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Indirubin-3'-(2,3 dihydroxypropyl)-oximether (E804) is a Potent Modulator of LPS-Stimulated Macrophage Functions

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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-3 β) 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 molecular structure. This study examined the effects of indirubin-3'-(2,3 dihydroxypropyl)-oximether (E804), a novel indirubin with potent STAT3 inhibitory properties, on basal and LPS-inducible activities in murine RAW264.7 macrophages. Using a focused commercial qRT-PCR array platform (SuperArray®), the effects of E804 on expression of a suite of genes associated with stress & toxicity were determined. Most genes up-regulated by LPS treatment were suppressed by E804; including LPS-induced expression of pro-inflammatory cytokines and receptors, apoptosis control genes, and oxidative stress response genes. Using qRT-PCR as a follow up to the commercial arrays, E804 treatment suppressed LPS-induced COX-2, iNOS, IL-6 and IL-10 gene expression, though the effects on iNOS and COX-2 protein expression were less dramatic. E804 also inhibited LPS-induced secretion of IL-6 and IL-10. Functional endpoints, including iNOS and lysozyme enzymatic activity, phagocytosis of fluorescent latex beads, and intracellular killing of bacteria, were also examined, and in each experimental condition E804 suppressed activities. Collectively, these results indicate that E804 is a potent modulator of pro-inflammatory profiles in LPS-treated macrophages.

Introduction

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 and 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-3 β

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Conflict of Interest:

The authors declare no conflict of interests.

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(GSK-3 β), 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.

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.*, 2002; Hu *et al.*, 2007; 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).

As with CDK and GSK-3 β inhibitory activities, indirubin-related AhR activities cover a wide range of potency, with EC₅₀ values between 5 nM – to over 10 μ M for CYP1A1 enzymatic activity in hepatoma cells, and 6 nM – to over 100 μ M in a yeast reporter assay system (Knockaert *et al.*, 2004; Polychronopoulos *et al.*, 2004). In comparison, the EC₅₀ 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.

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 repair 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 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). 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 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 μ M 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. Moreover, E804 lacks significant AhR-associated CYP1A1 activity in RAW264.7 cells, possibly indicating weak AhR-associated properties of this indirubin derivative, or that this particular cell line lacks necessary components of AhR signaling.

Materials and Methods

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 μ g/ml streptomycin, 110 μ g/ml sodium pyruvate, 1% non-essential amino acids (100 X stock), 4.5 g/L glucose, and 1.5 g/L of NaHCO₃, each from Sigma (Sigma Aldrich). Cells were typically grown and maintained at 37°C with 5% CO₂ in Corning 75 cm² 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 μ M E804 alone, 1 μ g/ml LPS from *E. coli*, Serotype 0127:B8 (Sigma Aldrich), or E804 and LPS in combination.

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 10⁵ cells per well in 100 μ l 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 μ M per well in a total volume of 200 μ l, and the plates incubated for 24 h. Four hours before termination of the assay, each well received 20 μ l 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 μ l 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 arc sine 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 μ M 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 μ M 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 1.5×10^6 cell/well in 6 well plates over 4 h for adherence, and treated with 0.01% DMSO carrier, 1 $\mu\text{g/ml}$ 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 FACScanTM (BD Biosciences), with 10,000 cells being scored in each run.

Commercial qRT-PCR arrays for stress and toxicity

Macrophages were seeded at 1.5×10^6 cells/ml in 75 cm² 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 $\mu\text{g/ml}$ 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 $12,000 \times g$ 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 $12,000 \times g$ 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 RT² Easy First Strand Kit (SABioscience Corporation) as described by the manufacturer. A predesigned Stress & Toxicity ProfilerTM array of 96 genes was purchased from SuperArray Bioscience Corporation for use with RT² 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 $\Delta\Delta\text{Ct}$ 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) and. The quantity of these mRNAs was expressed as fold-changes in gene expression compared to β -actin expression. For data analysis, the $\Delta\Delta\text{Ct}$ method was used. Expression data were compared between treatment groups using ANOVA, followed by a Bonferroni's post- test using GraphPad5 statistical package.

Immunoblotting for iNOS and COX-2 protein expression

Triplicate experiments were performed in which 1.5×10^6 macrophages were seeded in T-25 flasks in 3 ml of media containing 0.01% DMSO carrier control, 1 μ M E804, 1 μ g/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 μ l 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 $1000 \times g$ for 10 min. The overlying supernatant was removed and centrifuged again for 20 min at $14,000 \times g$; the overlying supernatant was again removed and its protein content quantified. Fifty μ g of lysate protein from each sample were separated by SDS-PAGE on 10% gels and transferred overnight onto 0.45 μ M nitrocellulose membranes at 4° 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 μ g 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 secretion, 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 μ M E804, 1 μ g/ml LPS, or both in a final volume of 200 μ l. 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 μ l 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 μ l 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 incubate for 24 h. Cells were lysed and the protein concentration normalized to 1 mg/ml. One hundred μ l of cell lysate, in triplicate, were added onto 96 well plates followed by the addition of 200 μ l 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 μ M 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_2HPO_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 μ l 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 μ l 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 arc sine 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 μ l 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 removed by extensive washing with PBS, and further treated with 200 μ l of 1000 U Penicillin and 10 μ g/mL streptomycin solution (Sigma Aldrich) for 15 min. Cells were then permeablized with 100 μ l 0.1% triton-x100 in PBS for 15 minutes to release the engulfed bacteria. The triton-x100 solution was removed by aspiration and the viable bacteria in each well were grown in 200 μ l 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 arc sine transformed prior to statistical analysis using ANOVA, followed by a Bonferroni's post- test using GraphPad5 statistical package.

