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The Effects of Indirubin-3'-(2,3 dihydroxypropyl)-oximether (E804) on Inflammation Profile in Macrophages

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ABSTRACT

Indirubin is a deep-red bis-indole isomer of indigo blue, both of which are biologically active ingredients in Danggui Longhui Wan, an ancient Chinese herbal tea mixture used to treat neoplasia and chronic inflammation, and to enhance detoxification of xenobiotics. Multiple indirubin derivatives have been synthesized and shown to inhibit cyclin-dependent kinases (CDKs) and glycogen-synthase kinase (GSK-3β) with varying degrees of potency. Several indirubins are also aryl hydrocarbon receptor (AhR) agonists, with AhR-associated activities covering a wide range of potencies, depending on structure-activity-relationships (SAR). In this study, the effects on indirubin-3'-(2,3 dihydroxypropyl)-oximether (E804), a novel indirubin derivative with potent effects on STAT3 signaling, on LPS-stimulated inflammatory profiles in RAW264.7 murine macrophages were examined. Most genes, proteins, and biological functions up-regulated by LPS treatment were suppressed by E804, including LPS-induced expression and secretion of pro-inflammatory cytokines. Additionally, E804 enhanced HO-1 expression, which may promote antioxidant responses to control inflammation. However, RAW264.7 cells showed only modest CYP1A1 induction following treatment, thus at this time it is not clear if E804 modulates inflammatory responses in RAW264.7 cells through AhR signaling. To further explore the possible effects of E804 on AhR signaling, THP-1 human monocytes were differentiated to macrophages by a 48 hr treatment with phorbol-12-myristate-13-acetate (PMA), then treated with E804 and select other indirubin derivatives, as well as PCB-126 - a well-characterized AhR ligand

and potent inducer of CYP1A1. CYP1A1 gene and protein expression were significantly induced by 7-bromoindirubin-3'-oxime (7BIO), another novel indirubin, and PCB-126, but not E804. Furthermore, both 7BIO and E804 suppressed IL-6 and IL-10 secretion, thus confirming that AhR signaling is not a requirement for the anti-inflammatory properties of this compound. Of particular note, 7BIO is a potent inducer of COX-2, another potent pro-inflammatory mediator known to be activated through the AhR. Taken together, this study demonstrates potent anti-inflammatory properties of E804 without the toxicities historically associated with AhR activity. These findings can be applied to development anti-inflammatory drugs that may replace current methods for topical inflammation control.

DEDICATION

To Ami, Angela and Paul: your encouragement, support, love, and unwavering belief in me have made this possible.

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LITERATURE REVIEW

History

Many plants have been used in Chinese medicine for over four millennia. One particular remedy, Danggui Longhui Wan, a mixture of eleven herbal medicines, has been used as a treatment of chronic myelocytic leukemia, as well as an antipyretic, anti inflammatory, and for detoxification (Zhu 1998). In 1966, Chinese researchers identified the active herbal component as Qing Dai (*Indigo Naturalis*), made from the leaves of *Baphicacanthus cusia*, *Polygonum tinctorium*, or *Isatis indigotica* (Wu, *et al*. 1979). The active compound was found to be indirubin, a dark-blue 3, 2' isomer of indigo. Indirubin, together with indigo, are indigoids, both derived from a chemical family of bis-indoles. Indirubin is derived from the spontaneous, non-enzymatic dimerization of instain and indoxyl. These precursors are found in many natural sources, such as mollusks (*Muricidae* and *Thaididae*) (Cooksey 2001), bacteria (Gillam, *et al*. 2000), indigo-producing plants (Maugard, *et al*. 2001), and in mammalian urine (Adachi, *et al*. 2001). Indigo has been used as dye since the Bronze Age to produce the dark blue pigment indigo. Natural indigoids can be obtained from the fermentation of indigo producing plants, from which indirubin is produced as a by-product.

Anti-proliferative effects of indirubin

Indirubin exerts its anti-proliferative effects by inducing cell cycle arrest in the G1/S or G2/M phases, resulting in inhibition of cell proliferation, and ultimately,

induction of apoptosis (Hoessel, *et al*. 1999, Marko, *et al*. 2001). Indirubin and many of its derivatives act as potent inhibitors of a variety of cyclin dependent kinases (CDKs) and glycogen synthase kinase-3β (GSK-3β), thereby preventing cell cycle progression (Hoessel, *et al*. 1999, Leclerc, *et al*. 2001, Marko, *et al*. 2001, Meijer, *et al*. 2003). They also affect apoptosis signaling and cell cycling via the *wnt*-signaling pathway (Meijer, *et al*. 2003). Indirubins inhibit cyclin-dependent kinase (CDK)1/cyclin B, CDK2/cyclin A, CDK2/cyclin E, CDK5/p25 and glycogen synthase kinase 3β (GSK-3β), one of the evolutionarily closest enzymes to the CDK family (Hoessel, *et al*. 1999, Leclerc, *et al*. 2001, Lovestone, *et al*. 1996, Marko, *et al*. 2001). In addition, indirubin-3′-monoxime has been shown to inhibit c-Jun NH2-terminal kinase, an important regulator of neuronal apoptosis (JNK1, and JNK3) *in vitro* (Xie, *et al*. 2004).

Kinase binding activity

Indirubins exhibit a wide range of inhibition activities (IC_{50}: 50 nM—100 μM) for both cyclin-dependent kinases and glycogen synthase kinase-3β (Hoessel, *et al*. 1999, Leclerc, *et al*. 2001, Polychronopoulos, *et al*. 2004). They have a high affinity for the enzyme ATP binding site and compete with ATP for the catalytic subunit of the enzyme (Eisenbrand, *et al*. 2004). A structure-activity relationship study suggests that indirubins bind to GSK-3β's ATP binding pocket in a way similar to their binding to CDKs (Leclerc, *et al*. 2001). Indirubins form three hydrogen bonds formed in the ATP-binding pocket with their NH, CO, and N'H groups, which are essential for binding to the peptide backbone

(Davies, *et al*. 2001, Hoessel, *et al*. 1999). Modifications at this region of the molecule cause indirubin to lose its affinity for the ATP binding pocket inhibition of CDKs. However, modifications at the 5' and 3' positions can be made to modify inhibitory potency, while methylation at N1 position renders indirubins kinase-inactive (Davies, et al. 2001, Knockaert, et al. 2004).

AhR-binding

Indirubin is also an agonist of the aryl hydrocarbon receptor (AhR), also called the dioxin receptor, a member of the bHLH/PAS family of transcriptional regulators found in most cell and tissue types of the body (Denison and Nagy 2003). The AhR mediates the effects of many xenobiotics such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), polyaromatic hydrocarbons and polychlorinated biphenyls and indole-containing compounds (Gonzalez and Fernandez-Salguero 1998, Hankinson 1995, Okey, *et al*. 1994). Xenobiotic ligand binding to the AhR triggers its translocation from the cytoplasm to the nucleus, where it forms a complex with the aryl hydrocarbon nuclear translocater (Arnt) (Hoffman, *et al*. 1991). The resulting heterodimer binds to xenobiotic response element (XRE) sequences upstream of cytochrome P450s, $p27^{kip1}$, and other AhR-responsive genes, inducing their transcription (Kolluri, *et al*. 1999, Rowlands and Gustafsson 1997, Santini, *et al*. 2001). While structural modifications at certain positions can cause indirubins to lose affinity for the kinase ATP-binding pocket, these modified

indirubins are still potent AhR agonists, and no kinase-inhibiting indirubins have been found to be inactive on the AhR (Knockaert, *et al*. 2004).

Dual mechanisms of cell-cycle inhibition

While indirubin inhibits the cell cycle though the direct inhibition of cyclin dependent kinases, indirubin may also control the cell cycle though kinase-independent inhibition of cycling (Adachi, *et al*. 2004, Knockaert, *et al*. 2004). The AhR was implicated in cell cycle arrest at G1 through the down-regulation of cyclins and cyclin-dependent kinases, as well as by induction of cyclin-dependent kinase inhibitors such as p21(CDKN1A) and $p27^{Kip1}$ (CDKN1B) (Adachi, *et al*. 2001, Cover, *et al*. 1999, Kawanishi, *et al*. 2003, Knockaert, *et al*. 2004, Ma and Whitlock 1996). The kinase-inactive 1-methyl-indirubins, cause an AhR-dependent cell cycle arrest in G1, that appears to be directly linked to the AhR-dependent expression of $p27^{KIP1}$ (Knockaert, *et al*. 2004). While direct kinase inhibition seems to represent the main mechanism of cytotoxicity, AhR activation is responsible for the cytostatic effects induced by some indirubins.

AhR activity

As with kinase inhibitory activities, the AhR activities of different indirubin derivatives cover a wide range of potency, with EC_{50} values between 5 nM – to over 10 µM for CYP1A1 enzymatic activity in hepatoma cells and 6 nM – to over 100 µM in a yeast reporter assay system (Knockaert, *et al*. 2004). In comparison to indirubin with an

EC_{50} of 6 nM for both assays, TCDD-induced CYP1A1 activity was 15 pM in the hepatoma cell line and 25 nM in a yeast reporter assay, but it has been shown that yeast reporter systems underestimate the potency of TCDD (Knockaert, et al. 2004, Miller 1999). There also appears to be a species-specific effect as indirubin had a 35-140 times higher EC_{50} value in yeast with human genes than mouse genes (Kawanishi, *et al*. 2003). While good screening tools, reporter systems have limited use, therefore, they can be poor substitutes for quantitatively accessing AhR potency.

The discovery of the interaction of indirubin with AhR has raised questions about its potential use since AhR activation has been directly linked to carcinogenesis, immune suppression, as well as reproductive and developmental disorders (Kaminski, *et al*. 2008, Safe 2001, Shimizu, *et al*. 2000). Gene expression studies have shown similar patterns of expression between indirubin and TCDD, though indirubin appears to be more potent. The lowest observed effective concentration (LOEC) for CYP1A1 and CYP1A2 mRNA induction in human cells was 100 pM for TCDD but only 1 pM for indirubin, the lowest of any AhR ligand (Adachi, *et al*. 2004). However, indirubin induced CYP1A1 and 1B1 expression only transiently in human breast cancer cells and the potency of indirubin increased when cells were treated with the CYP inhibitor (Spink, *et al*. 2003). While the binding affinity of the PAH benzo[a]pyrene was similar to that of indirubin, the long-term induction of cyp1a1 expression seen with B[a]P was not observed with indirubin (Adachi, *et al*. 2004). Together these studies suggest that while indirubin is a potent AhR ligand, the negative long-term effects of AhR activation commonly associated with toxic

environmental ligands may not be seen with indirubin. Strong AhR agonists like TCDD, which bind AhR with high affinity and are recalcitrant to metabolism, may have far lasting toxic effects on cell cycling and whole organ/organism toxicities than would indirubins, which are much more quickly metabolized by the P450s induced as a consequence of AhR-activation. Lacking the stability of highly toxic AhR ligands make indirubins an attractive alternative as potential therapeutic agents.

Indirubin derivative E804

One particular indirubin derivative, indirubin-3'-(2,3 dihydroxypropyl)-oximether (E804), has been shown to directly and potently inhibit c-Src kinase activity (IC_{50}:0.43 µM) *in vitro*, thus blocking downstream Stat3 and Stat5 signaling (Nam, et al. 2005, Nam, et al. 2012). Src kinase as an upstream regulator of Stat3 plays a key role in cell proliferation, tumorigenesis, and metastasis by phosphorylating cellular substrates that results in activation of oncogenic signal transduction pathways (Courtneidge and Fumagalli 1994, Parsons and Parsons 2004, Yeatman 2004). Stat3 is also a critical regulator of effector functions of the activated innate immune system, as it is the primary means by which IL-10 mediates its anti-inflammatory actions in peritoneal resident macrophages (Matsukawa, *et al*. 2005), as well as being a transcriptional mediator for the IL-6 family cytokines (Hirano, *et al*. 2000). E804 inhibits Src kinase with an IC_{50} of 0.43 µM, similar to IC_{50} values with which E804 inhibits CDK1/cyclin B, CDK2/cyclin A, and CDK1/cyclin E complexes (1.65, 0.54, and 0.21 µM, respectively),

causing G2/M cell cycle arrest (Nam, *et al*. 2005). It is thought that E804 competitively binds to the ATP-binding site of Src tyrosine kinase, similar to the mechanism by which indirubin inhibits CDKs (Nam, *et al*. 2005). To a lesser extent, E804 also inhibits Jak1 kinase activity (Nam, *et al*. 2005), which works together with Src kinase to mediate constitutive activation of Stat3 (Campbell, *et al*. 1997, Garcia, *et al*. 2001). Studies have suggested that E804 may block this cooperation of Src and Jak1 kinases involved in tyrosyl phosphorylation of Stat3 and Stat5 (Nam, et al. 2005, Nam, et al. 2012).

Stat3 also physically interacts with NF-κB, suppressing constitutive and LPS-induced NF-κB activation (Hoentjen, *et al*. 2005, Welte, *et al*. 2003, Yu and Kone 2004). NF-κβ is from a family of Rel domain-containing proteins activated by wide range of stimuli, including pathogens, carcinogens, stress signals and pro-inflammatory cytokines (Li and Verma 2002). NF-κβ is kept in the cytoplasm bound to inhibitors of NF-κβ (Iκβ). Upon activation, Iκβ proteins are phosphorylated by Iκβα kinase (IKK), ubiquitinated and degraded. NF-κβ is translocated to the nucleus, where it regulates genes involved in antiapoptosis, cell cycle regulation, and inflammation.

Conversely, indirubin has been shown to directly suppress NF-κβ activation through inhibition of IKK, leading to suppression of Iκβα phosphorylation and degradation (Sethi, *et al*. 2006). The suppression of NF-κβ by indirubin leads to down-regulation of gene products involved in cell proliferation, anti-apoptosis, and immune responses. In microglial cells, indirubin-3′-oxime reduced LPS-elicited NF-κβ activation, inhibiting nitric oxide release and reducing production of pro-inflammatory cytokines

(Jung, *et al*. 2011). Another study in microglial cells showed that indirubin-3′-oxime was effective at suppressing the expression of iNOS, IL-6, but not COX-2 (Yuskaitis and Jope 2009). Indirubin-3′-monoxime significantly inhibited COX-2 activity in a variety of cells, preferentially affecting COX-2-catalyzed prostaglandin synthesis (Danz, *et al*. 2001, Jung, *et al*. 2011, Sethi, *et al*. 2006). However, one study using the same indirubin derivative showed increased COX-2 in activated macrophages (Springs and Rice 2006).

Role of indirubins in mediating inflammation

Inflammation occurs in response to tissue damage, pathogens, or pathogen products and is mediated by a variety of immunocompent cells. Macrophages are important in innate immunity as phagocytes, in production of inflammatory cytokines, and in initiating the adaptive response through presentation of antigens to effector T-lymphocytes (Janeway, *et al*. 2001, Laskin and Pendino 1995). Macrophages are activated when their pattern recognition receptors (PRRs) recognize pathogen-associated molecular patterns (PAMPS), such as toll-like receptors (TLRs) binding lipopolysaccharide (LPS) from the gram-negative cell wall (Gordon 2002, Imler and Hoffmann 2000). Activation of TLR4 signaling pathways by bacterial endotoxin lipopolysaccharide (LPS) leads to NF-κβ signaling as the primary transcription factor driving the expression of iNOS, IL-6, and COX-2 and various other genes involved in inflammatory responses in macrophages (Tak and Firestein 2001, Yu and Kone 2004). Macrophages treated with LPS also produce the anti-inflammatory cytokine IL-10 to

limit their response, which is mediated through TLR4/MAPKinases and Sp-1-dependent signaling (Chanteux, *et al*. 2007).

While acute inflammation is critical for pathogen clearance, inflammation resulting from chronically activated macrophages is associated with multiple diseases including autoimmunity, cardiovascular disease, and tumor promotion and growth (Coussens and Werb 2002, Doria, *et al*. 2012, Libby, *et al*. 2002). Therefore, safe and effective interventions are needed to modulate macrophage functions during chronic inflammatory states. Lacking the stability of highly toxic AhR ligands make indirubins an attractive alternative as potential therapeutic agents. This study seeks to examine the effects of indirubin-3'-(2,3 dihydroxypropyl)-oximether (E804) and 7-bromoindirubin-3'-oxime (7BIO) on inflammatory profiles, including gene and protein expression, cytokine production, and functional endpoints, using bacterial endotoxin lipopolysaccharide (LPS) as stimulation towards a highly pro-inflammatory state. The studies described herein are the first to determine the potential anti-inflammatory properties of novel indirubin derivatives using mammalian macrophage cell lines, and to determine if being an active AhR ligand is necessary for anti-inflammatory properties. Much of the previously published literature on natural indirubin and its derivatives has been focused on anti-tumor and cell-cycle inhibition properties, but not immune function(s).

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The Effects of Indirubin-3'-(2,3 dihydroxypropyl)-oximether (E804) on Inflammation Profiles in Macrophages

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INTRODUCTION

Background

Indirubin is a deep-red bis-indole isomer of indigo blue, two biologically active ingredients in Danggui Longhui Wan, an ancient Chinese herbal tea used to treat neoplasia , chronic inflammation, and to enhance detoxification and detoxication of xenobiotics (Wu *et al.*, 1979; Hoessel *et al.*, 1999; Leclerc *et al.*, 2001). Natural indirubin and its multiple synthetic derivatives inhibit cyclin-dependent kinases (CDKs) and glycogen synthase kinase-3β (GSK-3β), thereby preventing cell cycle progression (Hoessel *et al.*, 1999; Leclerc *et al.*, 2001; Marko *et al.*, 2001; Meijer *et al.*, 2003), and also affecting apoptosis signaling and cell cycling via the *wnt*-signaling pathway.

