activity at higher a higher concentration. (D) STATTIC showed no AHR agonist activity. Data represents mean luminescence units ± standard error (n = 3 individual experiments).

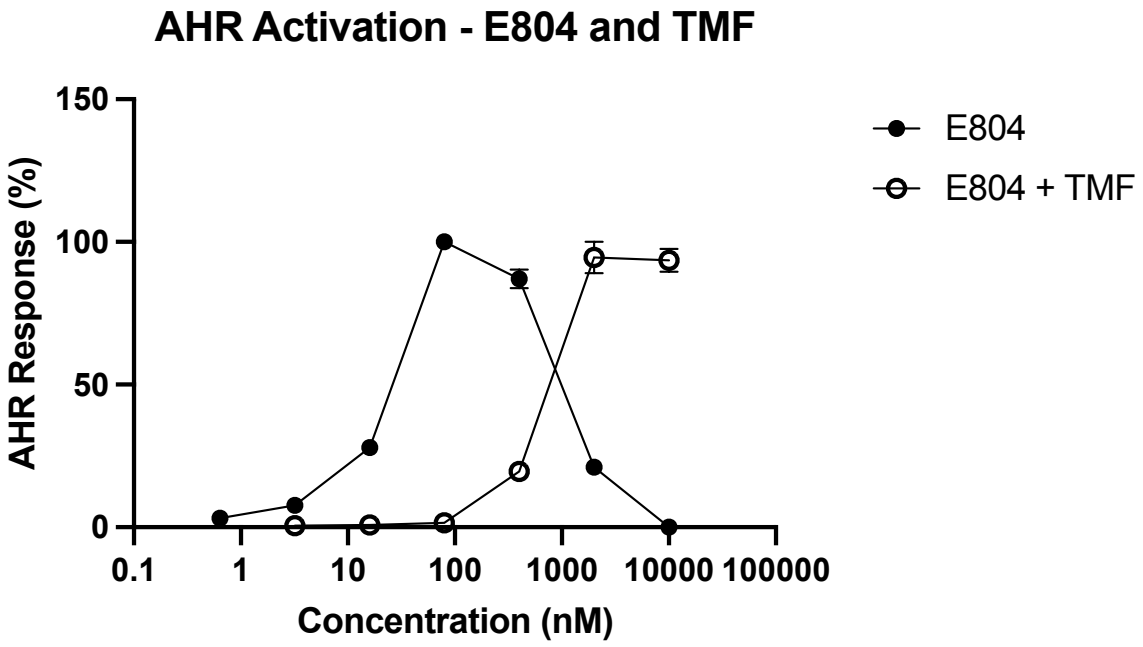

Figure 4: Effect of 24-h treatment with AHR antagonist 6,2',4'-trimethoxyflavone (TMF), on the AHR activity of indirubin-3'-(2,3 dihydroxypropyl)-oximether (E804) (EC85 76 nM) in a human AHR cell-based genetic reporter assay. TMF inhibited E804-dependent AHR activation at lower concentrations. At higher concentrations, TMF showed partial agonist activity. Data represents normalized mean luminescence units ± standard error (n = 3 individual experiments).

Evaluation of the effects of E804, STATTIC, and TMF on NO production

iNOS enzymatic activity is readily induced in macrophages stimulated with LPS. With this in mind, I initially assessed the effect of TMF on iNOS enzymatic activity following stimulation of RAW264.7 macrophages with 0.1 μg/ml of LPS. iNOS activity was suppressed ~50% by 10 mM of TMF (Figure 5). Next, I examined the impact of combining TMF with E804 and STATTIC on LPS-induced iNOS activity. E804 and STATTIC alone significantly reduced NO production in a dose-dependent manner

(Figure 6). Strikingly, the addition of TMF markedly increased the inhibitory effects of E804 and STATTIC (Figure 6). This was readily detected at low concentrations (0.1 mM) of E804 and STATTIC; namely 10 mM of TMF increased the inhibitory effect of STATTIC and E804 by ~3 and 2-fold, respectively (Figure 6). Under the present experimental conditions, it can be suggested that E804, STATTIC, and TMF play a role in mediating NO production in LPS-stimulated macrophages.

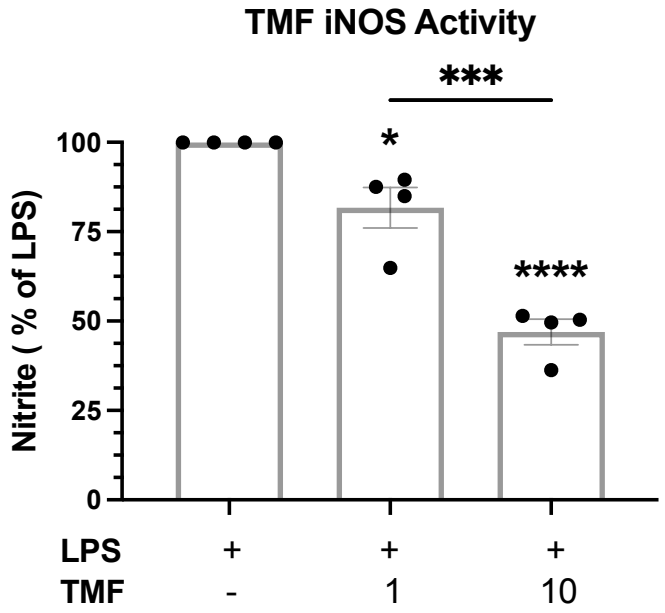

Figure 5: Effects of 6,2',4'-trimethoxyflavone (TMF) on iNOS enzymatic activity in RAW264.7 macrophages stimulated with 0.1 μg/ml lipopolysaccharide (LPS) using the Greiss reagent assay. TMF reduced iNOS activity in a dose-dependent manner. Compound concentrations are in μM. (*$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$). Data represent percent change in O.D. compared to LPS, shown as mean ± standard error (n = 3 individual experiments).

