Compounds that Inhibit Cytokine-Induced Interleukin-6 Expression in a Monocytic Cell Line

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Abstract

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Compounds that Inhibit Cytokine-Induced Interleukin-6 Expression in a Monocytic Cell Line

Director of Thesis: Douglas J. Goetz

Inhibiting Interleukin (IL)-6, a cytokine associated with inflammation, is an established therapeutic target for inflammatory diseases. In this study, we probe the hypothesis that two novel small organic compounds inhibit Tumor Necrosis Factor-alpha (TNF-α) and Interferon-gamma (IFN-γ)-induced IL-6 expression in an undifferentiated human monocytic cell line (THP-1) and freshly isolated monocytes from human peripheral blood. TC_{50}, IC_{50}, and TI values are determined. TC_{50} is the compound concentration at which the metabolic signal is decreased by 50% relative to carrier (DMSO) control treated cells. IC_{50} value is the compound concentration at which IL-6 production is inhibited by 50% relative to carrier control treated cells. Therapeutic index (TI) is the TC_{50} value divided by the IC_{50} value.

The combination of TNF-α and IFN-γ induced IL-6 expression in undifferentiated THP-1 cells. The compounds inhibited the cytokine-induced IL-6 expression at both the gene and the protein levels. The IC_{50} values for the first compound were 9.4 μM at the gene expression level and 5.8 μM at the protein expression level for 24-hour TNF-α and IFN-γ-induced IL-6 in undifferentiated THP-1 cells. The IC_{50} values for the second compound were 13.6 μM at the gene expression level and 10.5 μM at the protein expression level. The TC_{50} values were 79.8 μM and 312.2 μM respectively for undifferentiated THP-1 cells treated for 24 hours with the first compound and the second

compound respectively. The TI values for the first compound were 8.5 at the gene expression level and 13.8 at the protein expression level for 24-hour TNF-α and IFN-γ-induced IL-6 in undifferentiated THP-1 cells. The TI values for the second compound were 23.0 at the gene expression level and 29.7 at the protein expression level.

The combination of TNF-α and IFN-γ does not appear to induce IL-6 protein expression in freshly isolated monocytes from human peripheral blood. Flow cytometric analysis suggested that freshly isolated monocytes express little, if any, TNF receptor 1, TNF receptor 2, and perhaps a small amount of IFN-γ receptor. However, due to the high signal of IgG2a isotype control binding to the monocytes, it is difficult to draw a firm conclusion regarding the expression of these receptors.

In conclusion, this study provides evidence that two novel small organic compounds inhibit TNF-α and IFN-γ-induced IL-6 expression at both the gene and protein levels in undifferentiated THP-1 cells.

Dedication

I dedicate this thesis to my parents for supporting me with love.

Acknowledgments

I would like to express the deepest appreciation to my academic advisor, Dr. Douglas Goetz, for his professional guidance and persistent help in pursuit of my MS studies. I very much appreciate his enthusiasm and immense knowledge in regard to research and teaching. I would also like to thank Dr. Stephen Bergmeier and his lab for providing the compounds used in this research. My sincere thanks extend to the rest of my committee members Dr. Kelly McCall and Dr. Monica Burdick for offering mentorship and assistance in my thesis research. In addition, a thank you to all blood donors, Jean Thuma and Dr. Ramiro Malgor for helping with blood draw conducted in this research. I would also like to thank my lab mates Anuja Alapati and Chunyan Qi for their guidance and alliance in my MS study and research over the past two years.

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Chapter 1: Introduction

1.1 An overview of inflammation and cytokines

Inflammation happens when the body's immune system responds to pathogens, irritants, or damaged cells. It is a signal-mediated attempt to eliminate the harmful stimuli and activate the healing process (Coussens & Werb, 2002). Inflammation can be classified as either acute or chronic (Feghali & Wright, 1997). An acute inflammation responds to the stimuli within hours and days, which is the initial response from the immune system, accompanied with pain, redness, and swelling (James, Gibson, & Cleland, 2000). Typical acute inflammation exists in common diseases, such as acute bronchitis, acute dermatitis, acute tonsillitis, and even intense exercise (Smith & Miles, 2000). Chronic inflammation, however, is sustained over a longer period, involves a persistent process of destruction and healing, and sometimes demonstrates few outward symptoms (Abdo & Sanai, 2008). Chronic inflammation includes severe diseases, such as asthma (Gerard & Rollins, 2001), rheumatoid arthritis (Firestein, 2003), and can lead to cancer (Shacter & Weitzman, 2002).