AhR binding

Several indirubin derivatives are strong aryl hydrocarbon receptor (AhR) ligands (Adachi *et al.*, 2001; Kawanishi *et al.*, 2003; Adachi *et al.*, 2004; Knockaert *et al.*, 2004; Springs and Rice, 2006). The AhR is a member of the basic helix-loop-helix (bHLH-PAS) superfamily of proteins and functions as a ligand-activated transcription factor (Gu *et al.*, 2000) for a suite of responsive genes. Most notable of these genes in the fields of toxicology and pharmacology include phase I and II drug-metabolizing enzymes and

transporters (Nebert *et al.*, 2000; Denison *et al.*, 2002a; Hu *et al.*, 2007b; Kohle and Bock, 2007). Adverse effects of exposure to persistent environmental AhR ligands include immune suppression, as well as reproductive and developmental disorders (Kaminski *et al.*, 2008).

CDK and AhR activities

As with CDK and GSK-3β inhibitory activities, indirubin-related AhR activities cover a wide range of potency, with EC_{50} values between 5 nM – to over 10 μM for CYP1A1 enzymatic activity in hepatoma cells, and 6 nM – to over 100 μM in a yeast reporter assay system (Knockaert *et al.*, 2004; Polychronopoulos *et al.*, 2004). In comparison, the EC_{50} for TCDD-induced CYP1A1 activity in the Knockaert *et al* study was 15 pM in the hepatoma cell line and 25 nM in a yeast reporter assay. Taken together, these observations indicate that strong AhR agonists like TCDD, which bind AhR with high affinity and are recalcitrant to metabolism, may have far lasting toxic effects on cell cycling and whole organ/organism toxicities than would bis-indole derivatives like indirubins, which are much more quickly metabolized by the P450s (Spink *et al.*, 2003). Lacking the stability of highly toxic AhR ligands make indirubins an attractive alternative as potential therapeutic agents for adverse immune responses, including chronic inflammation.

Indirubin as an anti-inflammatory

Inflammation is a physiological response to both tissue damage and breaching of physiological barriers by pathogens and their products, and involves a complex interplay between tissue-fixed macrophages, mast cells, and the recruitment of monocytes, granulocytes, and dendritic cells for wound and tissue repair. Macrophages, in particular, play a significant role in coordinating the balance between wound repair and pathogen clearance, and do so through cytokines, growth factors, and reactive oxygen species (Mantovani, 2006; Dall'Asta *et al.*, 2012). Inflammation resulting from chronically activated macrophages is associated with multiple disease types including autoimmunity, cardiovascular disease, and tumor promotion and growth. Therefore, safe and effective interventions are needed to modulate macrophage functions during chronic inflammatory states.

Indirubin-3'-(2,3 dihydroxypropyl)-oximether (E804) is a recent indirubin derivative possessing novel activity against Stat3 signaling (Nam *et al.*, 2005). Stat3 inhibition by E804 is also associated with impaired normal vascular development as well as reduction of tumor vasculature development (Chan *et al.*, 2012; Shim and Kim, 2012). Stat3 signaling is significant in the context of inflammatory processes because signaling through Stat3 is the primary means by which IL-10 mediates its anti-inflammatory actions in peritoneal resident macrophages (Matsukawa *et al.*, 2005). Furthermore, IFN-γ induced MHC-II expression and antigen presentation in human blood monocyte/macrophages are suppressed by IL-10 through Stat3 signaling (Chan *et al.*, 2010). The suppressive effects of at least one indirubin derivative, indirubin-3'-

monoxime, on activated macrophages is suppressive for activated and differentiating human U937 monocytes (Springs and Rice, 2006), and for LPS-activated microglia (Jung *et al.*, 2011).

Role of macrophages in inflammation

To date, no published studies have examined the effects of E804 on macrophage functional endpoints and pro-inflammatory responses to LPS. Using RAW264.7 murine macrophages, the first study described herein tested the hypothesis that E804 is a potent modulator of key genes, proteins, and functions in macrophages exposed to LPS. We found that 1 µm E804 suppresses LPS-induced iNOS, COX-2, IL-6, and IL-10 expression, which is associated with reduced phagocytosis, cellular lysozyme activity, and intracellular killing of bacteria without cytotoxicity, apoptosis, or necrosis.

Despite the potent anti-inflammatory properties of E804 in RAW264.7 cells, this compound lacks significant AhR-associated CYP1A1 activity, at least in these cells, possibly indicating weak AhR-associated properties of this indirubin derivative, or that this particular cell line lacks necessary components of AhR signaling. Based on this unanswered question, other macrophage cell lines were evaluated. THP-1 cells are a human acute monocytic leukemia line (Tsuchiya *et al.*, 1980) that can be differentiated along a monocyte-macrophage direction in response to PMA (Schwende *et al.*, 1996). These cells are phagocytic, express lysozyme, and express cytokines, proteins, and markers typical of primary macrophages (Daigneault *et al.*, 2010), and have been used in

immunopharmacology and toxicology (Ustyugova *et al.*, 2007). Moreover, as described in the methods section (this dissertation), PMA-differentiated cells THP-1 cells easily express CYP1A1 in response to a variety of AhR ligands, including PCB-126, and prototypic AhR ligand used in toxicological research.

Role of neutrophils in inflammation

One of the key events in inflammatory processes is the recruitment of neutrophils into extravascular tissues in response to both pathogens and tissue debris (Futosi *et al.*, 2013). Sentinel macrophages, upon activation by pathogen-associated molecular patterns (PAMPs), and damage-associated molecular patterns (DAMPs), including various TLRs, innately secrete IL-1β, TNF-α, IL-6, IL-8, and GM-CSF. IL-8 is a chemokine principally directed towards neutrophils to extravagate between capillary endothelial cells (diapedesis), and migrate towards the source of IL-8 (Tsirogianni *et al.*, 2006). GM-CSF initiates increased granulocyte and monocyte hematopoiesis to further a source of monocytes and neutrophils towards the site of inflammation and tissue damage (Egea *et al.*, 2010). This wave of neutrophil influx towards local inflammatory events allows for rapid phagocytosis, intracellular and extracellular killing of pathogens through oxygen-independent and oxygen-dependent mechanisms, respectively (Edwards *et al.*, 1987; Rigby and DeLeo, 2012). In the face of high levels of TNF-α at the site of inflammation, neutrophils can be "primed" (El-Benna *et al.*, 2008) for a

heightened state of phagocytosis and oxidative burst to produce superoxide anion and other reactive oxygen intermediates (Babior, 1999; Babior *et al.*, 2002; Cathcart, 2004).

The phagocyte oxidative burst is produced by the membrane associated NADPH oxidase system, a complex of dissociated units located in the membrane and cytosol in the resting state, but rapidly assembled upon activation (Babior, 1999). Units p40PHOX, p47PHOX, p67PHOX, and Rac2 reside in the cytosol, while Rap1A and cytochrome b558, and a p22PHOX/gp91PHOX unit reside in the membrane. Upon activation, p47PHOX is phosphorylated and cytosolic units migrate to the membrane and complex with cytochrome b558, thereby assembling the oxidase system. Increases in intracellular Ca^{++} may lead to activation of phospholipase A2 activity, followed by arachidonate release Protein kinase C (PKCα) which can regulate the translocation of p47PHOX. Phagocytosis events can lead to activation of phospholipase C, and subsequent release of DAG and IP3, thus activating PKCδ and releasing Ca++ from the endoplasmic reticulum, respectively. PKCδ rapidly phosphorylates p67PHOX and p47PHOX, and assembles the NADPH oxidase system. NADPH oxidase converts molecular oxygen to superoxide anion – the major anti-microbial oxygen radical. Under experimental conditions, phorbol mysristate acetate (PMA), a molecular mimic of DAG, can rapidly induce the NADPH oxidase system to produce superoxide anion. Furthermore, calcium inophores (A23187, ionomycin) can increase intracellular calcium. Under most circumstances, both PKCα and PKCδ are involved (Cathcart, 2004).

Considering the importance of neutrophil/monocyte/phagocyte oxidative burst in inflammatory processes, it may be a molecular target for a variety of pharmacological and toxicological agents. Oxidative burst activities are routinely evaluated as an endpoint in monitoring phagocyte integrity in virtually all multi-cellular organisms, from plants (Torres and Dangl, 2005), to invertebrates (Park *et al.*, 2012; Di *et al.*, 2013), fish (Rice and Weeks, 1991; Kelly-Reay and Weeks-Perkins, 1994; Palić *et al.*, 2006), amphibians (Graham *et al.*, 2012), birds (Kogut *et al.*, 2001), and to all species of mammals (Keogh *et al.*, 2011). Because of their short life span, typically only a few days post-migration from hematopoiesis (Simon and Kim, 2010), there are no suitable and functional mature neutrophil cell lines. However, HL-60 human promyelocytic leukemia cells (Gallagher *et al.*, 1979) can be differentiated to neutrophils over a 6 day period with retinoic acid (Breitman *et al.*, 1980; Hauert *et al.*, 2002). HL-60 derived neutrophils are phagocytic and have the full array of assembled NADPH oxidase system when activated (Nordenfelt *et al.*, 2009).

In this study, retinoic acid-differentiated HL-60 cells were used as a model for understanding the possible effects of indirubins on neutrophil oxidative burst. This model was used under two conditions; as un-primed cells lacking exposure to supernatants containing pro-inflammatory cytokines, and as primed cells following a 24 hr exposure to supernatants from the human mast cell line HMC-1 (Butterfield *et al.*, 1988; Nilsson *et al.*, 1994), a generous gift to the lab from Dr. Joseph H. Butterfield, Mayo Clinic, Rochester, MN, and known to produce large amounts of TNF-α and IL-6

(Chi *et al.*, 2004; Sohn *et al.*, 2013). In this model, PMA is used to stimulate the oxidative burst in the presence of H_2DCFA, a common fluorescing probe for reactive oxygen intermediates produced during burst activity (Possel *et al.*, 1997; Eruslanov and Kusmartsev, 2010).

Role of E804 in mediating inflammation

Taken together, and by using three different cell lines, RAW264.7, THP-1, and HL-60 cells, it was found that E804 is a very weak AhR agonist, but very potent in suppressing LPS-inducible pro-inflammatory genes and proteins in macrophages. On the other hand, 7-bromoindirubin-3'-monoxime (7BIO) is a very potent AhR agonist, and is not as strong as E804 in reducing LPS-stimulated events in macrophages. Neither of these two indirubins significantly modulates the oxidative burst activity of neutrophils. The thesis of this dissertation is that E804 is an excellent candidate for developing anti-inflammatory preparations (creams, tablets etc.) for use in clinical medicine, while having the added benefit of not being a potent AhR ligand.

MATERIALS AND METHODS

I. RAW264.7 cells

Cells and cell culturing

The murine macrophage cell line RAW 264.7 was obtained from ATCC (Manassas, VA USA) and maintained in Dulbecco's modified Eagle's medium (DMEM; CellGro Mediatech) supplemented with 10% heat-inactivated fetal calf serum (FCS) with iron (Atlas; Fort Collins CO USA), 20 mM HEPES, 10 mM L-glutamine, 100 U/mL penicillin, 100 µg/ml streptomycin, 110 µg/ml sodium pyruvate, 1% non-essential amino acids (100 X stock), 4.5 g/L glucose, and 1.5 g/L of $NaHCO_3$, each from Sigma (Sigma Aldrich). Cells were typically grown and maintained at 37°C with 5% CO2 in Corning 75 cm^2 culture flasks.

Cytotoxicity assays

Indirubin-3'-(2,3 dihydroxypropyl)-oximether (E804; Alexis Biochemical) was solubilized in DMSO (Sigma Aldrich) to a stock solution of 10^{-2} M and stored at -20° C in sealed vials until ready for use. Stock preparations were diluted to a final working concentration using supplemented DMEM just prior to use. The chemical structures for natural indirubin and E804 are shown in Figure 1. Unless otherwise noted, cells were treated

with 0.01% DMSO carrier, 1 μM E804 alone, 1 μg/ml LPS from *E. coli*, Serotype 0127:B8 (Sigma Aldrich), or E804 and LPS in combination.

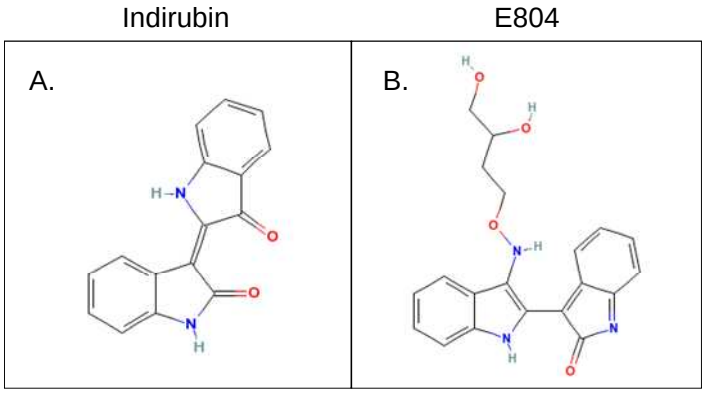


Figure 1

Molecular structures of (A) natural indirubin and (B) indirubin-3'-(2,3 dihydroxypropyl)-oximether (E804) used or referenced within the study.

Cellular respiration as an indication of viability was measured by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. 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 for adherence, compounds and DMSO carrier control (0.01% final) were added in triplicate over serial dilutions beginning with 50 µM per well in a total volume of 200 µl, and the plates incubated for 24 h. Four hours before termination of the assay, each well received 20 µl of a 5 mg/mL MTT solution in DMEM. After centrifugation, the supernatant for each well was discarded and cells containing reduced MTT were solubilized with 100 µl of acidified isopropanol (4 mM HCl, 0.1% NP-40 in isopropanol). Following a brief period of shaking the optical density (O.D.) for each well was recorded at 550 nm. Each experiment was repeated three times and the data averaged from each triplicate, then expressed as percentage of the control O.D. values for each experiment. For statistical purposes, percentage data were arcsine transformed and compared by ANOVA and Bonferroni's multiple contrast post-hoc tests using Graphpad5. Prior to experiments, an α value of 0.05 was established as statistically significant for cytotoxicity determinants and throughout this study.

Annexin V labeling assays

Results from MTT assays indicated that 1 µM and less of E804 were not cytotoxic, yet MTT assays quantify only cellular respiration-related viability and not

necessarily ongoing cell death due to apoptosis or necrosis. Potential apoptosis following exposure to 1 µM E804 was subsequently evaluated using an ApoScreen™ Annexin V Apoptosis Kit (Southern Biotech). Since the study herein would also involve LPS treatments in combination with E804, the potential interactions between compound and LPS in terms of possible apoptotic effects were also evaluated. Briefly, macrophages were seeded at 1.5 x 10^6 cell/well in 6 well plates over 4 h for adherence, and treated with 0.01% DMSO carrier, 1 µg/ml LPS, 1 µM E804, or both in combination for 24 h. Cells were then washed x 3 with ice-cold PBS and 500 µl of Annexin V binding buffer. Binding buffer was added to all wells, along with Annexin V-FITC, and the mixture incubated for 15 min on ice in the dark. Without washing, 380 µL of cold binding buffer and propidium were added to each well. After extensive wash steps, cells were gently scraped from attachment and aliquots processed by flow cytometry using FACScan™ (BD Biosciences), with 10,000 cells being scored in each run.

Commercial qRT-PCR arrays for stress and toxicity

Macrophages were seeded at 1.5 x 10^6 cells/ml in 75 cm^2 Corning culture flasks and allowed to adhere for 4 h. Cells were then treated for 24 h with 0.01% DMSO carrier, 1 µM of E804, 1 ug/ml LPS alone, or both in combination. This experimental regimen was repeated three times and the adhered macrophages scraped from the flask surfaces and pooled (n = 3) per treatment regimen, then harvested by centrifugation to

have one pooled pellet from each of the 3 replicated experiments. TRI-reagent® was added (1 ml) to cell pellets and the mixture homogenized by gentle pipetting several times. The homogenate was incubated at room temperature for 5 min and 0.2 ml chloroform added. After incubating the mixture for 15 min, samples were centrifuged at 12,000 x *g* for 15 min at 4°C. The aqueous phase was transferred to a new 1.5 ml micro centrifuge tube and the total RNA precipitated with 0.5 ml isopropyl alcohol/isopropanol over a 10 min period, then centrifuged again at 12,000 x *g* for 15 min, and re-dissolved in DEPC-treated water.

After collecting RNA from each tube, genomic DNA contamination was removed using elimination mixture supplied by the manufacturer, and first strand cDNA synthesis was carried out using the RT^2 Easy First Strand Kit (SABioscience Corporation) as described by the manufacturer. A predesigned Stress & Toxicity Profiler™ array of 96 genes was purchased from SuperArray Bioscience Corporation for use with RT^2 Real-Timer SYBR Green/fluorescein qPCR master mixes purchased from the same supplier. Specific methods followed those suggested by the manufacturer. RT-qPCR was performed on a BioRad iQ5 real-time PCR detection system. For data analysis, the ΔΔCt method was used; for each gene fold-changes were calculated as difference in gene expression between untreated controls and treated cell cultures. Data were gathered and interpreted using software provided by SABioscience. The manufacturer suggests a two-fold change in gene expression to be of significance, but statistical significance was not determined from a single pooled sample – our goal from the pooled sample was to

determine which genes may be of interest to follow up with, confirm using regular qRT-PCR, or to guide choices of functional endpoints.

qRT-PCR

The above treatment regimen was repeated, but samples from the three replicated experiments were examined individually and not pooled. RNA was extracted and cDNA obtained from treated murine macrophages as described above, but for both 12 and 24 h. Gene expression was analyzed by quantitative real-time PCR with a BioRad iC5 detection system, RT² SYBR green/ fluorescein master mix, and primer sets designed using Integrated DNA Technology (IDT) software, and validated prior to use (Table 1). The quantity of these mRNAs was expressed as fold-changes in gene expression compared to β-actin expression. For data analysis, the ΔΔCt method was used. Expression data were compared between treatment groups using ANOVA, followed by a Bonferroni's post- test using GraphPad5 statistical package.