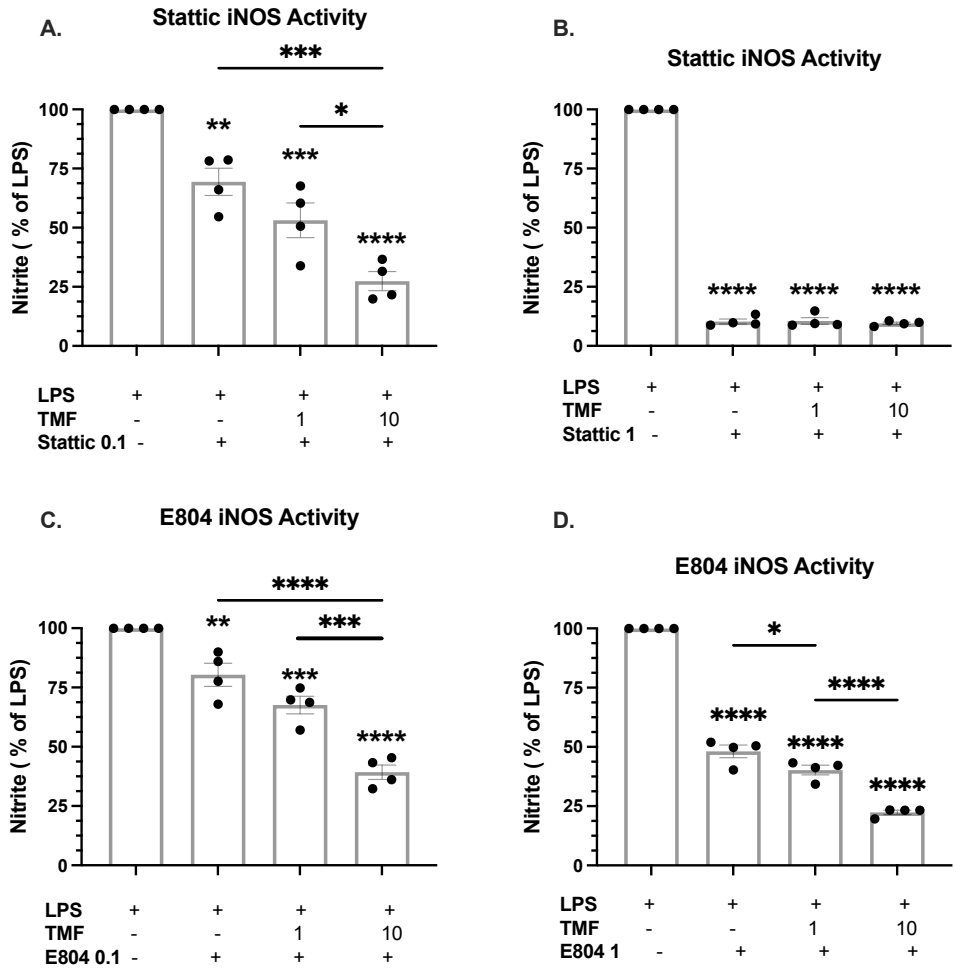

Figure 6: Effects of 24-h treatments with indirubin-3'-(2,3 dihydroxypropyl)-oximether (E804), 6-nitrobenzo[b]thiophene 1,1-dioxide (STATTIC), and/or 6,2',4'-trimethoxyflavone (TMF), and/or 0.1 μg/ml lipopolysaccharide (LPS), or no treatment (control) on iNOS enzymatic activity in RAW264.7 macrophages using the Greiss reagent assay (A, B) STATTIC reduced iNOS activity in a dose-dependent manner, and co-treatment with TMF enhanced the inhibitory effect. (C, D) E804 reduced iNOS activity in a dose-dependent manner, and co-treatment with TMF enhanced the inhibitory effect. Compound concentrations are in μM. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$). Data represent percent change in O.D. compared to LPS, shown as mean ± standard error (n = 3 individual experiments).

Defining the effects of E804, STATTIC, and TMF on cellular iNOS protein expression

As expected, LPS was a potent inducer of iNOS protein expression (Figure 7). On the other hand, neither E804, STATTIC, nor TMF alone upregulated iNOS protein expression (Figures 7 and 8). STATTIC alone, however, was observed to significantly reduce LPS-stimulated iNOS expression in a dose-dependent manner (Figure 8). Interestingly, the addition of TMF (10 μM) failed to enhance the inhibitory effect of STATTIC on LPS-induced iNOS expression (Figure 8A, B). E804 reduced LPS-induced iNOS protein expression only at the high (1 μM) versus low (0.1 μM) concentrations tested (Figure 8C). Furthermore, co-treatment with TMF (10 μM) had no marked effect on E804 inhibition of iNOS expression (Figure 8D). These results indicate that E804 and STATTIC reduce iNOS protein expression, thereby reducing NO production. However, TMF does not reduce iNOS protein expression, suggesting an alternative mechanism in which TMF reduces NO production.

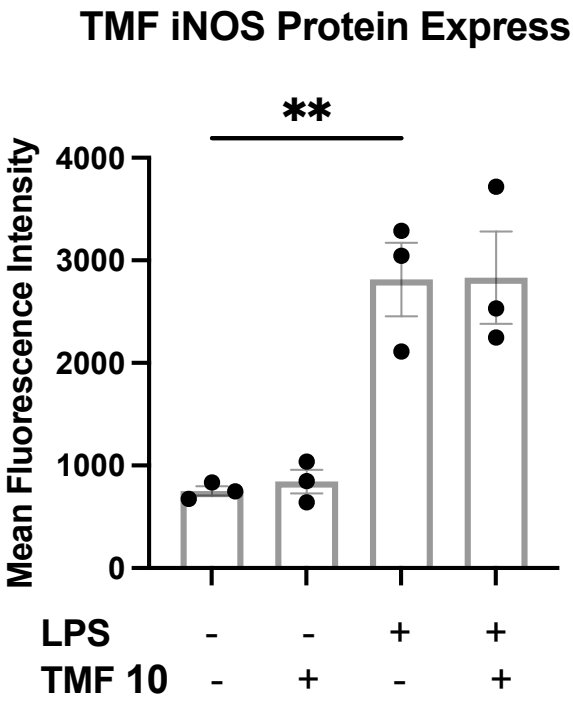

Figure 7: Effects of 24-h treatments with 6,2',4'-trimethoxyflavone (TMF), and/or 0.1 μg/ml lipopolysaccharide (LPS), or no treatment (control) on iNOS protein expression in RAW264.7 macrophages. LPS induced iNOS protein expression, TMF+LPS is comparable to LPS. Compound concentrations are in μM. (**$p < 0.01$). Data represents mean fluorescent units ± standard error (n = 3 individual experiments).