Inflammation has been widely studied. When a pathogen passes through the epithelial tissue of the human body, macrophages exhibit an innate immune response against the pathogen by recognizing it via surface receptors (Janeway, Travers, Walport, & Shlomchik, 2001). These receptors then bind with the pathogen and stimulate the macrophage to engulf the pathogen (Janeway, Travers, Walport, & Shlomchik, 2001). At the same time, the secretion of cytokines by macrophages is triggered (Janeway, Travers, Walport, & Shlomchik, 2001). Cytokines are proteins that affect cell behavior, such as cell growth, differentiation, migration, and apoptosis (Woo, Chen, & Ma, 2003).

Cytokines involved in the innate immune response include: Tumor Necrosis Factor (TNF)-α, Interleukin (IL)-1, IL-10, IL-12, Interferon (IFN)-α, IFN-β, and IFN-γ. TNF-α and IL-1 are secreted by macrophages in response to pathogens. As important mediators of acute inflammation, TNF-α and IL-1 stimulate endothelial cells to produce adhesion molecules to recruit neutrophils and more macrophages to the site (Kroon, Tol, van Amstel, Elias, & Fernandez-Borja, 2013). TNF-α can also act on the hypothalamus to produce fever, enhancing the production of acute phase proteins (Rummel, Gerstberger, Roth, & Hübschle, 2011). IL-10 is produced by activated macrophages (Antoniv & Ivashkiv, 2011) and Th2 cells (Neta et al., 2011). It inhibits the expression of IFN-γ expressed by Th1 cells (Liu, Tonkonogy, & Sartor, 2011). IL-12 stimulates the production of IFN-γ (Jaime-Ramirez et al., 2011). It induces the differentiation of T helper cells into Th1 cells (Zhang, Zhang, Gu, & Sun, 2014). IFN-α and IFN-β are Type I interferons that increase expression of class I MHC molecules (Garofalo et al., 1996). IFN-γ is primarily produced by Th1 cells (Schroder, Hertzog, Ravasi, & Hume, 2004). Prolonged hyper IFN-γ production can cause anemia and bone marrow failure (de Bruin, Voermans, & Nolte, 2014).

When innate immunity fails to eliminate the pathogens, it triggers the adaptive immune system that provides further protection, in which lymphocytes are the major involved cells (Janeway et al., 2001). The adaptive immune response is initiated when the foreign antigens expressed by the pathogen are recognized by dendritic cells. Cytokines involved in the adaptive immune system include: IL-2 and IL-5. IL-2, produced by T helper cells, is the major growth factor for T cells (Sim & Radvanyi, 2014). IL-5,

produced by T helper type 2 cells, functions in the growth and differentiation of B cells and eosinophils (Takatsu, 2011).

Cytokines associated with inflammation can be classified into pro-inflammatory and anti-inflammatory cytokines. Pro-inflammatory cytokines include IL-1 (Dinarello, 2000), IL-6, TNF-α (Oh, Kim, Lee, & Woo, 2013), and IFN-γ (Miyazoe et al., 2002). Anti-inflammatory cytokines include IL-4 and IL-10 (Zhou et al., 2013; Zingarelli, Yang, Hake, Denenberg, & Wong, 2001). Among patients with sepsis and patients with septic shock, patients with sepsis exhibited high pro-inflammatory cytokine levels and patients with septic shock exhibited even higher pro-inflammatory cytokine levels (Yilmaz et al., 2014). These pro-inflammatory cytokines include IL-1β, IL-6, and TNF-α. These pro-inflammatory cytokine levels in healthy people are significantly lower compared to patients with sepsis (Yilmaz et al., 2014). That said, high levels of these cytokines were found in healthy elderly individuals, indicating an inflammatory state among healthy elder people (Franceschi et al., 2000). TNF-α and IL-1β have also shown promotional effects in the induction of anorexia in mice (Wu & Zhang, 2014). Patients with depressive disorder expressed higher protein levels of pro-inflammatory cytokines compared to healthy people. These pro-inflammatory cytokines include IL-1, IL-6, and TNF-α [(Azar & Mercer, 2013); reviewed in (Young, Bruno, & Pomara, 2014)].

This thesis will particularly focus on the pro-inflammatory cytokine IL-6.

1.2 IL-6 and inflammation

IL-6 is a protein encoded by the IL-6 gene. The human IL-6 protein has a molecular weight of 24 kDa, and is formed as a single polypeptide chain that has a helical structure (Gadient & Otten, 1997). It can be secreted by different cells, such as T cells,

monocytes, endothelial cells, and some tumor cells (Hirano, 1998). It was discovered in 1986 as a B cell stimulatory factor 2 that regulates immunoglobulin G (Hirano, 1998).