Gene Name	Accession #	Primer Sequence (5' → 3')	Tm ^{0}C	Product size (bp)
GAPDH	NM_008084	F: TGT GAT GGG TGT GAA CCA CGA GAA R: ACC CTG TTG CTG TAG CCG TAT TCA	57	115
IL-6	NC_000071.5	F: TTG TAC AGT CCC AGT CAG GCA ACA R: TCA AGC TAC TGC AGG CCA GTT ACA	58	80
COX-2	NC_000067.5	F: GCC AGC AAA GCC TAG AGC AAC AAA R: TAC TGA GTA CCA GGC CAG CAC AAA	57.2	144
iNOS	NM_010927	F: CAA ACA CGA GTG CAG CTG GTT GAA R: AGG CAG GAC TGA GTT CAG TGT GTT	58	115
IL-10	NM_010548.2	F: ACTGCTATGCTGCCTGCTCTTACT R: ACTGGGAAGTGGGTGCAGTTATTG	57	90
CYP1A1	NM_009992	F: TAT GAC ACA GAG CAA CAG CAG GGT R: TAT GCC CTT CCA CTT CCT CCC AAA	57	154

Table 1

qRT-PCR primer sequences targeting murine IL-6, COX-2, iNOS, IL-10 and CYP1A1.

Immunoblotting for iNOS and COX-2 protein expression

Triplicate experiments were performed in which 1.5 x 10^6 macrophages were seeded in T-25 flasks in 3 ml of media containing 0.01% DMSO carrier control, 1 μM E804, 1 μg/ml LPS, or both compounds, and incubated for 12 and 24 h. Cells were scraped from each flask and pelleted by centrifugation and covered with 500 μl RIPA lysis buffer (50 mM Tris HCl, pH 8, 150 mM NaCl, 1 % NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing protease inhibitor cocktail (Pierce). The cell pellet was disrupted by gentle vortexing, and incubated on ice for 30 min, then centrifuged at 1000 x *g* for 10 min. The overlying supernatant was removed and centrifuged again for 20 min at 14,000 x *g*; the overlying supernatant was again removed and its protein content quantified. Fifty μg of lysate protein from each sample were separated by SDS-PAGE on 10% gels and transferred overnight onto 0.45 μM nitrocellulose membranes at 4^0 C. The membranes were washed three times with 0.01 M phosphate buffered saline (PBS) containing 0.1% tween-20 (PBS-tw20), covered with blocking buffer (10% FCS in PBS) for 1 h and again washed three times with PBS-tw20. Membranes were then probed for 2 h with goat anti-COX-2 antibody (C-20; Santa Cruz Biotechnology) at 1:750, rabbit anti-mouse iNOS (#610333; BD Transduction Labs) at 1:500, or anti-mouse β-actin (mAb AC-74; Sigma Chemical Company) at 2 μg per ml. Antibodies were diluted in PBS-tw20 containing 1% FCS. The blots were washed three times for 10 min with PBS-tw20, and probed for 2 h with either rabbit anti-goat IgGm goat anti-rabbit IgG, or goat anti-mouse

IgG secondary antibody conjugated with alkaline phosphatase (SouthernBiotech) diluted 1:1000 in PBS-tw20 containing 1% FCS. After extensive washings with PBS-tw20, alkaline phosphatase activity was determined using the substrate NBT-BCIP as means to visualize the relative amount of specific protein. Band densities for each protein were quantified using BioRad G-17 documentation system, and the relative amount of iNOS and COX-2 protein expression were normalized to the β-actin loading control. Data were expressed as relative expression compared to β-actin.

IL-6 and IL-10 secretion, iNOS and lysozyme enzymatic activity

For IL-6 and IL-10 secretion, and iNOS activity assessment, cells were seeded in 96 well plates at 10^5 cells per well in supplemented MEM-alpha lacking phenol red (#41061-029, Gibco). Following a 4 h incubation period for adherence, cells were treated for 24 h with 0.01% DMSO carrier control, 1 µM E804, 1 µg/ml LPS, or both in a final volume of 200 µl. Interleukin-6 was quantified by mouse READY-SET-GO IL-6 ELISA systems (eBioscience) following the directions provided by the supplier. Interleukin-10 was quantified by mouse IL-10 ELISA MAX systems (Biolegend). Briefly, 100 µl of supernatant were removed from each well 24 h after initiation of treatments and added to a pre-coated capture antibody Costar maxi-sorb plate provided by the kits, and incubated overnight at 4°C. The plates were washed and blocked using reagents provided by the vendor. Detection antibody was added for 1 h, followed by treatment

with avidin-HRP and a colorimetric substrate solution. A phosphoric acid stop solution was added, and the plate read at 450 nm. Concentrations of cytokines were plotted against the standard curve of known concentrations supplied by the manufacturer.

To quantify iNOS activity as NO secretion, 100 µl aliquots of supernatants were incubated in 96 well plates at room temperature for 15 min with an equal volume of Griess reagent (equal parts reagent 1; N-(1-naphthyl)-ethylenediamine dihydrochloride, and reagent 2; Sulfanilamide). The O.D. was measured at 550 nm and compared to a $NaNO_2$ standard curve. Values per replicate wells and per treatment replicates were averaged and then plotted. Cytokine and NO data were compared using ANOVA followed by a Bonferroni's post- test using GraphPad5 statistical package.

The enzymatic activity of lysozyme was measured in RAW 264.7 cells based on modifications of published procedures (Parry *et al.*, 1965; Burton *et al.*, 2002). Cells were seeded into 6 well plates at 1.5×10^6 per well in DMEM complete media and allowed to adhere for 4 h prior to treatment, then incubated for 24 h. Cells were lysed and the protein concentration normalized to 1 mg/ml. One hundred µl of cell lysate, in triplicate, were added onto 96 well plates followed by the addition of 200 µl of 2 mg/ml heat killed *Micrococcus lysodietikus* (Sigma Aldrich) in 0.01 M PBS, pH 4.0. The enzymatic reaction was carried out in an uGuant microplate reader at room temperature. The plates were read at wavelength 450 nm every 5 min for 30 min, then again at 10 min intervals for an additional 30 min. One unit of lysozyme activity was defined as a decrease in absorbance of 0.001 min^{-1}. Enzymatic activity data were

compared between treatment groups using ANOVA, followed by a Bonferroni's post-test using GraphPad5 statistical package.

Phagocytosis assays

Phagocytic capability of RAW264.7 cells following 24 h treatment was determined by their ability to phagocytize 1 µM fluorebrite carboxylate YG microspheres (Polyscience) using modifications of previously described methods (Bozzaro *et al.*, 1987; Peracino *et al.*, 1998). Washed beads, diluted in 50 mM Na_2HP0_4, were added to 10^5 treated cells in flat- bottom 96 well plates at a ratio of 100:1 (beads: cells) in a total of 200 µl media. Each treatment involved 12 wells of the plate, and the experiment was repeated three times. After adding beads to cells, the plates were centrifuged lightly for 5 min to maximize contact between cells and beads, and plates incubated at 37°C for 45min. Extracellular microspheres were then removed by 3 washes with PBS in a series of centrifugation steps (500 rpm for 5 min). Phagocytosis was stopped by the addition of 100 µl ice cold PBS with 1 mM $CaCl_2$ and $MgCl_2$. Trypan blue (2 mg/ml) dissolved in 20 mM citrate and 150mM NaCl, pH 4.5, was added to quench any fluorescence from adhered, but not internalized beads. Plates were then read on a FLx-800 fluorescent spectrophotometer at an excitation wavelength of 485 nm and emission wavelength 528 nm. Data from the 12 wells were gathered as fluorescence units, averaged for each experimental replicate, and compared to the

control as percentages and arcsine transformed prior to statistical analysis. Data were compared between treatment groups using ANOVA, followed by a Bonferroni's post-test using GraphPad5 statistical package.

Intracellular killing assays

The effects of treatment on intracellular killing of *E.coli* was measured based previously published methods (Boleza *et al.*, 2001). Briefly, RAW 264.7 cells were seeded onto 96 well plates at a concentration of 10^5 cells per well, allowed to adhere for 4 h, and treated in a total volume of 200 ul for 24 h. Each treatment utilized 12 wells of the plate. *E.coli* were then added at a ratio of 25:1 (bacteria:cells) and incubated for 180 min at 37°C. Extracellular bacteria were removed by extensive washing with PBS, and further treated with 200 µl of 1000 U Penicillin and 10 µg/mL streptomycin solution (Sigma Aldrich) for 15 min. Cells were then permeablized with 100 µl 0.1% triton-x100 in PBS for 15 minutes to release the engulfed bacteria. The Triton X-100 solution was removed by aspiration and the viable bacteria in each well were grown in 200 µl LB broth for 12 hours. Live bacteria were determined using the MTT assay, and results presented as a percentage of control treated cells. Percentage data were arcsine transformed prior to statistical analysis using ANOVA, followed by a Bonferroni's post-test using GraphPad5 statistical package.

II. THP-1 Cells

The human monocytic cell line THP-1 was obtained from ATCC (Manassas, VA USA) and maintained in RPMI-1640 (Cellgro) supplemented with 10% heat-inactivated FCS with iron (Atlas; Fort Collins CO), 20 mM HEPES, 10 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 110 µg/ml sodium pyruvate, 1% non-essential amino acids (100 X stock), 4.5 g/L glucose, and 1.5 g/L of NaCO3, each from Sigma (Sigma Aldrich, St. Louis MO, USA). Cells were grown and maintained at 37°C with 5% CO2 in Corning 75 cm^2 culture flasks. To differentiate THP-1 cells towards a macrophage phenotype, cells were incubated for 48 hr with 0.1 µM PMA.

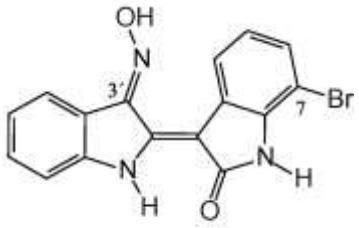
Treatment

Indirubin derivatives and phorbol-12-myristate-13-acetate were purchased from Alexis Biochemical (San Diego, CA), solubilized in DMSO (Sigma Aldrich) to a stock solution of 10^{-2} M and stored at -20° C in sealed vials until ready for use. Stock preparations were diluted to a final working concentration using supplemented RPMI just prior to use. The cells were stimulated with 0.1 µM PMA for 48 hours prior to treatment in order to induce differentiation. Cells were treated with 0.01% DMSO carrier, indirubins alone, 0.1 µg/ml lipopolysaccharide from E. coli, Serotype R515 (Re) (ultra pure,TLR4grade™) (Alexis Biochemical, San Diego, CA), or in combination,

depending on the experiment using approaches previously described for RAW264.7 cells.

Comparing the AhR activity of E804 to select indirubin derivatives

THP-1 cells were seeded at 10^6 cells/ml in 25 cm^2 culture flasks (Corning Inc., NY) and treated with 0.1 µM PMA for 48 hr for differentiation. Cells were then treated for another 24 hr with 1 µM of multiple available indirubins, as well as PCB-126, a potent AhR ligand to be used as a positive treatment for CYP1A1 induction. Cells were prepared for qPCR as described for RAW264.7 cells, but using human CYP1A1 specific primers (Table 2). This screening of indirubins for AhR activity revealed that E804 is a weak inducer of CYP1A1, while 7-bromoindirubin-3'-monoxime (7BIO) is very potent – more so than PCB-126 (data not shown). Additional experiments were conducted to determine if 12 hr vs 24, and 0.1 µM vs 1 µM E804 and 7BIO resulted in optimal CYP1A1 expression. Considering that 12 hr vs. 24 hr exposuures may lead to different results, IL-6 expression following 12 hr vs 24 hr exposure to LPS was examined. As described in the results section, 1 µM at 24 hr was optimal for both E804 and 7BIO, and there were no differences in IL-6 expression at 12 hr vs 24 hr. From this point onward, studies to further characterize the effects of 1 µM E804 on inflammation profiles also examined the effects of 1 µM 7BIO (Figure 2) as a means to evaluate two indirubins varying widely in their ability to activate AhR signaling.



7-bromoindirubin-3'-oxime

Figure 2

Molecular structure of 7-bromoindirubin-3'-oxime (7BIO) used or referenced within the study.

Cytotoxicity assays

Cellular respiration as an indication of viability was measured by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. Macrophages were plated into 96 well Costar plates at 10^5 cells per well in 100 µl of RPMI media. After 48 hr treatment with 0.1 µM PMA, compounds and DMSO carrier control (0.01% final) were added in triplicate over serial dilutions of compounds of interest, beginning with 50 µM per well, and the plates incubated for 24 hr. Developing the MTT assay and quantifying data followed the same procedures as described for RAW264.7 cells.

qRT-PCR for expression of IL-6, IL10, COX-2, iNOS, CYP1A1, TNFα, IL1β, IDO I&II, and ARG I&II

THP-1 cells were seeded at 10^6 cells/ml in 25 cm^2 culture flasks (Corning Inc., NY) and treated with 0.1 µM PMA for 48 hr. Cells were then treated for 24 hr with 0.1 µg/ml LPS, 1 µM E804 or 7BIO, or each indirubin and LPS in combination for 24 hr. This experimental regimen was repeated three times and the adhered macrophages scraped from the flask surfaces, then harvested by centrifugation. qPCR was carried out as described for RAW264.7 cells, but using human-specific primers (Table 2). Genes of interest were chosen based on results from RAW264.7 cells, and also to include indoleamine diooxygenases I & II, and arginine I & II as markers of possible polarization

of macrophages from typical MI (acute inflammatory) to M2 (more low-grade, chronic inflammation) polarized cells.

Gene Name	Accession #	Primer Sequence (5' ⟶ 3')	Tm ^{0}C	Product size (bp)
GAPDH	NM_001256799.1	F: AGCCTCAAGATCATCAGCAATGCC R: TGTGGTCATGAGTCCTTCCACGAT	57	105
CYP1A1	NM_000499.3	F: GACACAGTCACAACTGCTATCT R: GCTCCTCTTGGATCTTTCTCTG	53	82
IL1-β	NM_000576.2	F: AACAGGCTGCTCTGGGATTCTCTT R: ATTTCACTGGCGAGCTCAGGTACT	57	92
IL-6	NM_000600.3	F: TCAATGAGGAGACTTGCCTGGTGA R: TACTCATCTGCACAGCTCTGGCTT	58	123
TNF-α	NM_000594.2	F: AGGACGAACATCCAACCTTCCCAA R: TTTGAGCCAGAAGAGGTTGAGGGT	57	92
IDO1	NM_002164	F: CACTTTGCTAAAGGCGCTGTTGGA R: GGTTGCCTTTCCAGCCAGACAAAT	57	140
IDO2	NM_194294	F: TGGAAATTGGGAACCTGGAGACCA R: ATCCCAGGCACTGCTTCTTTCTCT	57	106
IL-10	NM_000572	F: TCCTTGCTGGAGGACTTTAAGGGT R: TGTCTGGGTCTTGGTTCTCAGCTT	57	109
iNOS	NM_000625.4	F: GTCAGAGTCACCATCCTCTTT R: GCAGCTCAGCCTGTACTTATC	52	129
COX-2	NM_000963	F: TACTGGAAGCCAAGCACTTT R: GGACAGCCCTTCACGTTATT	52	92
Arginase I	NM_000045.3	F: CAGGGCTACTCTCAGGATTAG R: CCGAAACAAGCCAAGGTTATT	52	115
Arginase II	NM_001172.3	F: CATGGACAGCCAGTTTCATTTC R: CCACGTCTCTCAGACCAATATAC	52	127

Table 2

qRT-PCR primer sequences targeting human IL-6, IL-10, COX-2, iNOS, CYP1A1, TNF-α, IL1-β, IDOI&II, and ARG I&II.

Immunoblotting for COX-2 and p21 protein expression:

Triplicate experiments were performed in which 1.5 x 10^6 macrophages were seeded in 25 cm^2 flasks in 3 ml of RPMI containing 0.1 μM PMA for 48 hr. Cells were then treated for 24 hr with 0.1 μg/ml LPS, 1 μM E804 or 7BIO, or each indirubin and LPS in combination for 24 hr. Cells were scraped from each flask and processed for SDS-PAGE and immunoblotting as described for RAW264.7 cells. Membranes were then probed for 2 h with goat anti-COX-2 antibody (C-20; Santa Cruz Biotechnology) at 1:750, mouse anti-p21 (556430; BD Transduction Labs) at 1:500, or anti-mouse β-actin (mAb AC- 74; Sigma Chemical Company) at 2 μg per ml. Antibodies were diluted in PBS-tw20 containing 1% FCS. The blots were washed three times for 10 min with PBS-tw20, and probed for 2 h with either rabbit anti-goat IgGm, or goat anti-mouse IgG secondary antibody conjugated with alkaline phosphatase (SouthernBiotech) diluted 1:1000 in PBS-tw20 containing 1% FCS. After extensive washings with PBS-tw20, alkaline phosphatase activity was determined using the substrate NBT-BCIP as means to visualize the relative amount of specific protein. Band densities for each protein were quantified using BioRad G-17 documentation system, and the relative amount of iNOS and COX-2 protein expression were normalized to the β-actin loading control. Data were expressed as relative expression compared to β-actin.

IL-6 and IL-10 secretion:

For IL-6 and IL-10 secretion, THP-1 cells were seeded in 96 well plates at $1x10^5$ cells per well in supplemented RPMI. Following a 48 hr treatment with 0.1 µM PMA for 48 hr for differentiation, cells were treated for 24 hr with 0.1 µg/ml LPS, 1 µM E804 or 7BIO, or each indirubin and LPS in a total 200 µl. Interleukin-6 and 10 were quantified by human IL-6 and IL-10 ELISA MAX systems (Biolegend) as described for RAW264.7 cells.