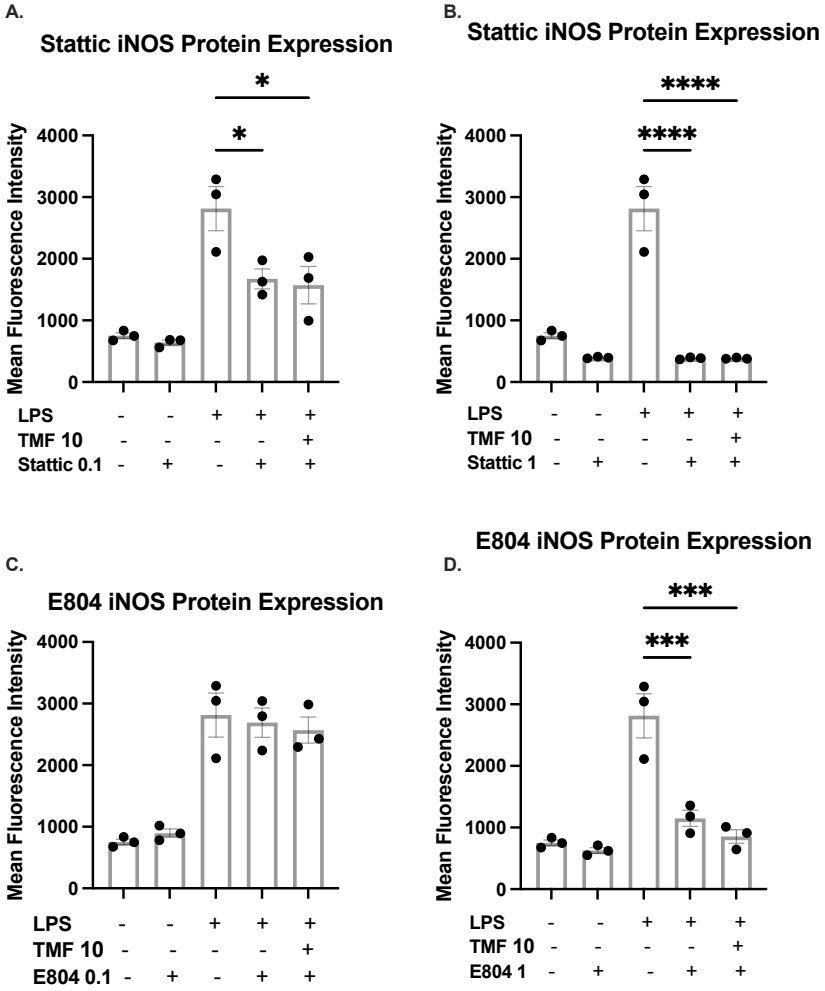

Figure 8: Effects of 24-h treatments with indirubin-3'-(2,3 dihydroxypropyl)-oximether (E804), 6-nitrobenzo[b]thiophene 1,1-dioxide (STATTIC), and/or 6,2',4'-trimethoxyflavone (TMF), and/or 0.1 μg/ml lipopolysaccharide (LPS), or no treatment (control) on iNOS protein expression in RAW264.7 macrophages. (A, B) STATTIC reduced iNOS protein expression in a dose-dependent manner. (C) E804+LPS, E804+TMF+LPS, iNOS protein expression was comparable to LPS. (D) E804 reduced iNOS protein expression comparable to control. The addition of TMF had no significant effect. Compound concentrations are in μM. (*$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$). Data represents mean fluorescent units ± standard error (n = 3 individual experiments).

Defining the effects of E804, STATTIC, and TMF on ROS production

To further assess the effects of E804, STATTIC, and TMF on the LPS-induced inflammatory function of RAW264.7 macrophages, total ROS production was measured using a fluorescence-based assay. Unstimulated RAW264.7 macrophages exhibited constitutively high ROS production, which was increased following LPS stimulation (Figure 9). TMF alone did not increase ROS production (Figure 9). Furthermore, in LPS activated RAW264.7 macrophages, TMF co-treatment appeared to limit the LPS-induced increase in ROS, although this effect was not statistically significant (Figure 9).

Both STATTIC and E804 had distinct effects on ROS production. The low (0.1 mM) but not the high (1.0 mM) concentration of STATTIC inhibited the LPS-induced increase in ROS synthesis (Figure 10A,B). On the other hand, E804 at the high (1.0 mM) but not the low (0.1 mM) concentration inhibited both constitutive and the LPS-induced increase in ROS production (Figure 10C, B). Co-treatment with TMF had no significant effect on STATTIC and E804-mediated ROS synthesis inhibition (Figure 10). These results suggest that E804 and STATTIC either inhibit NADPH-oxidase activities that lead to ROS production or inhibit the depletion of glutathione.