As a pro-inflammatory cytokine, IL-6 plays an important role in inflammation. For instance, IL-6 causes inflammation, while suppression of IL-6 can effectively inhibit inflammatory diseases, such as arthritis and colitis (Gabay, 2006). In an *in vivo* study of ulcerative colitic rats, IL-6 was shown to be a key indicator of inflammation (Wang, Zhou, Zhai, Wang, & Chen, 2013). Inhibition of IL-6 and its signaling pathway was found to be an effective way of preventing chronic inflammatory diseases [reviewed in (Gabay, 2006)]. In a study of Systemic Sclerosis (SSc), the successful blockade of IL-6 signaling has been suggested as a possible treatment (Barnes, Anderson, & Moots, 2011). Therefore, inhibition of IL-6 expression has become an established therapeutic target.

1.3 IL-6 signaling

Today, IL-6 and its signaling pathway is studied along with the IL-6 signaling transduction component, Glycoprotein 130 (gp130) and the IL-6 receptor (IL-6R) (Kishimoto, 2010). There are two types of IL-6 signaling pathways: the classical signaling pathway and the trans signaling pathway (Figure 1) (Kishimoto, 2006). In the classical signaling pathway, IL-6 binds to the IL-6 receptor called membrane-bound IL-6 receptor (mIL-6R) that is present on the outside of the cell membrane (Kishimoto, 2006; Rose-John, Waetzig, Scheller, Grötzinger, & Seegert, 2007). In the trans signaling pathways, IL-6 binds to soluble IL-6R (sIL-6R) (Kishimoto, 2006; Rose-John, 2012). When IL-6 binds to its receptor, they form a complex that later combines with gp130, which is then dimerized and activated (Kishimoto, 2006). This three component complex then initiates a down-stream signaling pathway (Kishimoto, 2006).

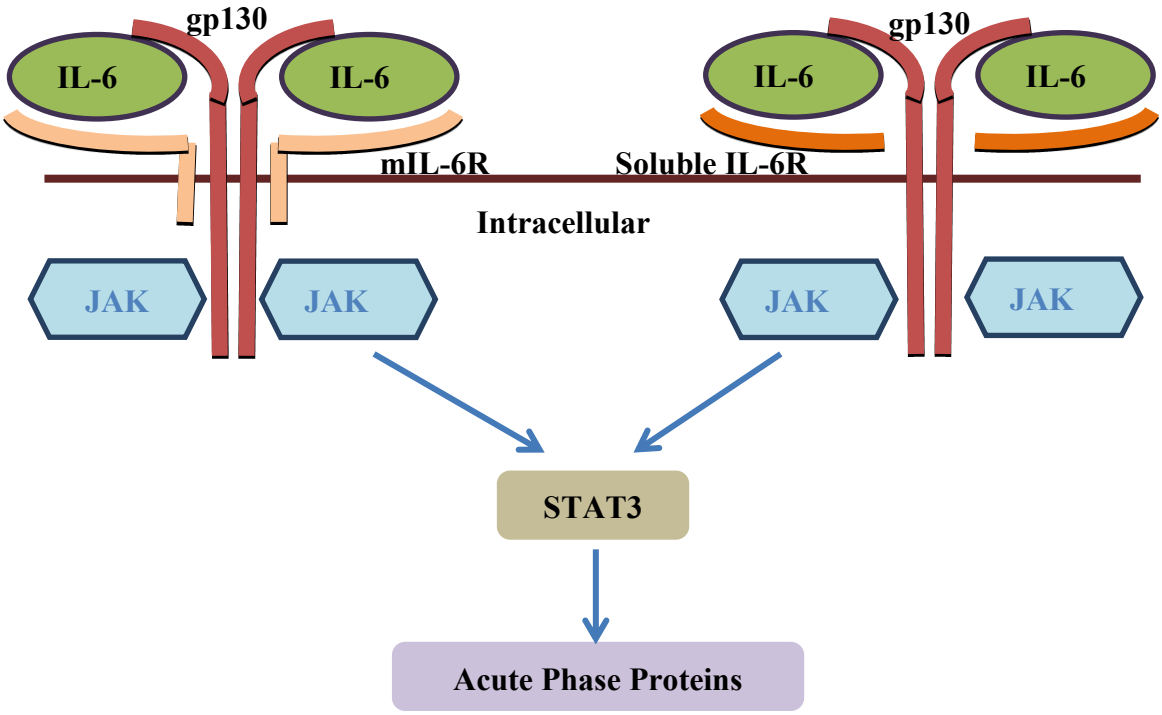

Figure 1. IL-6 signaling pathways: classical signaling pathway and trans signaling pathway. Adapted from: Kishimoto, T. (2006). Interleukin-6: discovery of a pleiotropic cytokine. *Arthritis Research and Therapy, 8*(2), S2.