Intracellular lysozyme activity as an indicator of lysozyme protein content:

THP-1 cells were seeded in 96 well plates at $1x10^5$ cells per well in supplemented RPMI. Following a 48 hr treatment with 0.1 µM PMA for 48 hr for differentiation, cells were treated for 24 hr with 0.1 µg/ml LPS, 1 µM E804 or 7BIO, or each indirubin and LPS in a total 200 µl. The enzymatic activity of lysozyme was measured as described for RAW264.7 cells.

Phagocytosis assays

THP-1 cells were seeded in 96 well plates at $1x10^5$ cells per well in supplemented RPMI. Following a 48 hr treatment with 0.1 µM PMA for 48 hr for differentiation, cells

were treated for 24 hr with 0.1 µg/ml LPS, 1 µM E804 or 7BIO, or each indirubin and LPS in a total 200 µl. Phagocytosis was measured as described for RAW264.7 cells.

III. Oxidative burst in retinoic acid-differentiated HL-60 cells (neutrophils).

HL-60 cells were obtained from ATCC, and cultured in RPMI-1640 (Cellgro) supplemented with 10% heat-inactivated FCS with iron (Atlas; Fort Collins CO), 20 mM HEPES, 10 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 110 µg/ml sodium pyruvate, 1% non-essential amino acids (100 X stock), 4.5 g/L glucose, and 1.5 g/L of NaCO3, each from Sigma (Sigma Aldrich, St. Louis MO, USA). Cells were grown and maintained at 37°C with 5% CO2 in Corning 75 cm^2 culture flasks. Retinoic acid was purchased from Alfa Aesar, through VWR Scientific, and dissolved to 0.01 M in DMSO, then diluted in media prior to use. Cells were allowed to differentiate in 1 µM retinoic acid for 6 days prior to treatment with indirubins and LPS, then washed by centrifugation and plated out in black 96-well microtiter plates (Corning, CoStar #3904) at $1x10^5$ cells per well in 100 µl media. Cells were then treated for 24 hr with 0.1 µg/ml LPS, 1 µM E804 or 7BIO, or each indirubin and LPS in a total 200 µl. Some cells were treated with the above regimens that also included 25% supernatants from HMC-1 cells growing to near confluence in culture medium [Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal calf serum (FCS), 2mM L glutamine, 100 IU/ml

penicillin, 50 mg/ml streptomycin, 2 mM α-thioglycero]). The experiment was repeated three times, and treatments included three wells of each plate.

To initiate the oxidative burst assay, H_2DCFA at a final concentration of 10 µM was added to all wells of the plate and the cells incubated for 15 min, at which time the plates were read at excitation/emission wavelengths of 494/522 nM. PMA at a final concentration of 0.2 µM was added to each well, and the plates read again 90 min later. The data were recorded, and relative fluorescence units (RFU) converted to stimulation indices as a ratio of PMA-stimulated RFU to non-stimulated RFU.

RESULTS

RAW264.7 Model.

E804 at 1 µM and less are not cytotoxic, nor pro-apoptotic and pro-necrosis

Using the MTT assay, which measures succinate dehydrogenase activity (Tim, 1983), the effects of E804 on cellular respiration in RAW264.7 cells as an indication of toxicity were examined over a range of concentrations for 24 h. One µM and below did not affect respiration (Figure 3) and was chosen for further studies. To confirm that 1 µM was not only non-cytotoxic, but non-pro-apoptotic and non-pro-necrotic as well, annexin-V binding was quantified following treatment with 1 µM of E804, 1 µg/ml LPS, or both in combination. The results of annexin-V labeling reflect MTT assay results in that > 99 % of cells are viable, and not apoptotic or necrotic after 24 h exposure to treatments (Figure 4).

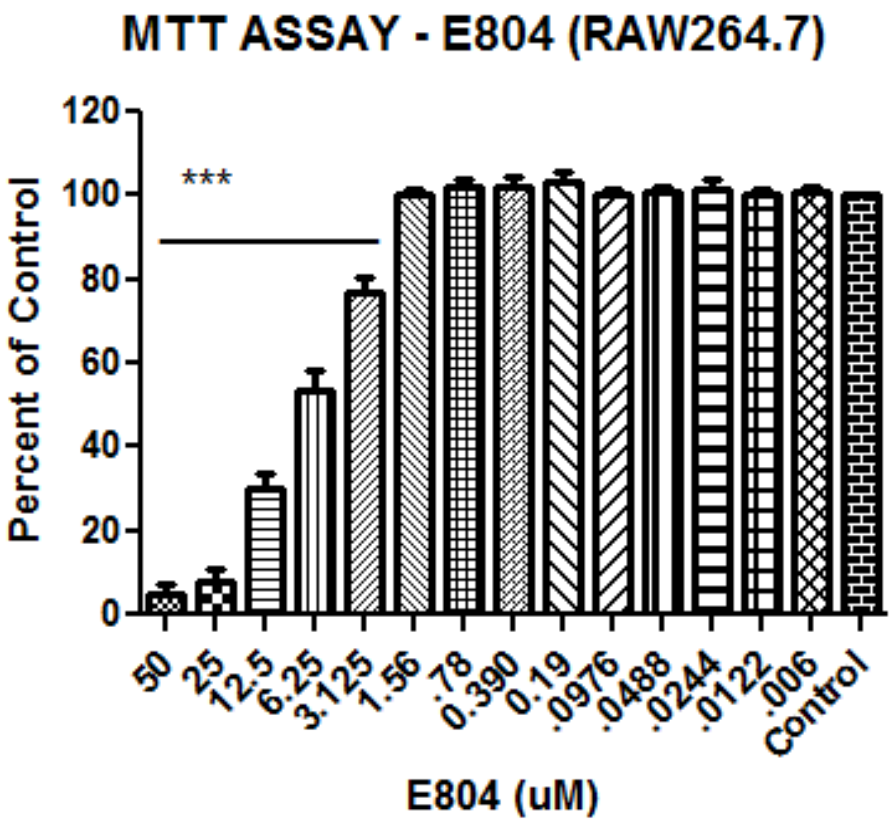


Figure 3

Effects of E804 on cellular respiration in RAW264.7 cells, as determined by the MTT assay. Data are presented as percentage of control and shown as the mean ± standard error of n = 3 independent experiments. * represents significance (P ≤ 0.001).**

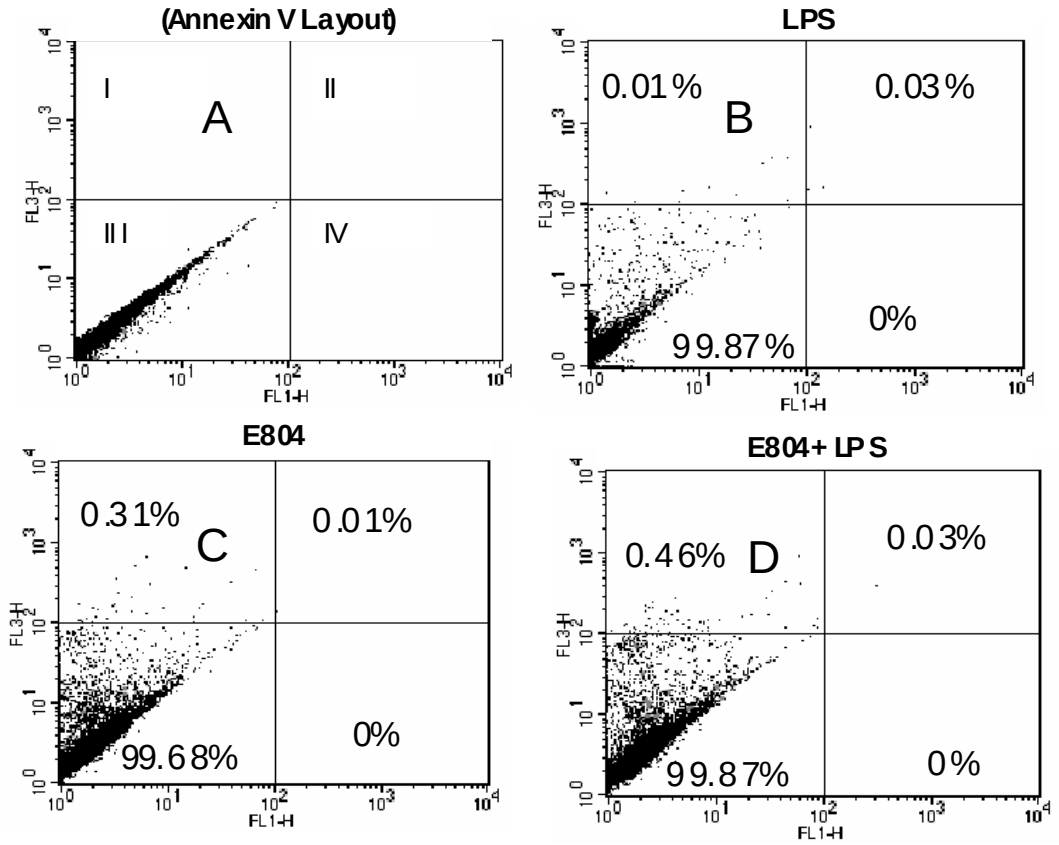


Figure 4

Annexin-V labeling of RAW246.7 macrophages following 24 hr exposure to 1μM E804, 1 μg/ml LPS, and E804 + LPS as measured by flow cytometry. Annexin V (FL1-H) and propidium iodide (FL3-H) labeling. (A) Guide figure of typical results: I- Early apoptosis; II-Late apoptosis; III-Viable cells, not undergoing apoptotoic events; IV- Early apoptosis. Effects of treatment are shown in other panels (B) LPS, (C) E804, (D) E804+LPS.

qRT PCR Arrays: LPS-induced proinflammatory genes and cell cycling are sensitive to E804 treatment

A commercially available qPCR array platform designed to screen for stress and toxicity was used as a first-line investigation to determine if E804 would modulate LPS-associated changes in gene expression in macrophages, and to explore possible effects of E804 alone. Data from such arrays are expressed as relative fluorescence units, not absolute fold change or mRNA copy number. These data do, however, provide an indication of relative magnitude of expression compared to controls, and with this particular array investigators are given a snap shot of genes associated with inflammation, apoptosis, cell cycle control, and oxidative stress.

As expected, LPS-activated macrophages exhibit an increased expression of several inflammatory genes available on this particular array (Table 3). In the case of Chemokine ligand 4, MMIF, and NF-κβ, E804 alone increased expression, but inhibited expression in combination with LPS. Otherwise, E804 was either non-effective or was inhibitory in terms of expression of genes examined using this approach. For example, cells treated with E804 alone exhibit only a slight increase in iNOS expression. On the other hand, E804 was a potent inhibitor of LPS-induced expression of iNOS, IL-1α, IL-1β, and IL-6 genes.

As part of the screening for stress and toxicity, select genes associated with apoptosis and anti-apoptosis were investigated. Expression of Caspase 1, Caspase 2,

and Annexin 5 were elevated by treatment with E804 alone, with similar levels of expression observed following LPS treatment alone. In combination, LPS and E804 completely shut down expression of these three genes. Likewise, Bcl2-associated-x and Bcl-2-like protein expression levels were strongly suppressed by combined treatment with LPS and E804, though treatments with each alone had little effect on expression. Two genes directly involved in initiating apoptosis, NF-κβ and TNR receptor 1a, were suppressed by E804, which was overcome in the presence of LPS.

Nine genes associated with cell cycle control and regulation were part of the stress & toxicity qPCR array. Expression of Cyclin C and Cyclin D1 were suppressed by E804 treatment, and LPS treatment restored Cyclin D1 expression, but not Cyclin C to levels comparable to vehicle-treatments. LPS treatment was associated with a strong expression of EGR-1, which is equally suppressed by E804. LPS treatment was associated with high expression of Mdm2, which is suppressed by E804, and E804 strongly suppressed expression of p53.

Genes associated with oxidative stress are also part of the commercial array platform, representing Cyp1a1, Fmo4 and Fmo5, and several associated with antioxidant responses. E804 did not induce Cyp1a1 gene expression under the experimental conditions described herein. However, Fmo5, HO-1, and SOD-1 genes were strongly increased. LPS treatment did not change the expression levels of HO-1 in the presence of E804, but SOD-1 expression was completely shut down in cells treated with both LPS and E804.

Table 3. Results from commercial qRT-PCR Stress & Toxicity™ arrays targeting select genes associated with inflammation, apoptosis, cell cycle control, and oxidative stress. RAW 264.7 cells were treated with 1 μM E804, 1 μg/ml LPS, E804 + LPS, or 0.01% DMSO as the carrier control for 24 h. Cells were harvested and processed for analysis of gene expression using designed commercial qRT-PCR arrays (SuperArray®) following instructions provided by the manufacturer. Data for gene expression represent x-fold changes in mean SYBER-green fluorescence units of 3 pooled samples compared to carrier control-treated cells, and normalized for house-keeping gene expression. Details are provided in the Methods and Materials section.

Gene Name	Genbank no.	Symbol	E804	LPS	E804 + LPS
		INFLAMMATION			
CSF	NM_009969	Csf2	+2.68	+27.29	-1.64
Chemokine ligand 10	NM_021274	Cxcl10	-72.64	+3.34	-928.16
Chemokine ligand 4	NM_013652	Ccl3	+633278	+190362	-38.6
IL-18	NM_008360	Il18	-1.16	-1.4	-1669.83
IL-1α	NM_010554	Il1a	+2.68	+169.9	-14.75
IL-1β	NM_008361	Il1b	-2.29	+35.45	-1.89
IL-6	NM_031168	Il6	+2.38	+67.89	-1.94
MMIF	NM_010798	Mif	+4862.56	+350089	-1.39
NF-Kβ (p105)	NM_008689	nfkb1	+6500	+16920	-1.46
iNOS	NM_010927	Nos2	+2.36	+10.19	-1.35
		APOPTOSIS			
Caspase 1	NM_009807	Casp1	+446703	+6562	-1.39
Capspase 8	NM_009812	Casp2	+265.75	+418.16	-1.37
Annexin 5	NM_009673	Anxa5	+173821	+91288	-1.09
Bcl2-assoc x	NM_007527	Bax	+3.95	-1.05	-21897
Bcl2-like protein	NM_009743	Bcl2l1	-302.91	+2.55	-21897.7
NFkappa B inhib	NM_010907	Nfkbia	-13.53	+2.88	+1.01
TNF receptor 1a	NM_011609	Tnfrs1a	-756	+1.2	+1.1

Table 3 (continued)

Gene Name	Genbank no.	Symbol	E804	LPS	E804 + LPS
		CELL CYCLE CONTROL AND REGULATION			
Cyclin C	NM_016746	Ccnc	-335.31	-1.21	-1072.65
Cyclin D1	NM_007631	Ccnd1	-3564	-1.51	+1.02
Cyclin G1	NM_009831	Ccng1	+7.2	-1.07	-11.9
E2f tf-1	NM_007891	E2f1	-968.65	-1.18	-3201.7
EGR-1	NM_007913	Egr1	+2.68	+8989	-1.81
PCNA	NM_011045	pcna	-5	+ 15.98	-1.27
CDKI-1α (p21)	NM_007669	Cdkn1a	-3.92	-1.06	+1.13
3T3 c.d.m.2	NM_010786	Mdm2	+2.68	+25458	-1.95
P53	NM_001640	Trp53	-6387	1.15	-1.35
		OXIDATIVE STRESS			
CYP1A1	NM_009992	cyp1a1	+2.69	+1.01	+1.46
Flavin moxy 4	NM_144878	fmo4	+2.68	+5.23	-7.58
Flavin moxy5	NM_010232	fmo5	-54.06	-1.75	-1.21
Glutathione Px1	NM_008160	GPx1	-32.58	-1.37	-1.48
Glutathione red	NM_010344	Gsr	+2.25	-1.05	+1.84
Glutathione trans	NM_010358	GSTm1	-507.0	-1.15	-1.36
Heme Ox 1	NM_010442	Hmox1	+1582.53	+1.01	+6448.8
Metallothionein 2	NM_008630	Mt2	-4.96	+1.52	-1.55
Polym II ppK	NM_023127	Polr2k	+8.9	-1.21	-1.06
P450 oxidored	NM_008898	Por	+1.81	+1.29	+1.1
Superoxide dis 1	NM_011434	Sod1	+323782	+66465	-1.49
Superoxide dis 2	NM_013671	Sod2	-457.67	+2.05	-1.69

qRT-PCR follow-up to arrays confirms array results

qRT-PCR was carried out to validate the effects of treatments on IL-6 gene expression as a keystone cytokine associated with inflammation and systemic acute phase responses. Also quantified, were expression profiles of COX-2, iNOS, IL-10, and CYP1A1. As expected, LPS was a strong inducer for expression of IL-6, iNOS, IL-10 and COX-2 genes (Figure 5). E804 inhibited LPS-induced IL-6, COX-2, and iNOS expression (Figure 5), with the most impact on IL-10 expression, but did not induce CYP1A1 gene expression (data not shown), thereby reflecting a rather anemic CYP1A1 response noted from the array data. Previous unpublished work from our lab shows that RAW264.7 cells are unresponsive to 3', 3', 4', 4', 5-pentachlorobiphneyl (PCB-126), a potent environmental AhR ligand, in terms of CYP1A1 gene expression.

Cellular iNOS and COX-2 protein expression is modestly affected by E804

As expected, LPS is a strong inducer of both iNOS and COX-2 at the protein level at both 12 and 24 h of treatment (Figure 6a). E804 did not induce visible protein, and modestly reduced LPS-induced expression (Figure 6b). When band densities were compared for all three replicate experiments, iNOS levels were more dramatically suppressed compared to COX-2. At least at the protein level, COX-2 induction is only slightly affected by E804.