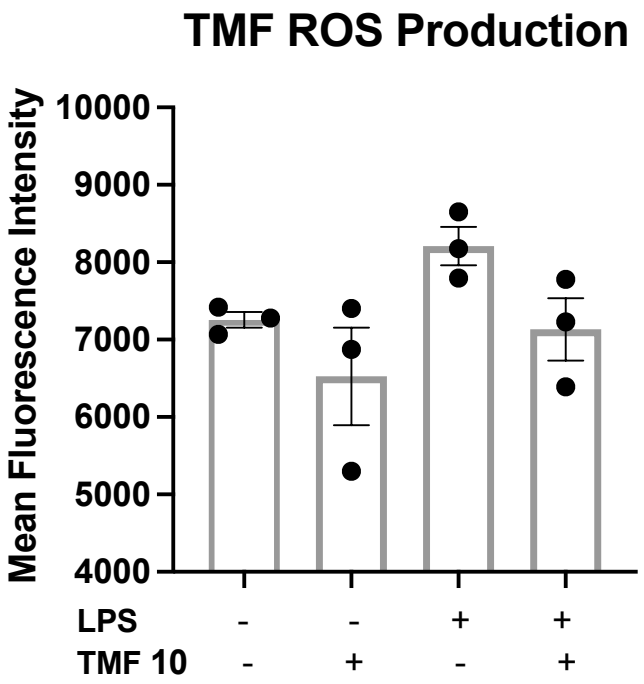

Figure 9: Effect of 24-h treatments with 0.1 μg/ml lipopolysaccharide (LPS), 6,2',4'-trimethoxyflavone (TMF) 10 μM, TMF+LPS, or no treatment (control) on general reactive oxygen species production in RAW264.7 macrophages. TMF had no significant impact on ROS production. Data represents mean fluorescent units ± standard error (n = 3 individual experiments).

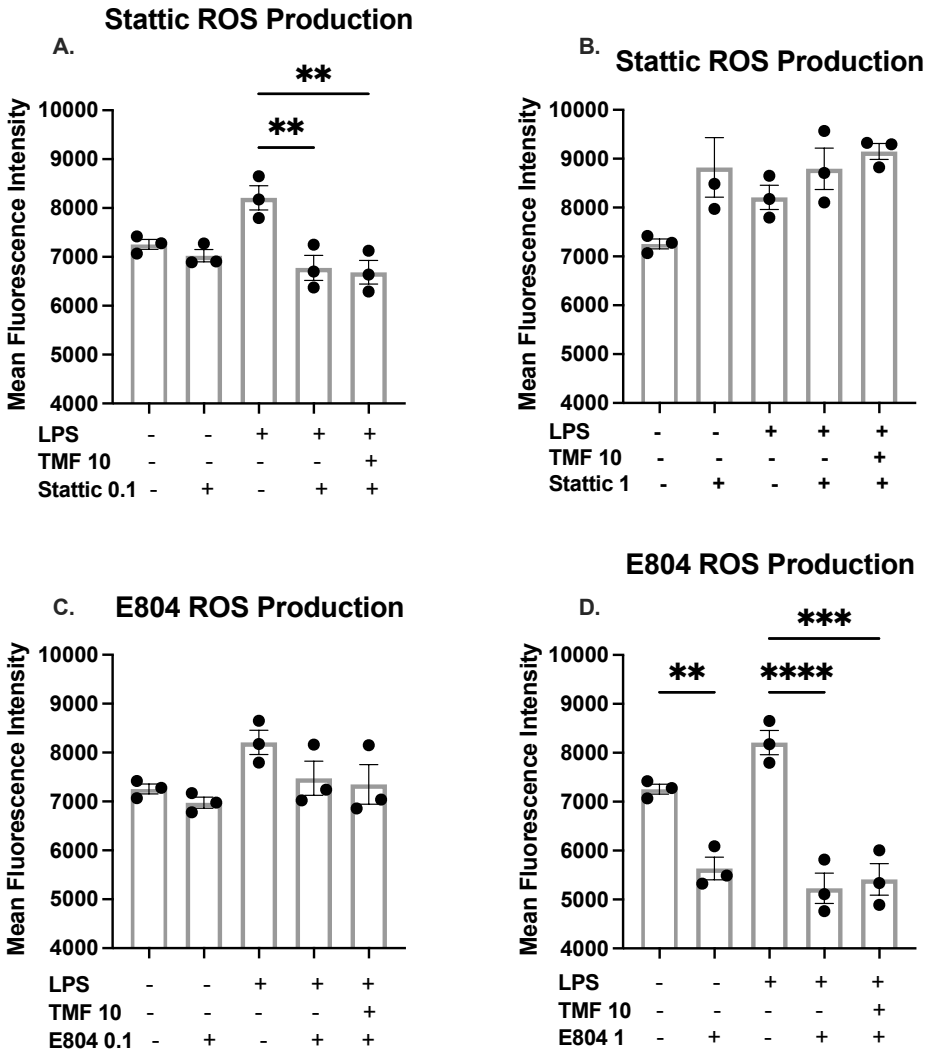

Figure 10: Effects of 24-h treatments with indirubin-3'-(2,3 dihydroxypropyl)-oximether (E804), 6-nitrobenzo[b]thiophene 1,1-dioxide (STATTIC), and/or 6,2',4'-trimethoxyflavone (TMF) 10 μM, and/or 0.1 μg/ml lipopolysaccharide (LPS), or no treatment (control) on general reactive oxygen species production in RAW264.7 macrophages. (A) STATTIC reduced oxidative stress comparable to control. (B) STATTIC induces oxidative stress comparable to LPS. (C) E804 induces oxidative stress comparable to LPS. (D) E804 reduced oxidative stress below control. Compound concentrations are in μM. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$). Data represents mean fluorescent units ± standard error (n = 3 individual experiments).

RAW264.7 macrophage phagocytosis is inhibited by E804 and STATTIC

To further characterize the effects of compound treatment on macrophage function, phagocytosis of latex beads was investigated. Phagocytosis was enhanced by treatment with LPS (Figure 11B). Treatment with either 0.1 or 1.0 mM of STATTIC alone suppressed RAW264.7 macrophages' ability to phagocytize latex beads (Figure 11A). Interestingly, at 0.1 μM, E804 increased macrophage phagocytosis, whereas at 1.0 μM, phagocytosis was significantly reduced (Figure 11A). TMF alone had no marked effect on bead phagocytosis (Figure 11A). In LPS stimulated macrophages, the addition of TMF did not further suppress macrophage phagocytosis in all treatments (Figure 11B). These results suggest that E804 and STATTIC impact macrophage function during the engulfment phase of phagocytosis.