1.4 Reducing IL-6 production in monocytes is an established therapeutic target for pathological inflammation

Monocytes, constituting 5–10% of the leukocytes, protect against blood-borne pathogens and react quickly to the sites of infection (Liaskou, Wilson, & Oo, 2012). Monocytes mature in the bone marrow and released into the blood stream (Owen, 1978). Subsequently, they circulate freely in the blood for about three days (Swirski et al., 2009). They have different roles depending on whether they are activated by an immune response. If there is no immune response, monocytes migrate to different tissues where they differentiate into macrophages that are the main resident phagocytes of the innate

immune system (Geissmann et al., 2010). If there is an immune response, monocytes migrate quickly to the site of the infection. On one hand, these monocytes and the resident macrophages protect the body from the infection within the blood stream and the tissues through an innate immune response. On the other hand, these monocytes differentiate within the tissue to create more macrophages to carry out the adaptive immune response (Tripp, Unanue, & Needleman, 1986). IL-6 hyper expression was found to be expressed in macrophages in rheumatoid arthritis (RA) patients (Grabiec, Korchynskyi, Tak, & Reedquist, 2012; Loebsch, Becker, Steiner, & Kloesch, 2013). Therefore, inhibition of monocyte-produced IL-6 is an attractive and established therapeutic target for pathological inflammation.

1.5 Current medication for IL-6 inhibition; Tocilizumab has significant side effects and motivates the search for small molecules that inhibit IL-6

Tocilizumab, an anti-IL-6 receptor antibody that can block IL-6 signaling, is a treatment for inflammatory diseases, such as rheumatoid arthritis (RA), colitis, Systemic Sclerosis (SSc), sepsis, and nerve pain (J. Guptarak et al., 2013; T. Hirano et al., 1988; Smolen et al., 2008). In people with rheumatoid arthritis (RA), IL-6 is over produced, particularly in the membrane that protects the joint (Toshio Hirano et al., 1988). Therefore, Tocilizumab has been introduced to RA patients as a treatment. At the same time, since Tocilizumab inhibits both IL-6 classical and trans signaling pathways (Figure 2), this drug has also been used as a positive treatment for Systemic Sclerosis (Barnes et al., 2011). Inhibition of IL-6 signaling with Tocilizumab is also regarded as an effective treatment of nerve pain (Jutatip Guptarak et al., 2013).

However, Tocilizumab does have certain side effects, including metabolic, respiratory, and nervous system disorders (Koike et al., 2011). Thus, there is a need for non-biological small molecules that inhibit IL-6.