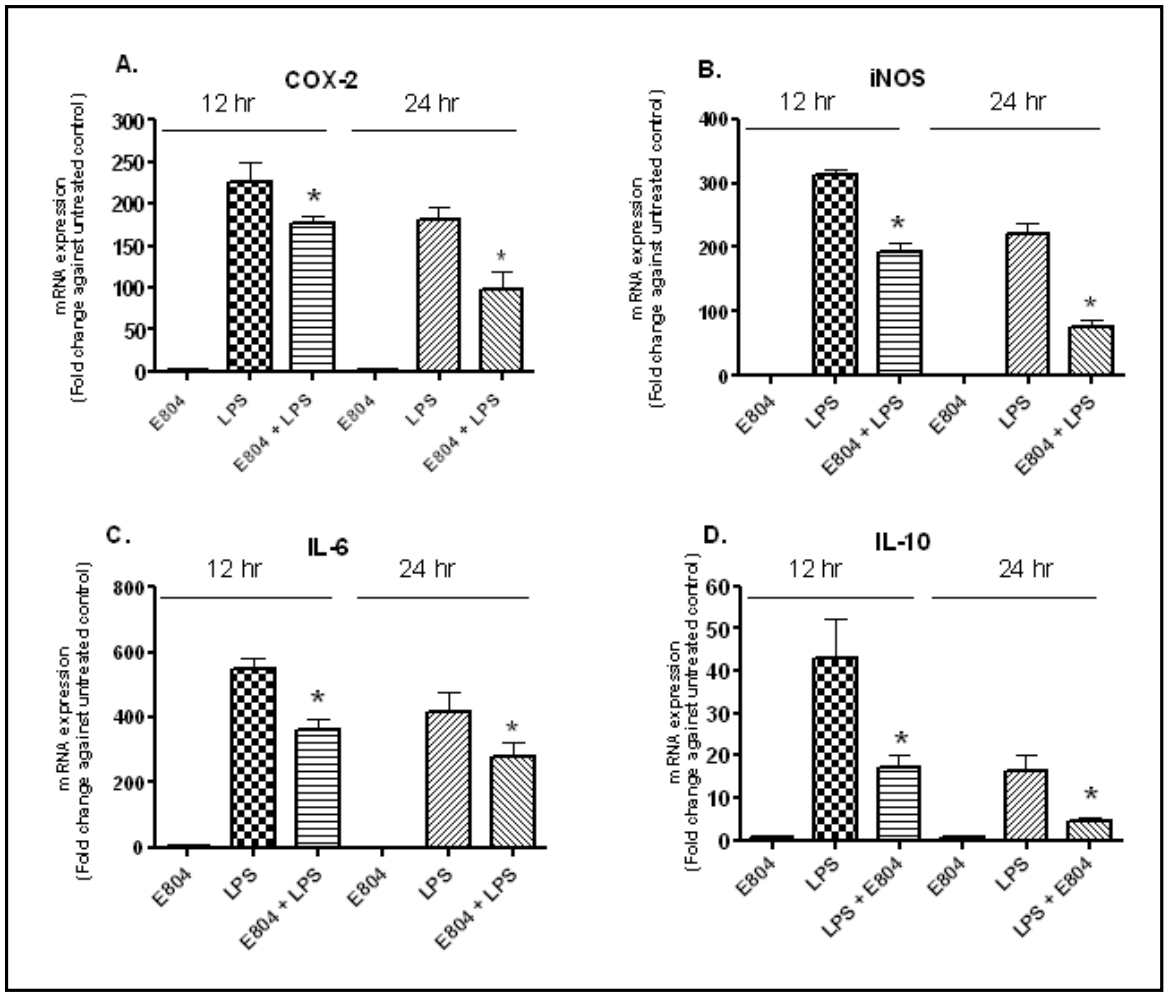


Figure 5

Real time quantitative PCR measuring the expression of COX-2, iNOS, IL-6, and IL-10 in RAW246.7 macrophages. Cells were treated 24 h with carrier control (0.01% DMSO), 1 µM E804, 1 µg/ml LPS, or LPS + E804 prior to quantifying gene expression. Data represent the mean ± standard error of n = 3 independent experiments in which specific gene expression was compared to controls and normalized as fold-difference compared to expression of β-actin as a housekeeping gene. * represents significance (P ≤ 0.05).

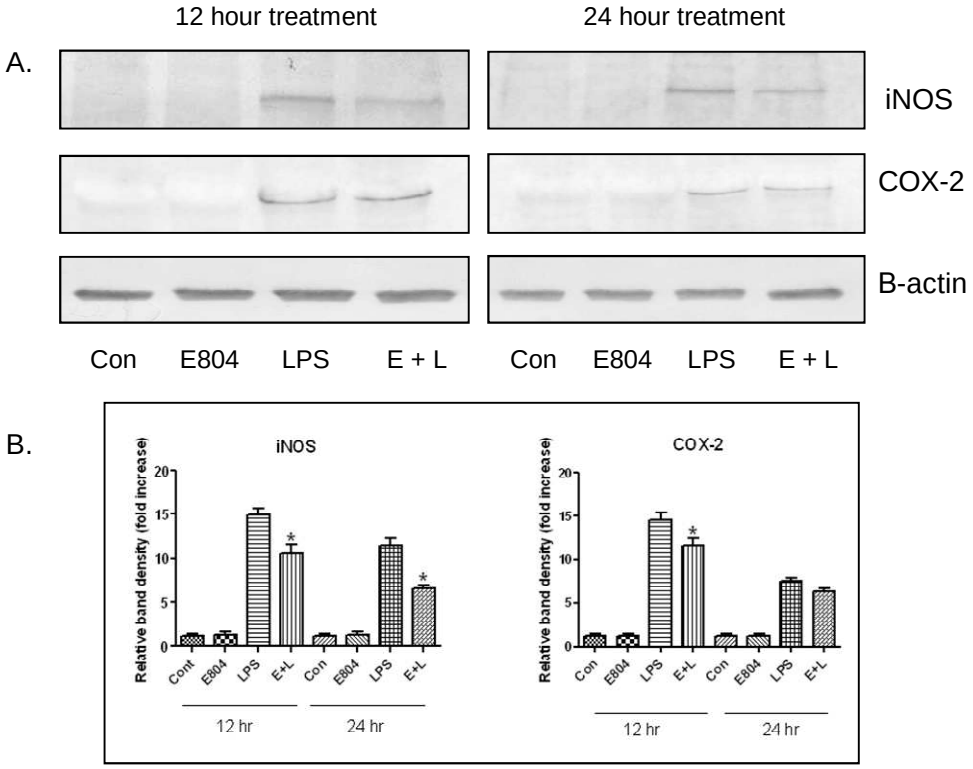


Figure 6

The effects of carrier control (0.01% DMSO), 1 µM E804, 1 µg/ml LPS, and LPS + E804 on iNOS and COX-2 in RAW 264.7 cells following 24 h treatments. Cell lysates were subjected to SDS-PAGE and immunoblotting. A. Representative immunoblots showing iNOS, COX-2, and β-actin as a loading control at 12 and 24 h treatment. B. Data represent the mean band densities ± standard error of n = 3 independent experiments. * represents significance (P ≤ 0.05).

Cellular lysozyme and iNOS enzymatic activity are reduced by E804 in the presence of LPS

Macrophages are a key source of iNOS and antimicrobial proteins, and both iNOS and lysozyme enzymatic activity are easily quantified using standard procedures. At least under these experimental conditions, lysozyme activity is not enhanced by treatment with LPS (Figure 7), nor is it modulated by E804 when give alone. However, E804 in combination with LPS suppressed lysozyme enzymatic activity by nearly 30%. iNOS activity (NO secretion into media) is highly induced by LPS, and E804 strongly inhibits the production of NO.

LPS-induced Interleukin-6 and IL-10 secretion are suppressed by E804

As demonstrated in the study described herein, LPS is predictably a potent inducer of IL-6 and IL-10 secretion in RAW264.7 macrophages (Figure 8). Under the present experimental conditions, E804 had no effect on basal IL-6 and IL-10 secretion, but suppressed secretion stimulated by LPS. IL-10 secretion was more dramatically affected by E804 than was IL-6.

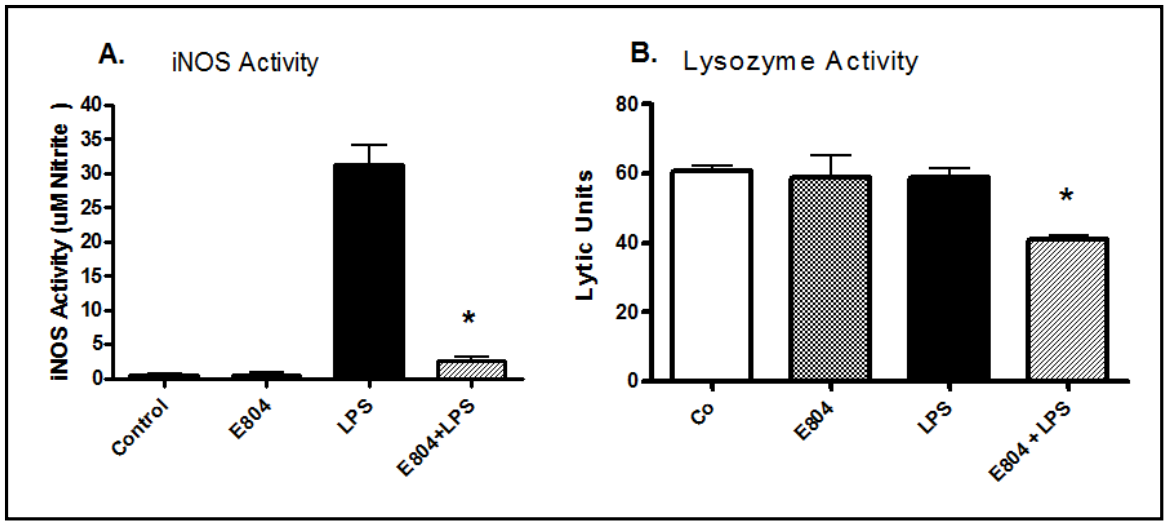


Figure 7

The effects of carrier control (0.01% DMSO), 1 µM E804, 1 µg/ml LPS, and LPS + E804 on cellular lysozyme and iNOS enzymatic activity in RAW 264.7 cells following 24 h treatments. Cell lysates were used as the source of lysozyme and cell supernatants were used as a source of nitric oxide. Data represent the mean enzymatic activity (NO production or lytic units) ± standard error of n = 3 independent experiments. * represents significance ($P \leq 0.05$).

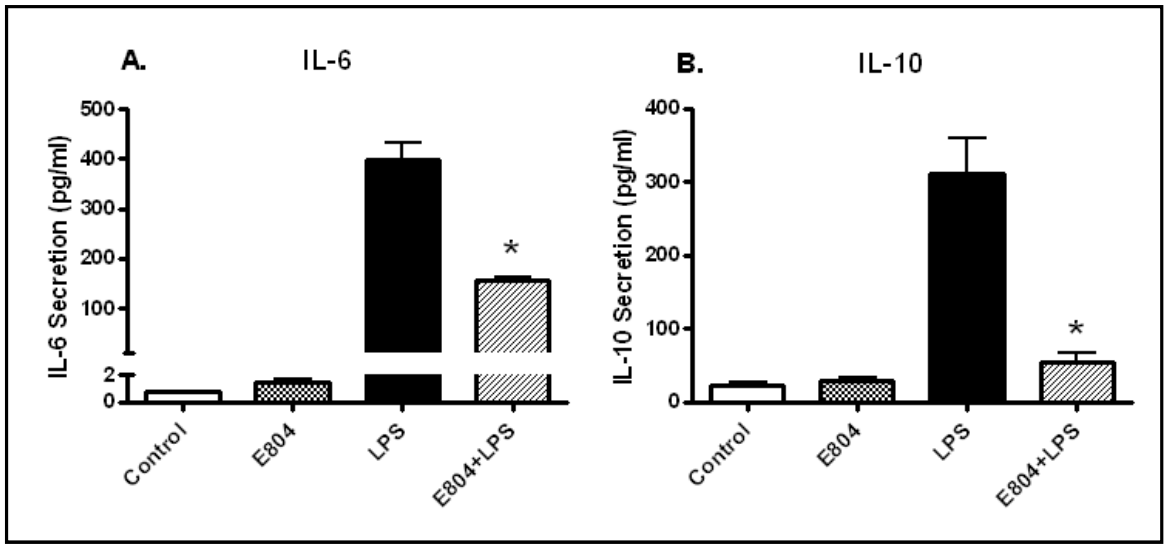


Figure 8

The effects of carrier control (0.01% DMSO), 1 µM E804, 1 µg/ml LPS, and LPS + E804 on the secretion of IL-6 and IL-10 by RAW 264.7 following 24 h treatments. Supernatants were removed and IL-6 and IL-10 quantified in supernatants by ELISA. Data represent the mean ± standard error of n = 3 independent experiments. * represents significance ($P \leq 0.05$).

Phagocytosis and intracellular killing are sensitive to E804 treatment

Gene expression, cytokine secretion, and lysozyme activity data from this study demonstrate that E804 is a potent indirubin derivative with significant potential in suppressing macrophage responses to LPS. To further characterize macrophage functions, we determined the effects of E804, LPS, and both in combination on two very important endpoints; phagocytosis and intracellular killing (Figure 9). Compared to treatment with the vehicle control, E804 suppressed the ability of macrophages to phagocytize latex beads. Phagocytosis was not enhanced by treatment with LPS, but was suppressed by E804 in combination with LPS.

Intracellular killing of *E. coli* was determined by quantifying survival of bacteria survival following the 3 h co-incubation with macrophages, and was not affected by pre-treatment with LPS. Indirubin E804 treatment alone enhanced bacterial survival, and thus indicating that macrophages were less able to kill intracellular bacteria as a direct result of treatment. Though LPS alone did not affect intracellular killing compared to the vehicle-control, LPS treatment seems to protect macrophages from E804-related suppression of intracellular killing.

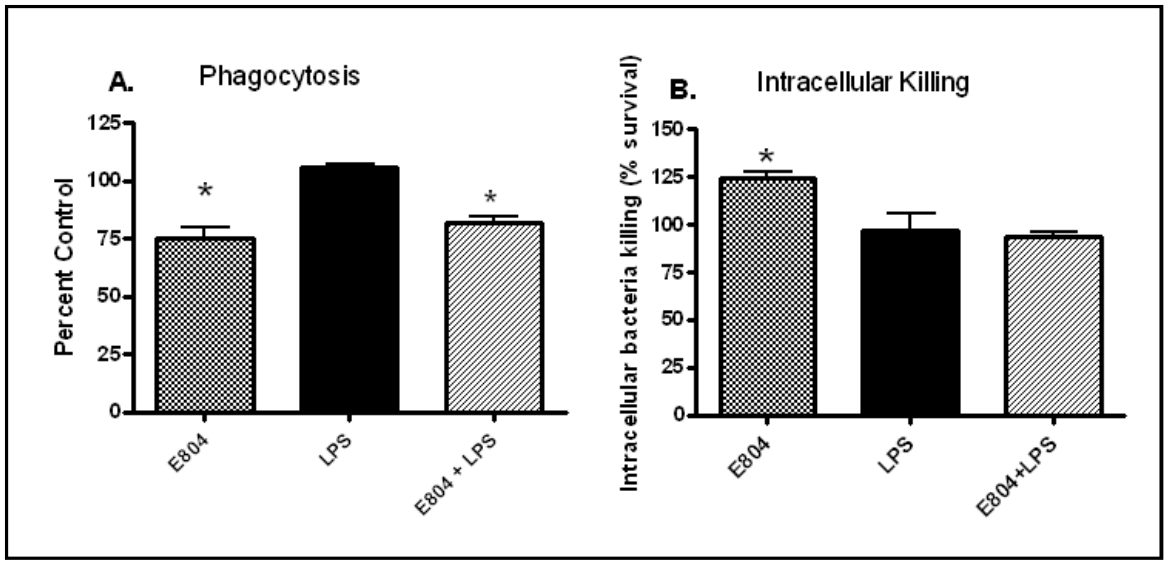


Figure 9

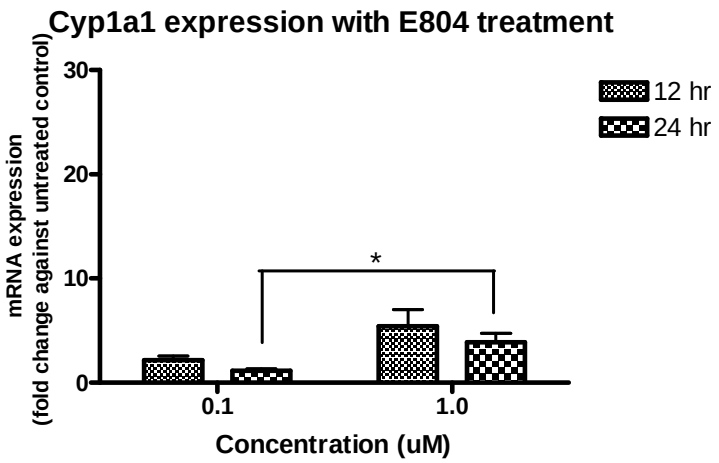
The effects of carrier control (0.01% DMSO), 1 µM E804, 1 µg/ml LPS, and LPS + E804 on phagocytosis of fluorescent beads and intracellular killing of E. coli by RAW 264.7 cells following 24 h treatments. Data are presented as percentage of control, and shown as the mean ± standard error of n = 3 independent experiments. * represents significance ($P < 0.05$).