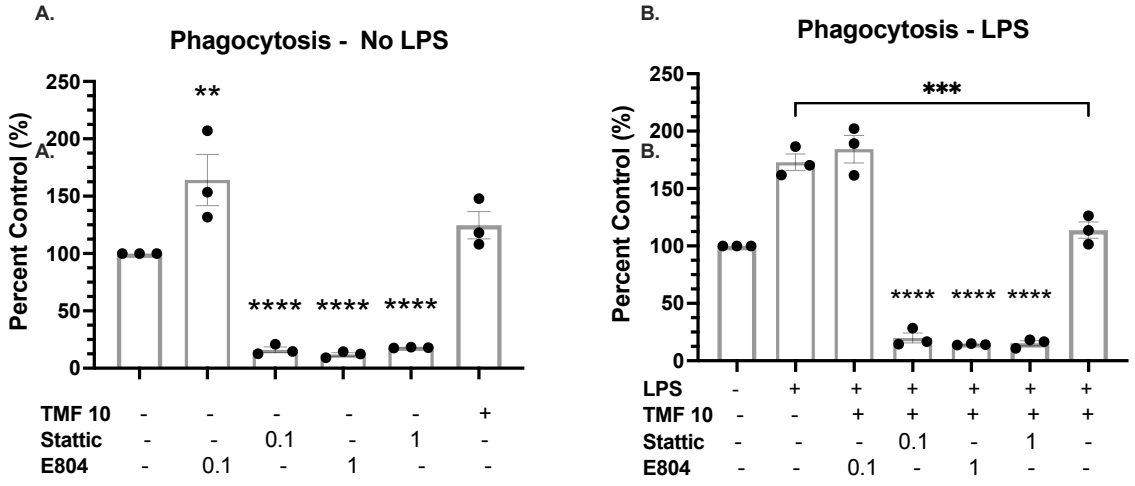

Figure 11: Effects of 24-h treatments with indirubin-3'-(2,3 dihydroxypropyl)-oximether (E804), 6-nitrobenzo[b]thiophene 1,1-dioxide (STATTIC), and/or 6,2',4'-trimethoxyflavone (TMF), and/or 0.1 μg/ml lipopolysaccharide (LPS), or no treatment (control) on phagocytosis activity in RAW264.7 macrophages. (A) phagocytosis is increased by E804 0.1 μM, while phagocytosis in inhibited by STATTIC and E804 1 μM. (B) Phagocytosis is stimulated by LPS and E804 0.1 μM+TMF, while phagocytosis is inhibited by STATTIC and E804 1 μM. Compound concentrations are in μM. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$). Data represent percent change in RFU compared to control, shown as mean ± standard error (n = 3 individual experiments).

LPS-induced IL-6 secretion is suppressed by E804 and STATTIC

IL-6 is a key cytokine that contributes to an inflammatory response. Accordingly, I assessed the effects of the compounds on LPS-stimulated IL-6 secretion by RAW264.7 macrophages. As expected, LPS induced IL-6 secretion in macrophages after 6 h and 24 h (Figures 12, 13). Alone, E804, STATTIC, and TMF did not induce IL-6 secretion (Figures 14, 15). In contrast, TMF alone after 24 but not 6 h significantly reduced IL-6 production elicited by LPS stimulation (Figures 12, 13). In addition, both STATTIC and E804 alone (Figures 14, 15) markedly reduced IL-6 production in LPS activated

RAW264.7 macrophages, and to a greater extent than TMF (Figure 13). Of note, TMF combined with 0.1 μM E804 reduced IL-6 secretion 4-fold at 24 h (Figure 15C). However, under the other co-treatment conditions tested, TMF had no effect on E804 and STATTIC inhibition of IL-6 secretion. Under the present experimental conditions, E804 and STATTIC may mediate the production of IL-6 through inhibition of a common transcription factor such as NF-κB.

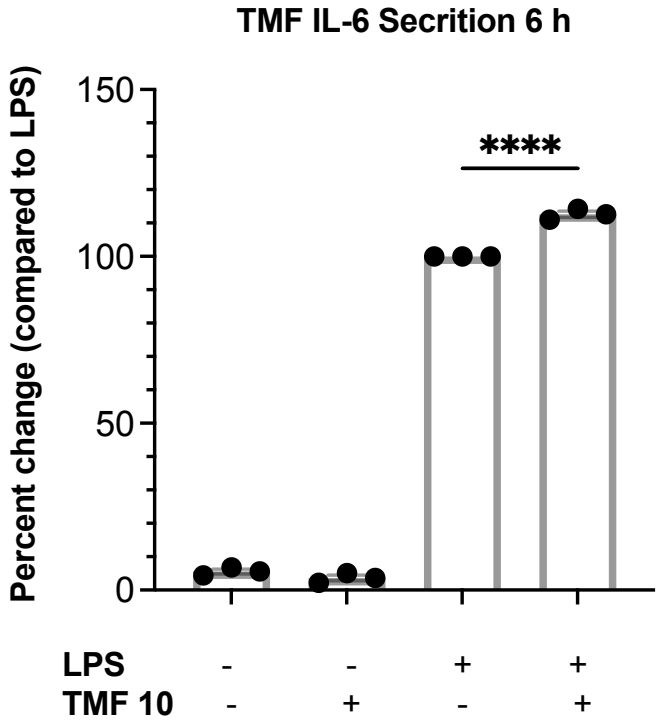

Figure 12: Effects of treatments on the secretion of IL-6 in RAW264.7 macrophages. Cells were stimulated with 0.1 μg/ml lipopolysaccharide (LPS) 24 h and then treated with either control (no treatment) or 6,2',4'-trimethoxyflavone (TMF) 10 μM alone for 6 h. LPS stimulated IL-6 secretion above control, TMF alone did not stimulate IL-6 secretion, TMF+LPS increased IL-6 secretion compared to LPS alone (****$p < 0.0001$). Data represent percent change compared to LPS (300 pg/mL), shown as mean ± standard error (n = 3 individual experiments).