Results

E804 at 1 μ M 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 μ M and below did not affect respiration (data not shown) and was chosen for further studies. To confirm that 1 μ M was not only non-cytotoxic, but non-pro-apoptotic and non-pro-necrotic as well, annexin-V binding was quantified following treatment with 1 μ M of E804, 1 μ g/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 (see supplemental data).

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 1). In the case of Chemokine ligand 4, MMIF, and NF- κ B, 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-1 α , IL-1 β , 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- κ B and TNF 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.

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 3). E804 inhibited LPS-induced IL-6, COX-2, and iNOS expression (Figure 2), 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-pentachlorobiphenyl (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 3a). E804 did not induce visible protein, and modestly reduced LPS-induced expression (Figure 3b). 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.

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 4), 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 5). 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.

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 6). 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.

Discussion

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 μ M levels, or higher, we found that concentrations above 1 – 2 μ M were cytotoxic. In our study, 1 μ M 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- κ B 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 was 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- κ B pathways that drive the expression of proinflammatory cytokines, IL-10 expression in response to LPS is mediated through TLR4/MAPKs 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- κ B- 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 IKKs associated with NF- κ B 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; Cancelli 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- κ B 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-1 β , 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- κ B 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 protective against further oxidative stress through the formation of biliverdin, 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- κ B 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- κ B only when treated together with LPS (array data), 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-3 β 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.

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 indirubin derivative may be a safe alternative to strong ligands that are often associated with severe toxicity.

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Highlights

264.7 macrophages were treated with 1 μ M Indirubin E804, 1 μ g/ml LPS, or both.

E804 suppresses LPS-induced expression of iNOS, IL-6, COX-2, and IL-10 in macrophages.

E804 suppresses LPS-induced iNOS and lysozyme activity, as well as IL-6 and IL-10 secretion.

E804 suppressed phagocytosis and intracellular killing by macrophages.

Figure 1. Molecular structures of (A) natural indirubin and (B) indirubin-3'-(2,3 dihydroxypropyl)-oximether (E804) used or referenced within the study.

Figure 2.

Real time quantitative PCR measuring the expression of COX-2, iNOS, IL-6, and IL-10. RAW246.7 macrophages were treated 24 h with carrier control (0.01% DMSO), 1 μ M E804, 1 μ g/ml LPS, or LPS + E804 prior to quantifying gene expression. Data represent the mean $\pm$ 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 3.

The effects of carrier control (0.01% DMSO), 1 μ M E804, 1 μ g/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 ($P < 0.05$).

Figure 4.

The effects of carrier control (0.01% DMSO), 1 μM E804, 1 $\mu\text{g/ml}$ 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 ($P < 0.05$).

Figure 5.

The effects of carrier control (0.01% DMSO), 1 μ M E804, 1 μ g/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 $\pm$ standard error of $n = 3$ independent experiments. * represents significance ($P < 0.05$).

Figure 6.

The effects of carrier control (0.01% DMSO), 1 μ M E804, 1 μ g/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 ($P < 0.05$).

Table 1

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 μ M ES04, 1 μ g/ml LPS, ES04 +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	ES04	LPS	ES04 + 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-1 α	NM_010554	Il1a	+2.68	+169.9	-14.75
IL-1 β	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- κ B(p105)	NM_008689	nfb1	+6500	+16920	-1.46
iNOS	NM_010927	Nos2	+2.36	+10.19	-1.35
APOPTOSIS					
Caspase 1	NM_009807	Casp1	+446703	+6562	-1.39
Caspase 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
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 G2	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

Gene Name	Genbank no.	Symbol	E804	LPS	E804 + LPS
PCNA	NM_011045	pca	-5	+15.98	-1.27
CDK1-1 α (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	MM_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	IMM_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 dis2	NM_013671	Sod2	-457.67	+2.05	-1.69

Table 2

qRT-PCR primer sequences targeting IL-6, COX-2, iNOS, IL-1D and CYP1A1

Gene Name	Accession #	Primer Sequence (5' → 3')	Tm°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	NM_000071.5	F: TTG TAC AGT CCC AGT CAG GCA ACA R: TCA AGC TAC TGC AGG CCA GTT ACA	53	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	144
iNOS	NM_010927	F: CAA ACA CGA GTG CAG CTG GTT GAA R: AGG CAG GAC TGA GTT CAG TGT GTT R: GGC AAT GTT CTC ACA CAG GCG TTT	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