THP-1 Macrophage Model

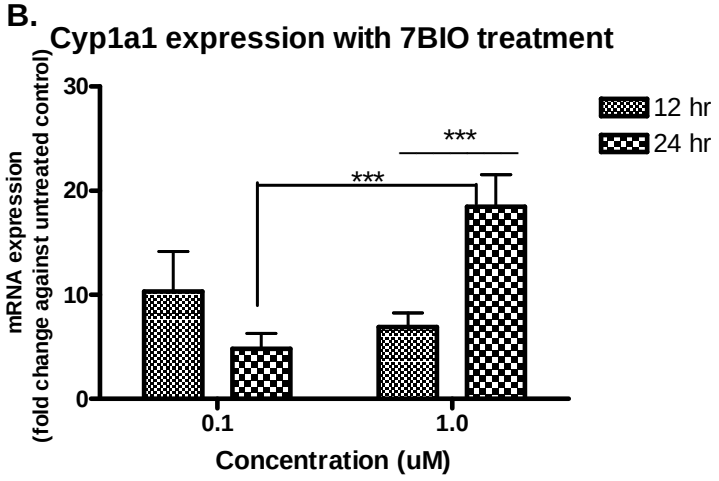
Time vs. concentration comparison for CYP1A1 and IL-6 expression

Considering that the two indirubins with very different abilities to induce CYP1A1 (from preliminary studies) may have very different effects on inflammatory profiles, and especially in combination with LPS as the initiator of inflammatory responses, additional preliminary studies were carried out to determine an optimal (12 hr vs 24 hr), and if there may be a better response to a lower level of indirubin. There were no differences in E804-induced CYP1A1 expression between 12 and 24 hr treatment at 0.1 µM, but at 1 µM concentration the expression was greater at 24 hr (Figure 10A). Compared to 7BIO, E804 is a relatively weak inducer of CYP1A1. CYP1A1 expression at 1 µM concentration following 24 hr treatment with 7BIO was much higher than the other three combinations (Figure 10B). No differences were seen in LPS-induced expression IL-6 with respect to 12 vs 24 hours (Figure 10C).

A.


Cyp1a1 expression with E804 treatment
mRNA expression
(fold change against untreated control)
30
20
10
0
*
12 hr
24 hr
0.1
1.0
Concentration (uM)

B.


Cyp1a1 expression with 7BIO treatment
mRNA expression
(fold change against untreated control)
30
20
10
0
***
***
12 hr
24 hr
0.1
1.0
Concentration (uM)

C.

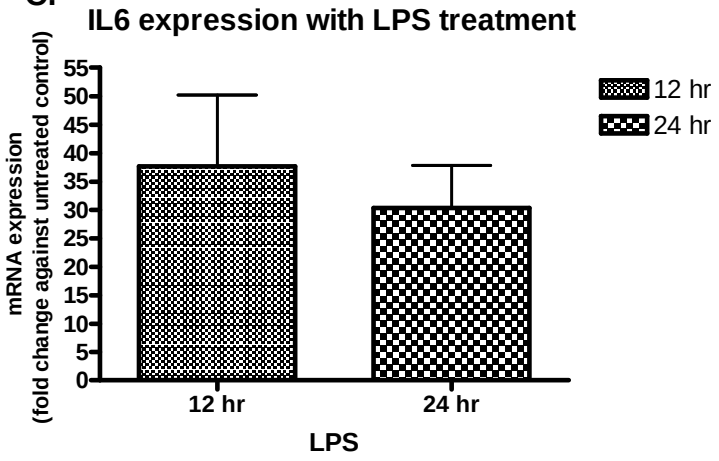

IL6 expression with LPS treatment
mRNA expression
(fold change against untreated control)
55
50
45
40
35
30
25
20
15
10
5
0
12 hr
24 hr
12 hr
24 hr
LPS

Figure 10

Real time quantitative PCR measuring the expression of CYP1A1 and IL-6 at 0.1 µM and 1 µM indirubins at 12 and 24 h. THP-1 macrophages were stimulated with 0.1 µM PMA for 48 hours and then treated with carrier control (0.01% DMSO), 0.1 µg/ml LPS , indirubins alone, or indirubins + LPS for 12 or 24 h prior to quantifying gene expression. Data represent the mean ± standard error of n = 3 independent experiments in which specific gene expression was compared to controls and normalized as fold-difference compared to expression of β-actin as a housekeeping gene. * represents significance (P ≤ 0.05), * (P ≤ 0.001).**

Effects of E804 and 7BIO on cell viability

The MTT assay was used to study the effects of E804 and 7BIO of cellular respiration in THP-1 human monocytes. This assay measures succinate dehydrogenase activity (Tim, 1983) as an indication of toxicity over a range of concentrations for 24 h. One μM and below of E804 and 7BIO did not affect viability (Figure 11). The effect on cellular respiration was then measured following treatment with carrier control (0.01% DMSO), 0.1 μg/ml LPS , 1 μM indirubins alone, or indirubins + LPS for 24 h (Figure 12).

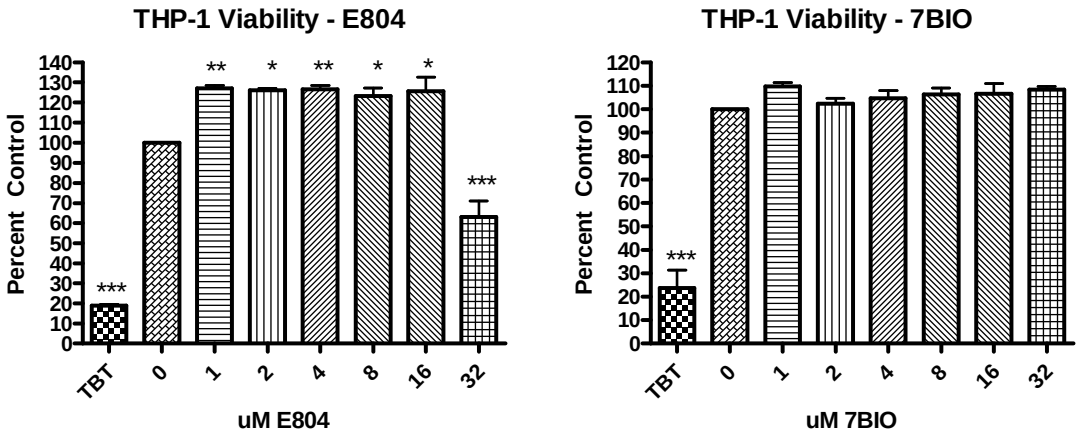


Figure 11

Effects of E804 and 7BIO on cellular respiration in THP-1 cells as determined by the MTT assay. THP-1 macrophages were stimulated with 0.1 µM PMA for 48 hours and then treated with concentrations of E804 and 7BIO from 32 µM to 1 µM for 24 hr. Tributyltin chloride (TBT) at a concentration of 10 µM was included as a positive control showing cytotoxicity. Data are presented as percentage of control, and shown as the mean ± standard error of n = 3 independent experiments. * represents significance (P ≤ 0.05), ** (P ≤ 0.01), * (P ≤ 0.001).**

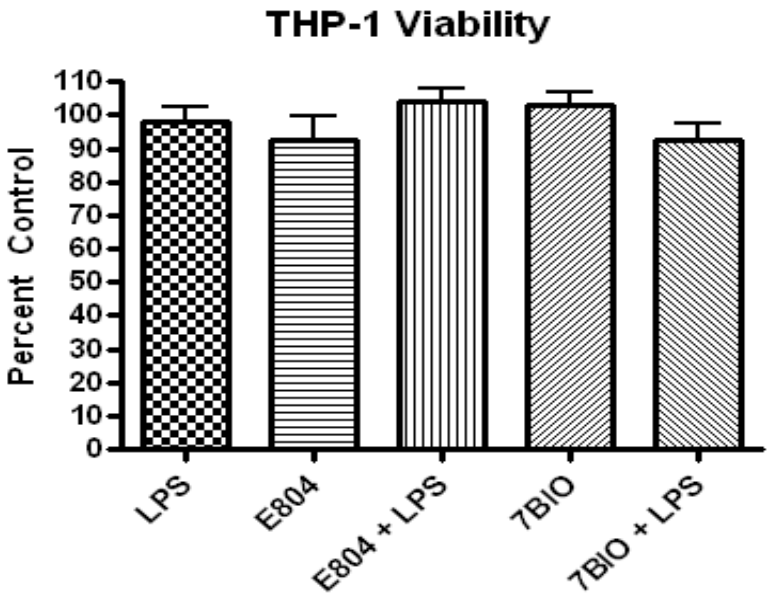


Figure 12

Effects of treatment on cellular respiration in THP-1 cells as determined by the MTT assay. THP-1 macrophages were stimulated with 0.1 µM PMA for 48 hours and then treated with carrier control (0.01% DMSO), 0.1 µg/ml LPS , 1 µM indirubins alone, or indirubins + LPS for 24 h. Data are presented as percentage of control, and shown as the mean ± standard error of n = 3 independent experiments.

qRT-PCR--IL-6, IL10, COX-2, iNOS, CYP1A1, TNFα, IL1β, IDOI&II, and ARG I&II

qRT-PCR was carried out to determine the effects of E804 and 7BIO on IL-6 expression as a key cytokine associated with inflammation and systemic acute phase responses. Also quantified, were expression profiles of COX-2, iNOS, IL-1β, TNF-α, and IL-10 for their involvement in the inflammatory response to LPS. CYP1A1 expression was also quantified as a means to determine if LPS treatments, alone and in combination with indirubins, affected this sentinel gene for AhR activity. Moreover, because macrophages can be functionally heterogeneous before stimulation and may be polarized to either a classical M1 or alternative M2 response, this study also quantified the expression of LPS-induced IDO-1, IDO-2, Arg-1, and Arg-2: gene products expressed by M2 macrophages.

LPS was a potent inducer for the expression of IL-6, IL1-β, and TNF-α genes (Figure 13), but not IL-10. With respect to LPS treatment, E804, but not 7BIO suppressed IL-6 expression. Unlike in RAW264.7 cells, LPS did not stimulate IL-10 secretion in THP-1 cells, yet both indirubins, alone and in combination with LPS stimulated secretion. Like IL-6, TNF-α is a hallmark proinflammatory cytokine secreted in response to LPS, and under these experimental conditions, E804 suppressed expression, while in combination with LPS 7BIO enhanced production. Interleukin-1β expression in response to LPS was not altered. In humans, macrophages do not easily express iNOS, even in response to LPS. However, both indirubins suppress baseline iNOS expression

levels (Figure 14A). LPS only modestly induced COX-2 under these experimental conditions, but the combination of 7BIO and LPS was a potent inducer, just as was observed for TNFα secretion (Figure 14B). IDO1 is generally expressed in response to LPS, and in this case E804 and not 7BIO suppressed expression (Figure 15A). IDO2 was not significantly altered by any of the treatments (Figure 15B). Arginase expression is generally considered a biomarker for M2 polarized macrophages, and thus would indicate that differentiated THP-1 cells are not pro-inflammatory. However, neither of the two arginases responded to LPS, suggesting that these cells are pre-programed towards an M1 lineage. E804, but not 7BIO suppresses baseline arginase-1 expression (Figure 15 C,D).

CYP1A1 is a hallmark gene associated with AhR activation. In this study using differentiated THP-1 cells, E804 only modestly induced CYP1A1 expression, while 7BIO and 7BIO + LPS increased CYP1A1 expression with respect to the control (Figure 16). Of particular note, 7BIO was a very potent inducer of CYP1A1 compared E804.

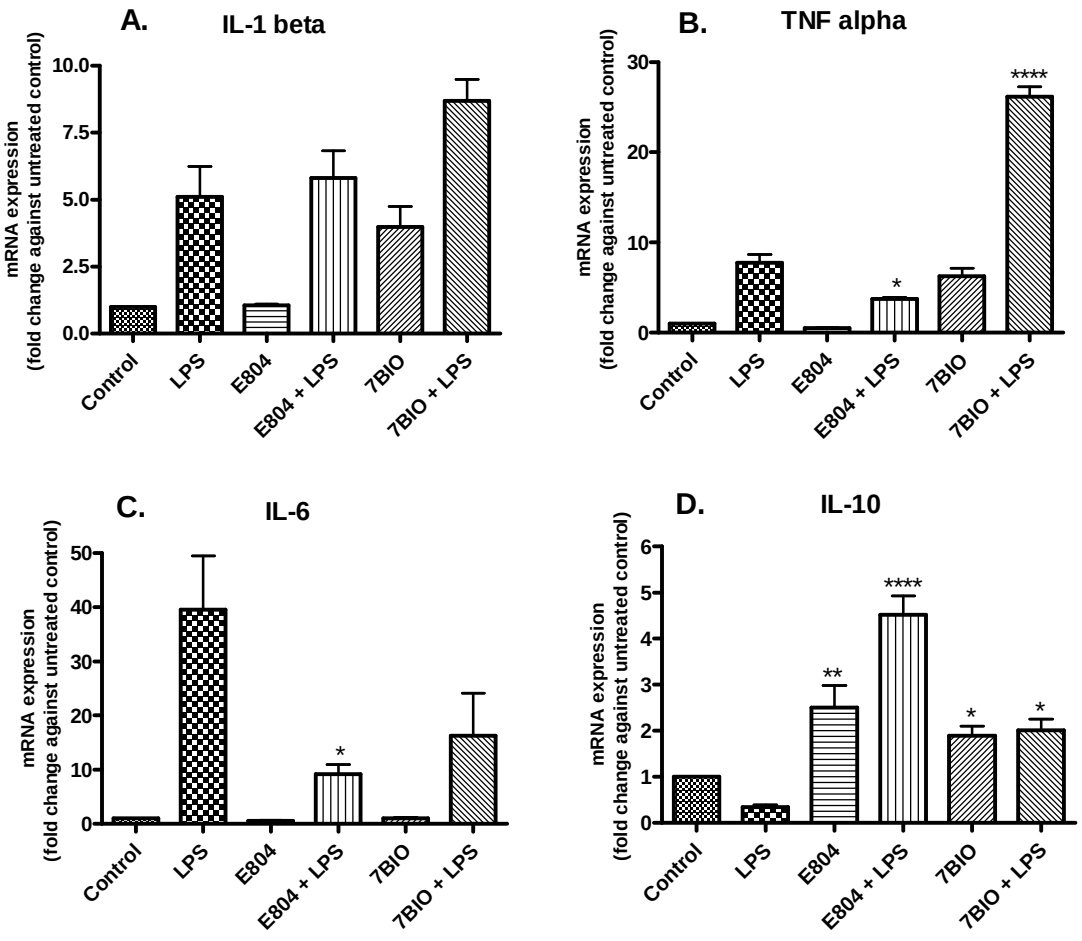


Figure 13

Real time quantitative PCR measuring the expression of IL-1β, TNF-α, IL-6 and IL-10 in THP-1 cells. THP-1 macrophages were stimulated with 0.1 µM PMA for 48 hours and then treated with carrier control (0.01% DMSO), 0.1 µg/ml LPS , 1 µM indirubins alone, or indirubins + LPS for 24 h prior to quantifying gene expression. Data represent the mean ± standard error of n = 3 independent experiments in which specific gene expression was compared to controls and normalized as fold-difference compared to expression of β-actin as a housekeeping gene. * represents significance (P ≤ 0.05), * (P ≤ 0.001).**

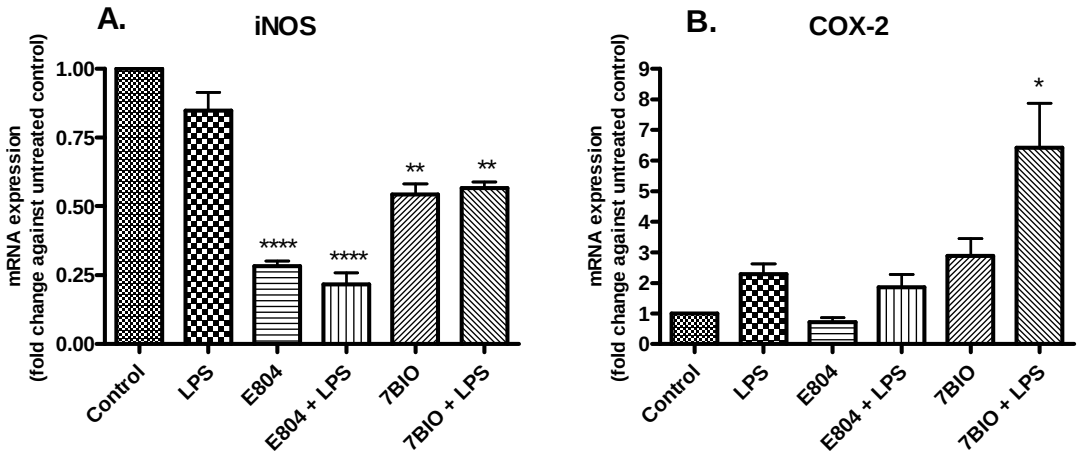


Figure 14

Real time quantitative PCR measuring the expression of iNOS and COX-2 in THP-1 cells. THP-1 macrophages were stimulated with 0.1 µM PMA for 48 hours and then treated with carrier control (0.01% DMSO), 0.1 µg/ml LPS , 1 µM indirubins alone, or indirubins + LPS for 24 h prior to quantifying gene expression. Data represent the mean ± standard error of n = 3 independent experiments in which specific gene expression was compared to controls and normalized as fold-difference compared to expression of β-actin as a housekeeping gene. * represents significance ($P \leq 0.05$), ** ($P \leq 0.01$), ** ($P \leq 0.0001$).**