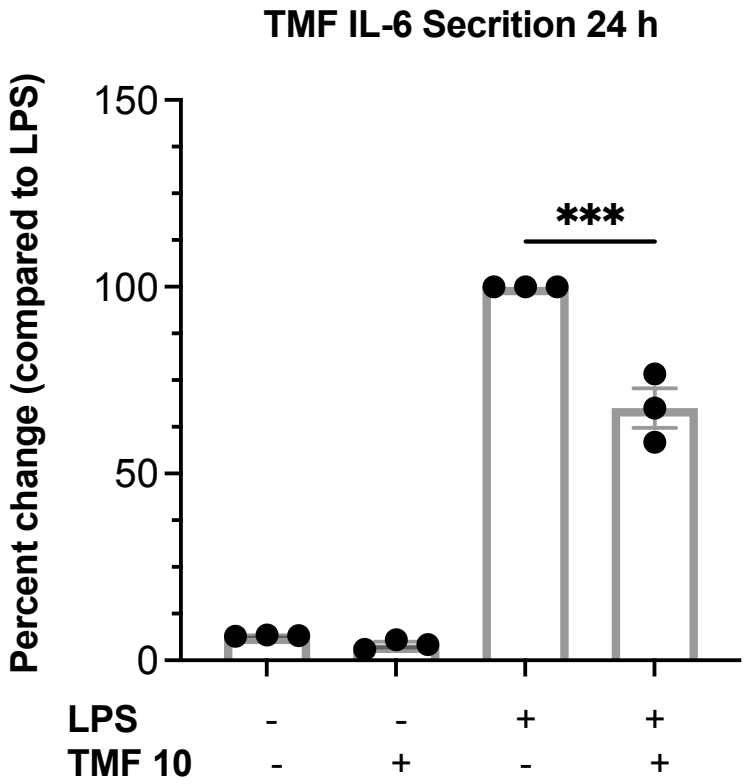

Figure 13: Effects of 24-h treatments with 0.1 μg/ml lipopolysaccharide (LPS), 6,2',4'-trimethoxyflavone (TMF) 10 μM, TMF+LPS, or no treatment (control) on IL-6 secretion in RAW264.7 macrophages. LPS stimulated IL-6 secretion above control, TMF alone did not induce IL-6 secretion, TMF+LPS reduced IL-6 secretion compared to LPS alone. (***$p < 0.001$). Data represent percent change compared to LPS (300 pg/mL), shown as mean ± standard error (n = 3 individual experiments).

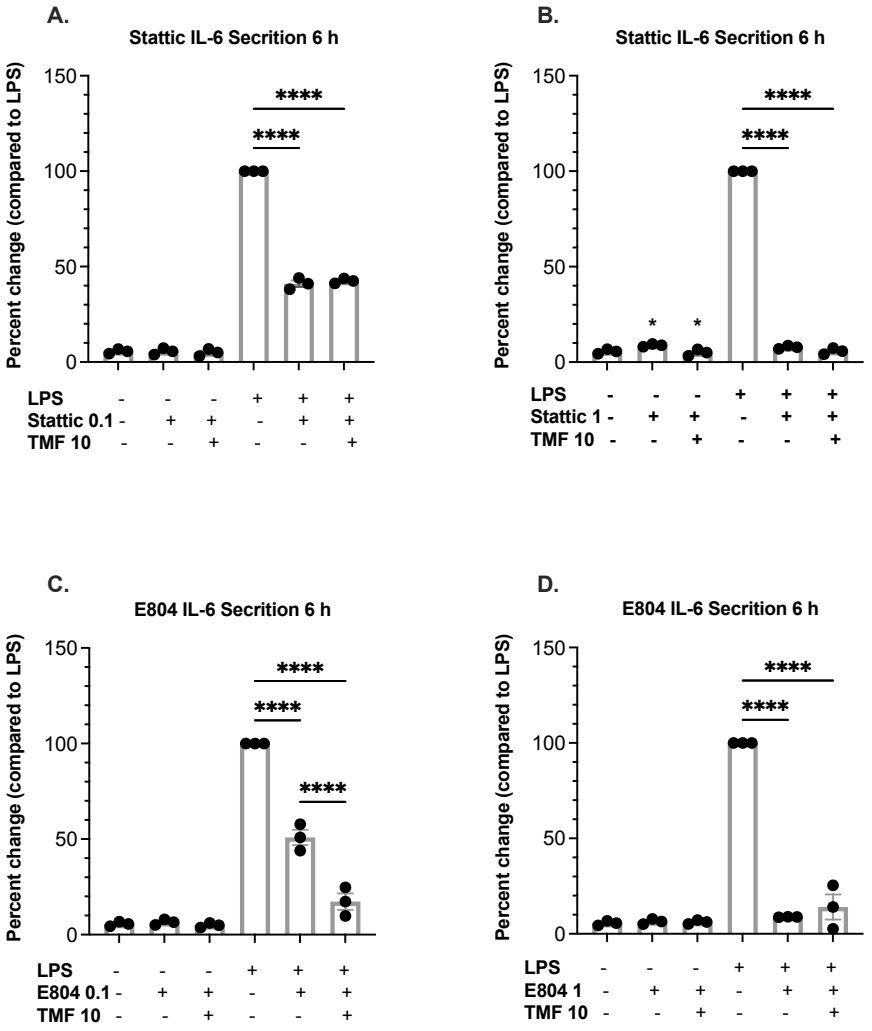

Figure 14: Effects of 6-h treatments with indirubin-3'-(2,3 dihydroxypropyl)-oximether (E804), 6-nitrobenzo[b]thiophene 1,1-dioxide (STATTIC), and/or 6,2',4'-trimethoxyflavone (TMF) 10 μM, and/or 0.1 μg/ml lipopolysaccharide (LPS), or no treatment (control) on IL-6 secretion in RAW264.7 macrophages. (A, B) LPS stimulated IL-6 secretion above control, while STATTIC reduced IL-6 in a dose-dependent manner. (C, D) E804 reduced IL-6 in a dose-dependent manner. Compound concentrations are in μM. (****$p < 0.0001$). Data represent percent change compared to LPS (300 pg/mL), shown as mean ± standard error (n = 3 individual experiments).

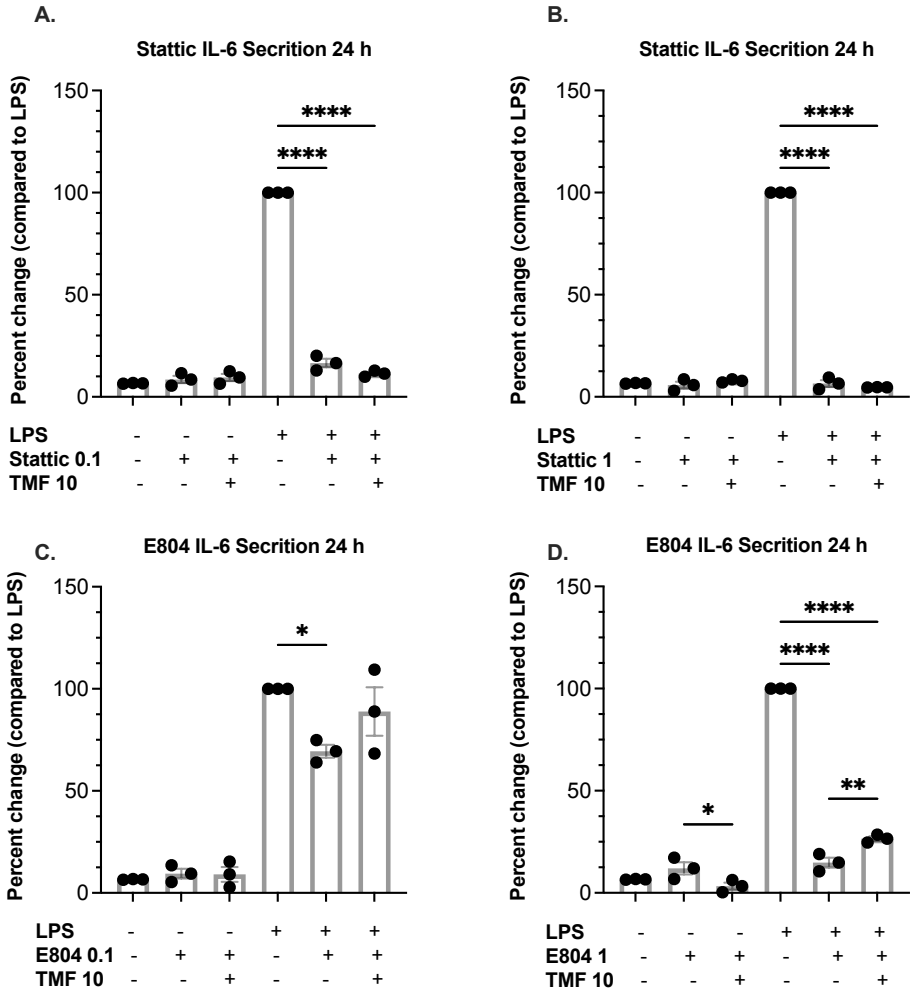

Figure 15: Effects of 24-h treatments with indirubin-3'-(2,3 dihydroxypropyl)-oximether (E804), 6-nitrobenzo[b]thiophene 1,1-dioxide (STATTIC), and/or 6,2',4'-trimethoxyflavone (TMF) 10 μM, and/or 0.1 μg/ml lipopolysaccharide (LPS), or no treatment (control) on IL-6 secretion in RAW264.7 macrophages. (A, B) LPS stimulated IL-6 secretion above control, while STATTIC reduced IL-6 secretion comparable to control. (C) E804+TMF+LPS IL-6 secretion was comparable to LPS alone. (D) E804+LPS was comparable to control. Compound concentrations are in μM. (*$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$). Data represent percent change compared to LPS (300 pg/mL), shown as mean ± standard error (n = 3 individual experiments).

DISCUSSION

In this study, I examined the effects of AHR antagonism on mediating the anti-inflammatory properties of the indole-derived compound E804. Similarly, I examined the potential use of the indole-derived compound STATTIC as an anti-inflammatory molecule and as an AHR ligand. For these analyses, the murine macrophage cell line RAW264.7 was employed. RAW264.7 cells provide a well-established model to study macrophage pro-inflammatory function.

Indole derivatives are important heterocyclic compounds in drug-discovery studies. This class of molecules play a significant role in cell biology and are naturally derived. Indole-containing compounds have gained significant interest in the use against cancer cells, inflammation, and a multitude of disorders in the human body (Kaushik et al., 2013). However, indole-containing compounds' interactions with the AHR and the resulting immune modulation have been well documented (Dvořák et al., 2020). Therefore, E804, STATTIC, and TMF were evaluated for AHR activity. As previously described, E804 induced the gene transcription of the drug-metabolizing enzymes CYP1A1 and CYP1B1, which suggested AHR activity (Scobie et al., 2019; Babcock et al., 2013). In the study described herein, I confirm that E804 is an AHR ligand using a human AHR reporter assay. E804 is an AHR agonist with a moderate binding activity. To our knowledge, there has been no previous research on STATTIC as an AHR ligand. This study determined that STATTIC has no AHR activity, indicating that it is not an AHR ligand. In previous studies, TMF was shown to exhibit no partial agonist capabilities,

exhibiting the functional characteristics of a true AHR antagonist through repressing AHR-mediated gene induction (Murray et al., 2010). However, in this study, I show that TMF acts as a partial agonist at higher concentrations.