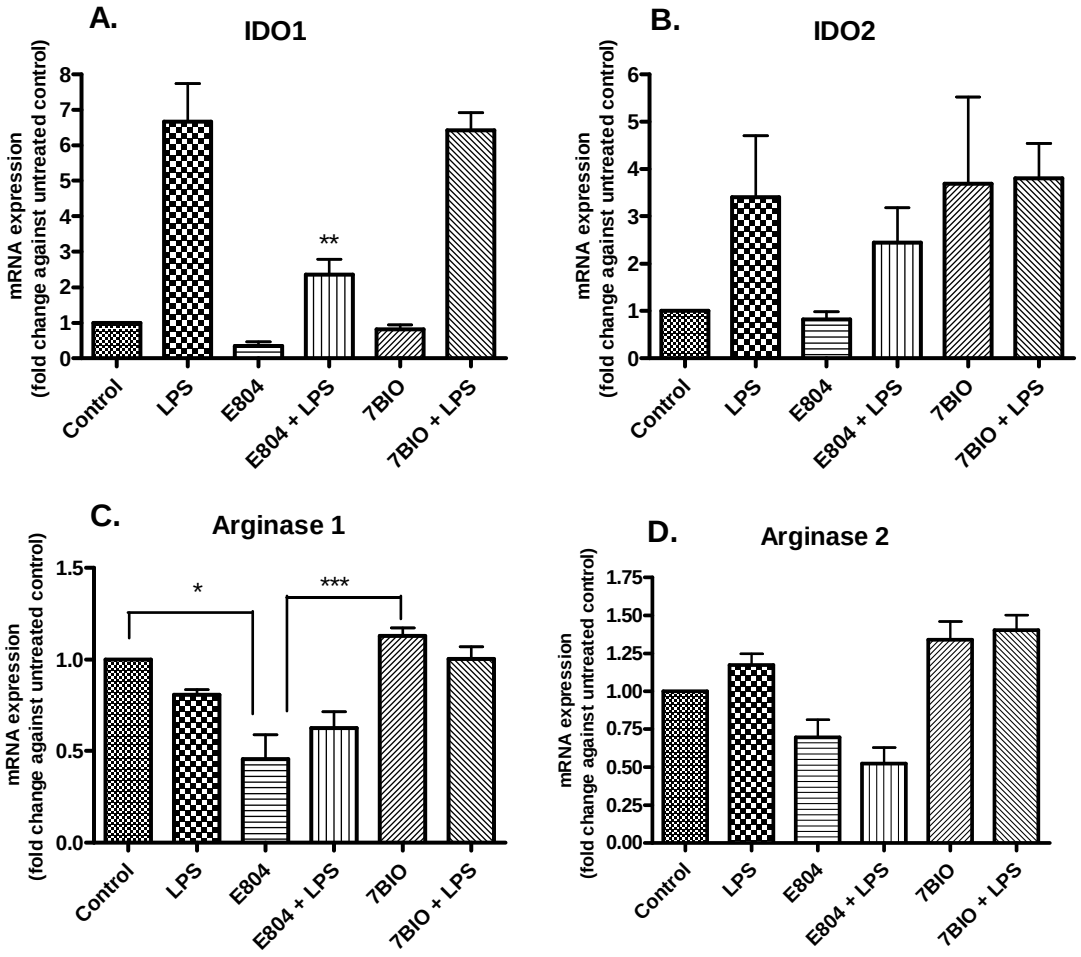


Figure 15

Real time quantitative PCR measuring the expression of IDO 1 and 2, and Arginase 1 and 2 in THP-1 cells. THP-1 macrophages were stimulated with 0.1 µM PMA for 48 hours and then treated with carrier control (0.01% DMSO), 1 µM 0.1 µg/ml LPS , indirubins alone, or indirubins + LPS for 24 h prior to quantifying gene expression. Data represent the mean ± standard error of n = 3 independent experiments in which specific gene expression was compared to controls and normalized as fold-difference compared to expression of β-actin as a housekeeping gene. * represents significance (P ≤ 0.05), ** (P ≤ 0.01), *, (P ≤ 0.001), ****(P ≤ 0.0001).**

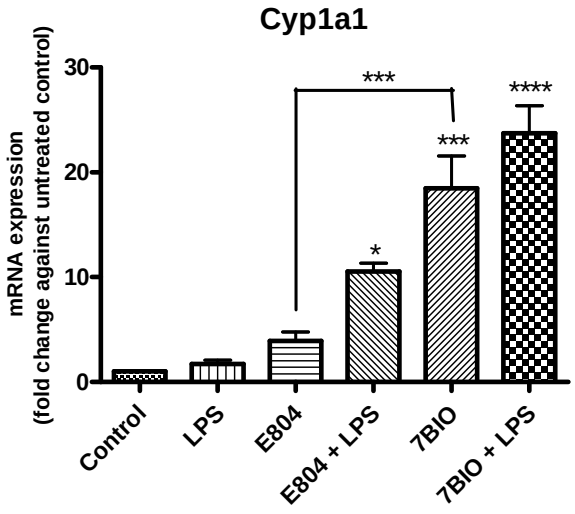


Figure 16

Real time quantitative PCR measuring the expression of CYP1A1 in THP-1 cells. THP-1 macrophages were stimulated with 0.1 μM PMA for 48 hours and then treated with carrier control (0.01% DMSO), 0.1 μg/ml LPS , 1 μM indirubins alone, or indirubins + LPS for 24 h prior to quantifying gene expression. Data represent the mean ± standard error of n = 3 independent experiments in which specific gene expression was compared to controls and normalized as fold-difference compared to expression of β-actin as a housekeeping gene. * represents significance (P ≤ 0.05), * (P ≤ 0.001).**

Cellular p21 and COX-2 protein expression

As expected, LPS induces COX-2 in THP-1 macrophages (Figure 17B). An increase in COX-2 gene expression was seen in 7BIO+LPS compared to both LPS and 7BIO alone. No difference in p21 expression was seen in any of the treatment groups.

LPS-induced Interleukin-6 and IL-10 secretion are suppressed by E804

While LPS is a potent inducer of both IL-6 and IL-10 secretion (Figure 18), neither E804 or 7BIO alone had an effect on secretion. When the compounds were treated together with LPS, IL-6 secretion significantly decreased compared to LPS-treatment, though this was not seen in IL-10. Gene and protein expression seem to be quite different in LPS-treated differentiated THP-1 macrophages, which may reflect the point in time when IL-10 protein is being regulatory on its own gene expression.

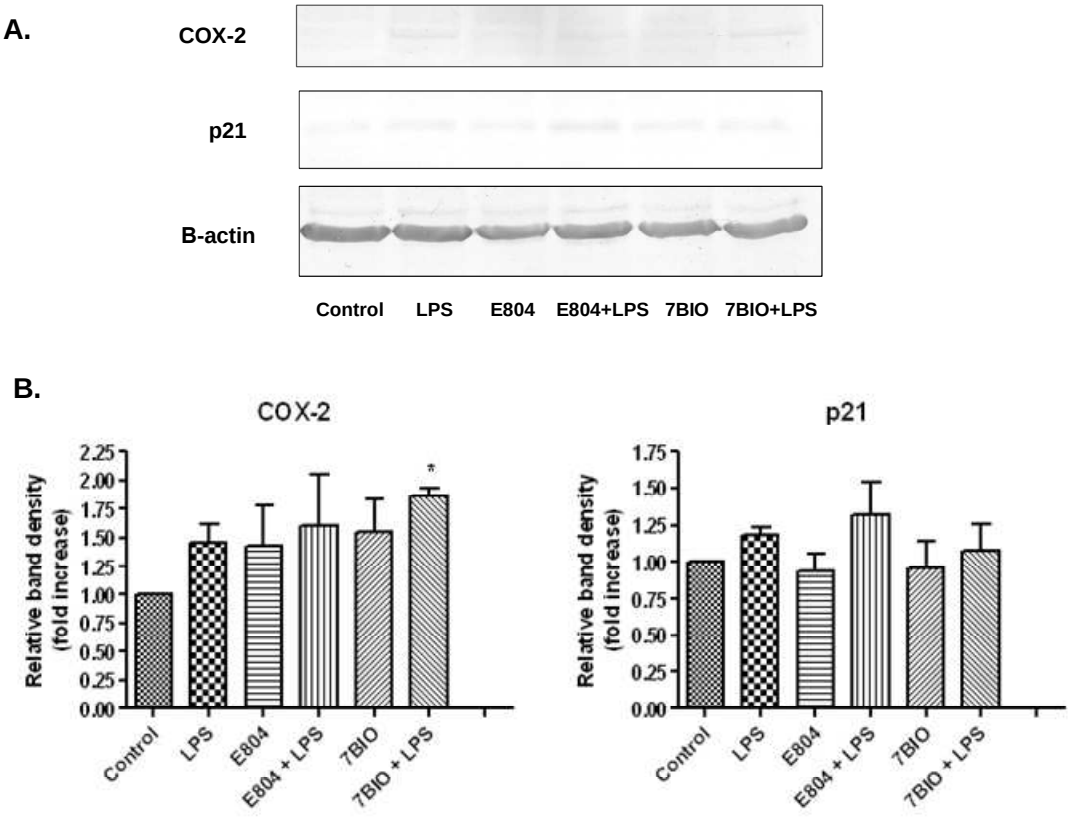


Figure 17

The effects of carrier control (0.01% DMSO), 0.1 µg/ml LPS, 1 µM indirubins alone, or indirubins + LPS on COX-2 and p21in THP-1 cells following 0.1 µM PMA stimulation for 48 hours and 24 h treatments. Cell lysates were subjected to SDS-PAGE and immunoblotting. A. Representative immunoblots showing COX-2and p21, with β-actin as a loading control at 24 h treatment. B. Data represent the mean band densities ± standard error of n = 3 independent experiments. * represents significance (P ≤ 0.05).

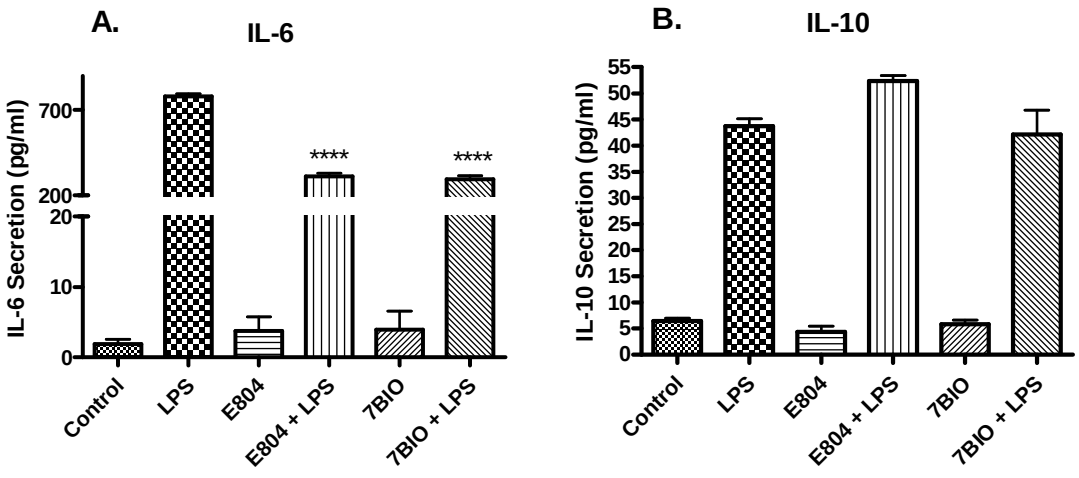


Figure 18

The effects of treatments on the secretion of IL-6 and IL-10 by THP-1 cells. Cells were stimulated with 0.1 μM PMA for 48 hours and then treated with either carrier control (0.01% DMSO), 0.1 μg/ml LPS, 1 μM indirubins alone, or indirubins + LPS for 24 h. Supernatants were removed and IL-6 and IL-10 quantified in supernatants by ELISA. Data represent the mean ± standard error of n = 3 independent experiments. ** represents significance (P ≤ 0.0001).**

Cellular lysozyme enzymatic activity

Lysozyme enzymatic activity was increased by LPS, but not by other treatments (Figure 19).

Phagocytosis is not sensitive to treatment

To determine if functional macrophage activity was affected by treatments, phagocytosis of fluorescent latex beads was measured. This particular function was not affected by either of the treatments (Figure 20).

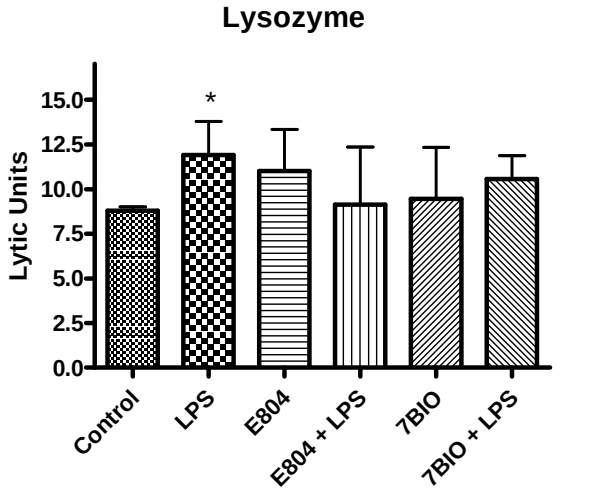


Figure 19

The effects of treatments on cellular lysozyme activity by THP-1 cells. Cells were stimulated with 0.1 µM PMA for 48 hours and then treated with either carrier control (0.01% DMSO), 0.1 µg/ml LPS, 1 µM indirubins alone, or indirubins + LPS for 24 h. Cell lysates were used as the source of lysozyme. Data represent the mean enzymatic activity (lytic units) ± standard error of n = 3 independent experiments. * represents significance (P ≤ 0.05).

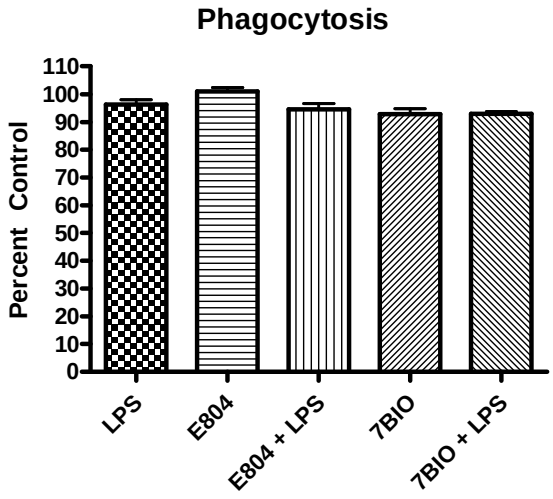


Figure 20

The effects of treatments on phagocytosis of fluorescent beads by THP-1 cells. Cells were stimulated with 0.1 µM PMA for 48 hours and then treated with either carrier control (0.01% DMSO), 0.1 µg/ml LPS, 1 µM indirubins alone, or indirubins + LPS for 24 h. Data are presented as percentage of control, and shown as the mean ± standard error of n = 3 independent experiments.

HL-60/Neutrophil Model

Phagocyte Oxidative Burst Activity

Under the conditions of experimental conditions described herein, retinoic acid-differentiated HL-60 cells exhibit the typical polymorphonuclear (PMN) phenotype associated with neutrophils. Upon stimulation with PMA and using the probe H_2DCFA, these cells reach maximum oxidative burst activity at 90 min, and this effect is somewhat enhanced by LPS and by exposure to cytokine containing supernatants (Figure 21). While neither of the treatments affected oxidative burst activity, this endpoint was different between E804- and E804 + LPS-treated cells. There were no differences between E804 and 7BIO in terms of affecting oxidative burst activity. Despite preliminary data showing that primed neutrophils have higher oxidative burst activity when activated with PMA, there were no treatment-related differences.

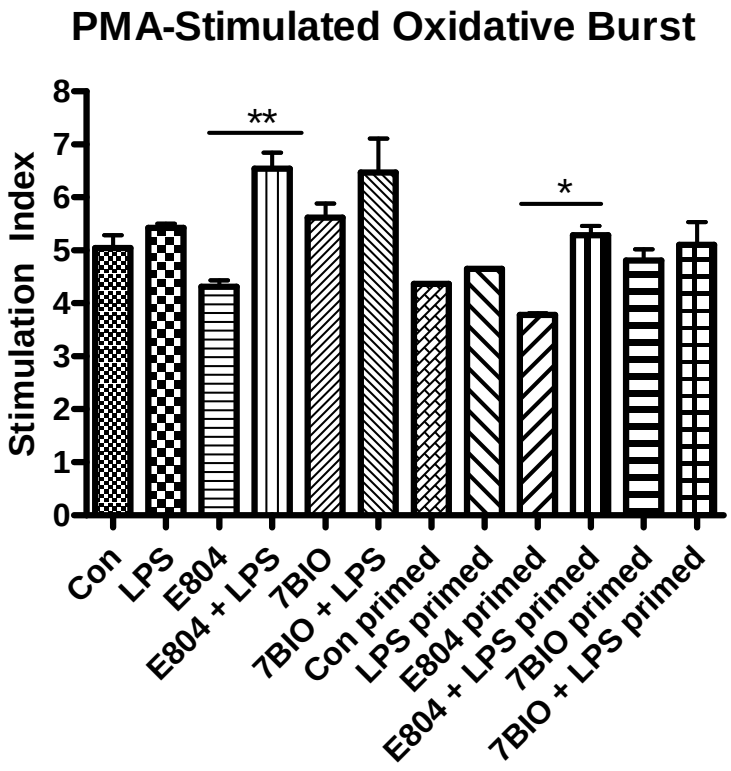


Figure 21

The effects of treatments on PMA-stimulated oxidative burst by HL-60 cells. Cells were differentiated with 1 µM retinoic acid for 6 days and then treated with either carrier control (0.01% DMSO), 0.1 µg/ml LPS, 1 µM indirubins alone, or indirubins + LPS for 24 h. H2DCFA was used to detect hydrogen peroxide intermediates. Data represent the mean ± standard error of n = 3 independent experiments in which relative fluorescent units (RFU) converted to stimulation indices as a ratio of PMA-stimulated RFU to un-stimulated RFU. * represents significance ($P \leq 0.05$), ** ($P \leq 0.01$).

DISCUSSION

RAW264.7 cells

To our knowledge, this is the first study to examine the effects of E804 on RAW264.7 macrophage responses to LPS, and we find that E804 is a potent modulator of inflammation-related physiological endpoints. While others have examined the anti-cancer and anti-inflammatory effects of E804 on various cell lines (Nam *et al.*, 2005; Nam *et al.*, 2012), those studies used 5 – 10 μM levels, or higher, we found that concentrations above 1 - 2 μM were cytotoxic. In our study, 1 μM was more suitable for determining immunopharmacological effects, and in line with previous work (Springs and Rice, 2006) using U937 human histiocyte/monocytes to determine the effects of indirubin-3'-monoxime at the same concentration on gene expression during activation and differentiation.