As shown in this study with E804 and previous research efforts, indole-derived compounds are commonly AHR ligands (Dvořák et al., 2021). However, AHR activation results in the expression of drug-metabolizing enzymes such as cytochrome P450 enzymes like CYP1A1 and CYP1B1, leading to the degradation of these ligands in an AHR-dependent manner (Spink et al., 2003). In this study, I showed that by inhibiting the activation of AHR and the resulting transcription of drug-metabolizing enzymes via TMF, rapid metabolism of E804 and STATTIC was inhibited in an AHR-dependent manner. Therefore, AHR antagonism should be further explored when evaluating the efficacy of future drug treatments. Additionally, knowing that STATTIC has no AHR activity can be used to eliminate concerns over unnecessary immune modulation via AHR activation in future studies.

Using LPS activated macrophages is a standard model used for understanding primary immune responses to Gram-negative bacteria. Typically, LPS initiates an immune response through TLR4 activation, initiating NF-κB signaling. In response, NF-κB drives the expression of potent pro-inflammatory mediators and cytokines such as iNOS and IL-6 (Bagaev et al., 2019). iNOS enzymatic activity contributes to oxidative stress through the production of NO. NO combined with superoxide anion yields peroxynitrite, a potent long-lived free radical capable of disrupting surrounding tissues.

In this study, I observed the reduction of LPS-induced iNOS protein expression, NO production, and IL-6 secretion by E804 and STATTIC in a dose-dependent manner. Notably, the reduction in the expression of these inflammatory mediators was not due to RAW264.7 macrophage death. The concentrations of E804 and STATTIC used in the respective assays were determined to have no effect on RAW264.7 macrophage viability. The reduction in these pro-inflammatory mediators by E804 and STATTIC suggests a mechanism of action targeting the NF-κB signaling pathway, which regulates TLR-4 signaling and expression of proinflammatory mediators such as iNOS activity, IL-6, IL-12, COX-2, TNF- α, and IL-β. It should be noted that E804 and STATTIC may have distinct effects on the NF-κB pathway that include, for instance, inhibition of IκBα phosphorylation (Miller et al., 2010). Additionally, with the inclusion of the AHR antagonist TMF, inhibition of NO production by STATTIC and E804 was enhanced, although this effect did not correlate with a further decrease in iNOS protein expression. Interestingly, AHR antagonism by TMF on LPS stimulated RAW246.7 macrophages increased secretion of IL-6 at 6 h and decreased secretion at 24 h, suggesting that AHR activity may be beneficial in early inflammatory responses. These results also indicate that AHR antagonism may have a role in mediating the anti-inflammatory properties of indole-containing compounds.

The complex biochemistry of NO production provides many potential sites for regulatory action. The biosynthesis of NO in macrophages is carried out as a byproduct from L-arginine to L-citrulline through the enzymatic activity of iNOS (Aktan, 2004). The reduction observed in NO production by TMF and not in iNOS protein expression

could be due to the alteration of iNOS enzymatic ability mediated by TMF interactions. Previous research efforts have indicated that flavones and flavone derivatives reduce NO production by directly scavenging NO radicles as well as directly inhibiting iNOS enzymatic activity (Sheu et al., 2001; Aktan, 2004). Overall, it is clear that future studies are needed to characterize the mechanisms in which TMF reduces NO production.

Oxidative stress plays an essential role during the inflammatory response. When cellular production of ROS overwhelms its antioxidant capacity, it leads to a state of oxidative stress, contributing to the pathogenesis of several diseases (Lingappan, 2018). The effect of E804, STATTIC, and TMF on oxidative stress was monitored by ROS assays in which LPS-induced superoxide radical production was quantified. Under the conditions explored in this study, E804 (1 μM) and STATTIC (0.1 μM) significantly reduced ROS production during macrophage activation by LPS. AHR antagonism by TMF however, did not affect ROS production. AHR has multiple roles in ROS production. Previous research has indicated that AHR promotes ROS production by inducing gene expression of $p40^{phox}$, a subunit for nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which is a crucial enzyme in ROS production (Vogel et al., 2020). Furthermore, it has been shown that activation of AHR transcriptionally activates nuclear factor erythroid 2-related factor 2 (NRF2), a critical transcriptional factor that mediates antioxidative enzymes such as superoxide dismutase (Ma et al., 2004). The apparent varying roles of AHR in ROS production may explain the minimal effect of TMF treatment. Future studies are needed to define the role AHR has in ROS production

fully. This data suggests that E804 and STATTIC should be further explored to mediate oxidative stress during the inflammatory response.

An indication of proper macrophage function includes the cell's ability to phagocytize particles properly. As demonstrated in the experiment described herein, macrophage phagocytosis was markedly reduced by both E804 and STATTIC. E804 in lower concentrations (0.1 μM) significantly increased phagocytosis in LPS-stimulated macrophages as well as non-stimulated macrophages. This result was interesting because at a higher concentration (1 μM), phagocytosis was inhibited completely. This inhibition may be attributed to E804's modulation of GSK-3β, which is involved in cytoskeletal changes during engulfment (Cabello et al. 2010). To our knowledge, this is the first study to examine the effects of STATTIC on the ability to mediate phagocytosis in RAW264.7 macrophages. STATTIC inhibited phagocytosis in RAW264.7 macrophages, and this inhibitory action is potentially due to STATTIC's high binding affinity to cysteine residues as seen in the inhibition of STAT3 (Heidelberger et al., 2013). STATTIC most likely binds to a cysteine residue in the target-binding cleft in actin, preventing actin filaments' formation during phagocytosis (Otterbein, 2001). However, STATTIC's direct mechanism of action in macrophage phagocytosis needs to be explored in future studies.

CONCLUSION

This study suggests that the indole-containing compounds E804 and STATTIC are promising immunomodulating compounds with potent negative effects on iNOS

protein expression, NO production, IL-6 secretion, and macrophage phagocytic mediating capabilities. Additionally, this paper supports the use of E804 and STATTIC in combination with TMF to antagonize the degradation of these compounds in an AHR-dependent manner. E804 and STATTIC may also possess valuable anti-inflammatory properties via inhibition of the NF-κB signaling pathway. However, future studies are needed to understand these compounds' therapeutic properties fully.

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