Treating macrophages with LPS is a common model for understanding basic physiological responses to gram negative bacteria, and especially those responses mediated through TLR4. In an effort to determine the likelihood of cell stress and toxicity resulting from E804 on the model, a targeted qRT-PCR array of select genes was employed, and highlights are as follows. Those genes associated with inflammatory responses to LPS were suppressed by E804. As previously demonstrated by others (Knockaert *et al.,* 2004; Polychronopoulos *et al.,* 2004), indirubins typically inhibit cell cycling control points and dependent kinases, and in this study E804 modulated the

expression of those genes. Oxidative stress genes SOD-1 and HO-1 were the two most affected by E804, and indicate perhaps an induction of anti-oxidative stress responses.

The observation that E804 suppresses LPS-induced iNOS and IL-6, and COX-2 gene expression in macrophages implies a common mechanism of action. TLR4 signaling pathways in macrophages leads to NF-κβ signaling as the primary transcription factor for driving the expression of iNOS, IL-6, and COX-2 in macrophages. In this study, iNOS, IL-6, and COX-2 expression at the gene level were sensitive to E804, but at the protein level this effect was less dramatic. However, macrophages treated with LPS also secrete IL-10, a Th2 cytokine, that suppresses inflammatory cytokines through STAT3 signaling (Chanteux *et al.*, 2007). Rather than through TLR4 and NF-κβ pathways that drive the expression of proinflammatory cytokines, IL-10 expression in response to LPS is mediated through TLR4/MAPKinases and Sp-1-dependent signaling (Chanteux *et al.*, 2007). Under physiological conditions, IL-10 expression by macrophages may be a means to self-limit aggressive inflammatory responses to stimulants like LPS, as well as to limit Th1 responses in general. In this study, E804 suppressed not only proinflammatory IL-6, iNOS, and COX-2, but IL-10 expression as well, at both the gene and protein level. A key observation was that the suppressive effect of E804 on IL-10 expression was greater than the effects on IL-6 and COX-2. These effects are not unique to E804, as indole-3-carbinol, the active indole ingredient in cruciferous vegetables, also inhibits both IL-6 and IL-10 in RAW264.7 macrophages (Tsai *et al.*, 2010), and SR31747A

(sigma ligand) inhibits both iNOS and IL-10 in the same cell line (Gannon *et al.*, 2001). A unifying mechanism of action for these compounds is not known.

In terms of mechanisms of action associated with E804, it is difficult to reconcile how E804 can inhibit both NF-κβ- and MAPKinase-associated signaling. However, most indirubins inhibit kinases, including cyclin-dependent kinases by blocking the ATP-binding pocket (Knockaert *et al.,* 2004; Polychronopoulos *et al.,* 2004), therefore one possible mechanism of action is that E804 inhibits both the IKKinase associated with NF-κβ activation and the kinases involved with MAPkinases, thus impacting both pathways, leading to suppression of both pro- and anti-inflammatory cytokine production in response to LPS.

Our finding that E804 suppresses both IL-6 gene expression and protein secretion is significant because this cytokine is a key player in systemic inflammatory processes. Interleukin-6 is produced by several cell types, including keratinocytes, adipocytes, smooth muscle cells, and macrophages, and is considered a potent inducer of the systemic acute phase response to infection, tissue damage, obesity, atherosclerosis, and variety of stimuli (Krueger *et al.*, 1990; Sugawara *et al.*, 2001; Jensen *et al.*, 2003; Cancello and Clement, 2006). IL-6 is also a growth factor for multiple myeloma, prostate, and basal cell carcinoma (Jensen *et al.*, 2003). It is not surprising, therefore, to appreciate the need and effort to find potent, yet safe means to modulate the production and activity of IL-6.

LPS-induced iNOS gene and protein expression, and NO production were suppressed by E804 treatment under the experimental conditions described herein. Inducible nitric oxide synthase (iNOS) is a potent inflammatory mediator and is strongly induced by LPS in macrophages (Moncada *et al.*, 1991; Marques *et al.*, 2008), but is also expressed in parenchymal cells of liver, colon, and muscle during systemic infection (HICKEY *et al.*, 2002). Moreover, while IL-6 production by macrophages may have systemic effects, direct NO production is a local response with local implications. Ultimately, nitric oxide (NO) produced by iNOS enzymatic activity contributes to oxidative stress, especially when combined with superoxide anion to yield peroxynitrite, a potent long lived free radical. The mechanism of iNOS inhibition by E804 is most likely related to its effects on NF-κβ signaling because this transcription factor is a key regulator of TLR-4 signaling, affecting not only iNOS activity, but IL-6, COX-2, TNF-α, IL-1β, and IL-12 as well. Modest induction of iNOS gene expression in cells treated with E804 alone may be related to a similar modest increase in NF-κβ gene expression noted in the array. Thus, iNOS activity is good biomarker for the effects of LPS on proinflammatory functions in macrophages and a reliable endpoint for screening potential anti-inflammatory compounds.

Oxidative stress is an important component of the biology of inflammation (Fernández-Sánchez *et al.*, 2011), and several key genes relative to oxidative stress are part of the commercial array used in this study. Oxidative stress under normal conditions is controlled by redox cycling of glutathione, which under normal conditions

is replenished with functioning glutathione reductases, peroxidases, and transferases. Hemoxygenase-1 (HO-1) gene expression stood out as a significant effect of E804 treatment, even in the absence of LPS. Since HO-1 is inducible by LPS, inflammatory cytokines, and hypoxia this may be an adaptive response in tissue injury, vasculature inflammation, and transplantation rejection models (Kapturczak *et al.*, 2004). At least under the conditions of this study, LPS alone did not induce HO-1 expression, and may be an intrinsic characteristic of the cell line. Under physiological conditions, induction of HO-1 leads to down regulation of Th1-affiliated (proinflammatory) responses and promotes Th2 responses (Sheikh *et al.*, 2011), and is protective against further oxidative stress through the formation of billiverdin, which is a reactive oxygen species scavenger (Naito *et al.*, 2004). Induction of HO-1 by E804, and especially the maintenance of HO-1 induction in the presence of LPS may be an important mechanism of feed-back control of aggressive inflammatory actions for this compound since carbon monoxide production through bilirubin production inhibits pro-inflammatory cytokine production. Though SOD-1 was induced by E804 or LPS alone, any benefit of induction appears to be lost or suppressed during co-treatments. However, it is possible that the reduction of HO-1 expression in cells treated with both E804 and LPS is a function of time.

Lysozyme is an antimicrobial peptide produced in abundance by myeloid cells, and secreted into body fluids and cell cultures as part of the innate antimicrobial response (Yehuda *et al.*, 2003). Lysozyme is easily quantified in serum/plasma and tears, and can be quantified in cell lysates. At least under the conditions explored in this

study, E804 decreased enzymatic activity, and presumably total lysozyme protein content, in the presence of LPS. The mechanism(s) associated with reduced lysozyme content of cells in these experiments are unknown, but the potent immunosuppressive glucocorticoid dexamethasone inhibits the secretion of lysozyme in monocytes (Yehuda *et al.*, 2003), and the mechanism is probably through inhibition of NF-κβ activation and signaling (Crinelli *et al.*, 2000). Yet the negative endocrine and metabolic side effects make potent steroids an unappealing option for routine treatment of inflammation. In this study, E804 suppressed NF-κβ only when treated together with LPS (Table 3), and the same is true for lysozyme activity. Thus, E804 may be a more appealing anti-inflammatory agent in comparison to steroid use.

Gene and protein expression, and enzymatic activity profiles in this study demonstrate potent effects of E804 on macrophage responses to LPS, but the ability to phagocytize particles and kill intracellular bacteria are indicative of cell functions. One of the more interesting observations from these phagocytosis and intracellular killing assays was the lack of effect by LPS treatment alone, which generally enhances expression of genes and proteins associated with inflammatory-like responses. A similar lack of response to LPS in macrophages has been reported for pinocytosis (Peppelenbosch *et al.*, 1999). To significantly enhance phagocytosis of beads and intracellular killing of *E. coli*, macrophages may require an additional signals such as IFN-γ or one provided by phorbol esters as a pharmacological stimulant (Vincenti *et al.*, 1992; Gordon *et al.*, 2005). But, regardless of the effects of LPS on phagocytosis, E804

suppresses the phagocytic ability of RAW264.7 cells, and this is likely due to modulation of GSK-3β pathways, which are involved in cytoskeletal changes during engulfment (Cabello *et al.*, 2010), and a key target of many indirubins (Meijer *et al.*, 2003; Knockaert *et al.*, 2004).

This study also demonstrated that E804-treated cells were compromised in their ability to kill intracellular *E. coli*, but not in cells also treated with LPS. Considering the induction of typical genes and proteins associated with LPS treatment, one would predict that cells treated with LPS would be more efficient at clearing intracellular bacteria. However, considering the above discussed similarity in effects on phagocytosis of beads, macrophages may require additional signals such as IFN-γ or pharmacological stimulation in addition to LPS for more efficient intracellular killing of bacteria. While we did not include IFN-γ or phorbol esters in this particular study, this may be a key point in future studies using E804 as a potential immunomodulating agent.

THP-1 Cells:

In this study, PMA-differentiated THP-1 cells towards a macrophage lineage was used to further test the effects of indirubin E804 on pro-inflammatory responses to LPS, but using a cell line model responsive to AhR activation. Indirubin 7BIO is very potent in inducing CYP1A1 gene expression, and from the preliminary screening of indirubins, even more so than PCB-126, a model environmental contaminant used to elicit toxicities

mediated primarily through AhR signaling (Rice and Schlenk, 1995; Chen, 2010; Zhang *et al.*, 2012). In comparison, E804 has very low AhR activity as evidenced by low expression of CYP1A1 (Hu *et al.*, 2007a), an observation that supports findings from the RAW264.7 study (this dissertation) that despite very weak AhR activity, or lack thereof, E804 holds promise as an anti-inflammatory indirubin, and may be safe compared to indirubins with high activity. It is well known that compounds with high AhR activity are associated with toxicities, including immune dysfunction, reproductive and developmental abnormalities, and tumorogenesis (Denison *et al.*, 2002b; Denison *et al.*, 2011; Jönsson *et al.*, 2012).

As with RAW264.7 cells, differentiated THP-1 cells response well to LPS in that key signature genes associated exposure to TLR-4 ligand (LPS), such as IL-1β, TNF-α, and IL-6 are induced. In both cell lines, E804 suppresses LPS-induced IL-6, yet IL-10 expression profiles are very different in the two lines; gene expression is greatly induced in RAW264.7 cells, but not THP-1, though the cytokine is readily secreted by both cells. Of note, E804 suppression of IL-10 is seen only in RAW264.7 cells in response to LPS. This difference is difficult to fully reconcile, but may be due to the level of differentiation of RAW264.cells, which are very similar to primary macrophages, while THP-1 cells must be treated with PMA to induce differentiation, and even then are referred to as “macrophage like”. Furthermore, macrophages secrete IL-10 as a means to self-regulate activation, and the time from gene expression to secretion of the protein may be different between these two cell lines at the time of use (no PMA

stimulation for the mouse macrophage, PMA-stimulation for the human cell line). PMA is a diacylglycerol mimic, and as such can stimulate PKCδ activity, and the long term effects of this treatment to induce differentiation may be revealed in altered cytokine secretion. With regards to systemic inflammation, IL-6 is a key initiation of the acute phase response, and at least in the in vitro models used herein, E804 and 7BIO suppress LPS-stimulated secretion, thus indicating anti-inflammatory properties.

While COX-2 expression in RAW264.7 cells is highly responsive to LPS, differentiated THP-1 cells are less responsive, in terms of fold-increase in expression. At least in differentiated THP-1 cells, COX-2 expression is increased by 7BIO + LPS, suggesting that whatever the mechanism is associated with a relatively small increase in expression (compared to RAW264.7 cells) is alleviated by 7BIO. Strong Ahr ligands, such as PCB-126 and especially TCDD, are known to induce COX-2, and probably through two different mechanisms. First, the COX-2 gene has an AhR response element (Vogel *et al.*, 2007), but equally important is the activation of c-src signaling in the cytosol associated with PLA2 activation and arachidonic acid metabolism to COX-2 (Vogel *et al.*, 2003), as well as non-canonical pathway to NF-κβ activation through RelB, and the canonical pathway of RelA.

One of the enigmas of human immunology is the near lack of iNOS activity in response to LPS so typically found in rodent models. iNOS is easily induced by TNF-α and IL-1β, but slow to react to LPS, and this may be due to feedback regulation in sensing NO by humans. Nitric oxide is quickly oxidized to nitrate in humans, therefore

nitric oxide reductase enzymes are required for simple assays like the Griess assay used in mouse cell lines (Marzinzig *et al.*, 1997). At least in differentiated THP-1 cells, LPS did not stimulate the expression of iNOS, but both indirubins further suppressed expression. This may indicated that iNOS expression in humans is not as tightly linked to RelA (p65) signaling and NF-κβ activation as it is in rodents (Karin *et al.*, 2004; Miller *et al.*, 2010).

Markers of possible polarization of differentiated THP-1 cells from M1 (classical inflammatory) to alternatively activated (low grade, chronically activated) M2 cells were examined. Of the four genes examined, IDO-1, IDO-2, Arginase-1, and Arginase-2, only IDO-1 was induced by LPS, and this was reduced by E804, but not 7BIO. If all four genes were altered by treatments, this would indicate a switch from producing iNOS, as an example, from L-arginine metabolism to L-citrullene for producing nitric oxide, towards L-arginine metabolism to urea and L-ornithine. Urea and ornithine are classical products of activated M2 macrophages (Mantovani *et al.*, 2013). Taken as a group of indicator genes, at least under the conditions of this study, differentiated THP-1 cells display an M1 phenotype, and serve as a good model for classical inflammatory processes.

To minimally address the question of genotypic vs phenotypic changes in differentiated THP-1 cells, cytosolic lysozyme activity and cellular phagocytosis were examined. Only LPS increased lysozyme activity, and presumably this is due to an increased expression of the protein, not increased activity of the enzyme. The lack of effect on lysozyme activity by either indirubin strongly suggests that pathways leading to increased expression (cellular content) are not targets. The same is true for

phagocytosis, in that the rate of uptake of beads was not affected by treatments. Phagocytosis involves a complicated interaction of membrane fusion and cytoskeleton contraction, yet neither of these seemed to be affected. It may be that E804 and/or 7BIO affect only "inducible" systems. In terms of inducibility, most strong AhR ligands not only induced CYP1A1 as a marker of exposure, but downstream gene products involved in cell cycle maintenance (Knockaert *et al.*, 2004), one of which is p21. At least in this study, even the strong AhR ligand 7BIO did not alter expression of p21, which this may be due to rapid metabolism of most indigoids (Guengerich *et al.*, 2004) and the time frame of this study may have missed any possible changes in expression.

Retinoic-acid differentiated HL-60 cells/neutrophils and oxidative burst activity

Oxidative burst activity in phagocytes is one of the most critical aspects of innate immunity in that any deficiency in the NADPH oxidase system will inhibit the ability of the cell to produce reactive oxygen species, namely superoxide anion, and is the underlying pathology associated with chronic granulomatous disease (CGD). In CGD, there is a loss or mutation on the X-chromosome coding for p91PHOX, and patients are overwhelmed with bacterial and fungal infections normally impeded by a functioning phagocyte (Cross *et al.*, 2000; Heyworth *et al.*, 2001). In the study described herein (this dissertation), HL-60 cells were differentiated to a neutrophil phenotype by retinoic acid over a 6 day period. Cells under these conditions respond well to PMA in terms of

initiation of NAPH oxidase activity, with stimulation indices typically 4-5 and beyond over un-stimulated cells. Only cells treated with both E804 and LPS were significantly altered compared to indirubin alone in how they responded to PMA, and the biological significance of this observation may be only in that E804 may synergize with LPS in terms of altering the membrane containing parts of the NADPH oxidase complex at the time of stimulation.

Not examined in this particular study was the possible effect of treatment on the ability of these cells to respond to increases in intracellular calcium. Normally, the oxidative burst response occurs during contact with a pathogen, such as during phagocytosis, and this contact initiates PLCδ activity to in turn release DAG that activates PKC to directly phosphorylate p67PHOX and p47PHOX. Elevated intracellular calcium results from IP3 release during PLCδ activity, and this in turn activates PLA2 to release arachidonate that leads to the translocation of p47PHOX to the membrane (Babior, 1999; Babior *et al.*, 2002). Pharmacologically, DAG can be mimicked with PMA, while IP3 can be mimicked with calcium ionophores, each given alone, or both in combination to give the strongest signal (Rice and Weeks, 1989; Rice and Weeks, 1991). Future studies examining oxidative burst activity and indirubins E804 and 7BIO should include a calcium ionophore, such as A23187, alone and in combination with phorbol ester.

Conclusion:

This study suggests that indirubin E804 is a novel and promising immunomodulating compound with potent negative effects on iNOS, IL-6, and COX-2 expression, while also suppressing IL-10 expression and enhancing HO-1 expression. Induced expression of HO-1 may promote antioxidant responses as a means to control inflammatory responses. Three key future studies with E804 related directly to immune responses are needed; 1. Determine the effects of E804 on phagocyte responses to opsonized and non-opsonized intracellular pathogens, and 2. Determine the *in vivo* effects of E804 on a battery of other immune responses, and 3. To determine the therapeutic potential of this anti-inflammatory agent for protection against endotoxin-induced animal lethality. In addition, if the findings that E804 is not an AhR ligand are consistent throughout additional studies using multiple models, then this particular immunomodulating indirubin derivative may be a safe clinical alternative to strong ligands that are often associated with severe toxicity.

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