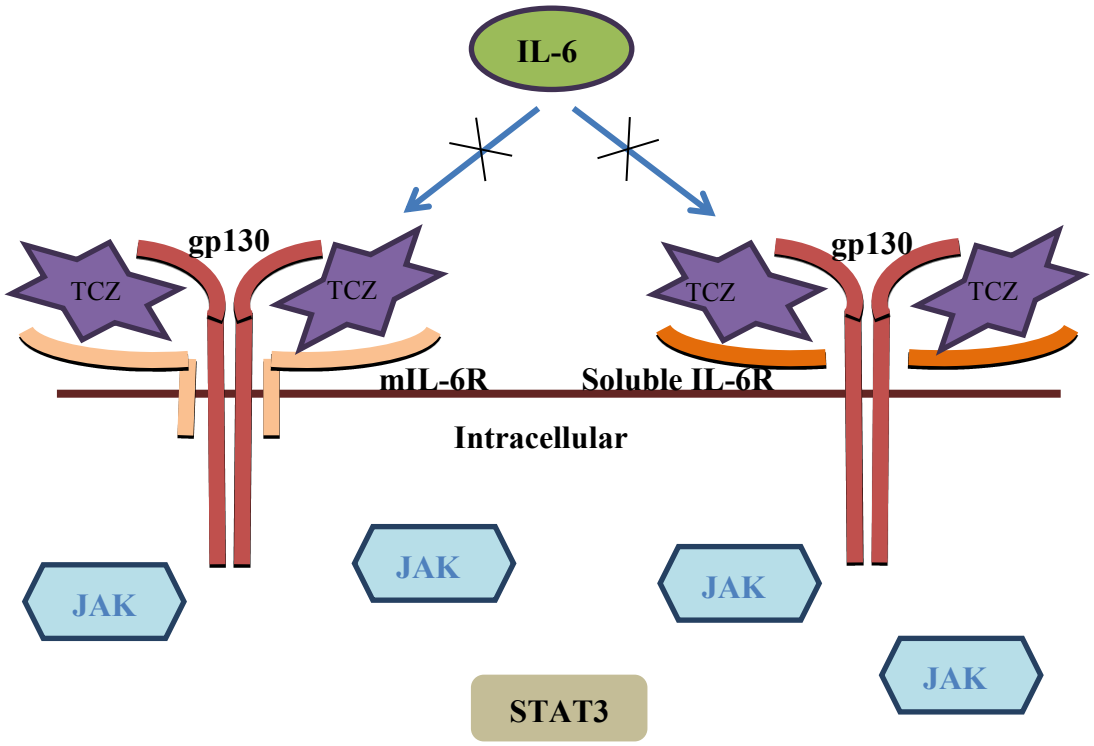

Figure 2. Blocking IL-6 signaling pathways using Tocilizumab (TCZ). Adapted from: Guptarak, J., Wanchoo, S., Durham-Lee, J., Wu, Y., Zivadinovic, D., Paulucci-Holthauzen, A., & Nesic, O. (2013). Inhibition of IL-6 signaling: A novel therapeutic approach to treating spinal cord injury pain. *Pain, 154*(7), 1115-1128.

1.6 Previous studies have identified a class of compounds that inhibit IL-6

In previous human breast cancer research, compounds synthesized at Ohio University were shown to inhibit IL-6 protein production in the human breast cancer cell line MDA-MB-231 (O'Brien, 2012). This finding leads to the hypothesis that compounds, such as COB-204 and COB-214 that were generated in the same study as the previous

compounds, may be effective at inhibiting IL-6. The goal of this thesis is to investigate this hypothesis using an *in vitro* model of inflammation involving IL-6.

1.7 Inducers of IL-6 in *in vitro* experimental models

In order to create an inflammatory IL-6 expression model for *in vitro* studies, different inducers have been introduced to various cell models. These inducers include LPS, IL-12, IL-18, TNF-α, and a combination of TNF-α and IFN-γ. IL-6 production can be induced by LPS in peripheral blood monocytes of patients with high or low levels of HDL-lipoprotein (Eggesbø et al., 1994) and patients with chronic liver disease and acute viral hepatitis (Muller & Zielinski, 1992). IL-6 can be induced in peripheral blood monocytes of healthy persons as well. In a previous study (Leoni et al., 2002), monocytes were isolated from healthy donors and then differentiated into macrophages. These macrophages successfully produced IL-6 after stimulation with LPS (Leoni et al., 2002). Later, this research group found that monocytes pre-incubated with a histone deacetylase inhibitor produced high levels of IL-6 upon stimulation with IL-12 and IL-18 (Leoni et al., 2005). Macrophages differentiated from monocytes isolated from RA patients also expressed high IL-6 protein after stimulation with TNF-α (Grabiec et al., 2010). A combination of TNF-α and IFN-γ induces IL-6 production in sclerotic fibroblasts (Antonelli et al., 2011).

THP-1 is a human monocytic leukemia cell line, which was isolated from a one year old male infant suffering from acute monocytic leukemia. This monocyte-like cell line has been used to gain insights into inflammation (Hsu, Lin, Chang, Hong, & Chen, 2013). For example, as low pro-inflammatory cytokine levels are related to a diet rich in vegetables, a recent study utilized LPS stimulated THP-1 cells to produce pro-

inflammatory cytokines that are inhibited by molecules isolated from broccoli (Folkard et al., 2014). Previous results also showed that the combination of TNF-α and IFN-γ (Sanceau, Wijdenes, Revel, & Wietzerbin, 1991) were able to induce IL-6 production in undifferentiated THP-1 cells. We have chosen to use this cell line in our studies. To test COB-204 and COB-214 in this model, this thesis will focus on TNF-α and IFN-γ induction of IL-6 in undifferentiated THP-1 cells. In addition, we will utilize freshly isolated monocytes from human peripheral blood.

1.8 Pathological relevance of using TNF-α and IFN-γ

As described above, TNF-α induces IL-6 in macrophages differentiated from monocytes donated by RA patients (Grabiec et al., 2010). At the same time, IFN-γ was found to activate monocytes in an immune response (Bell, 2014). TNF-α has been shown to enhance the effects of IFN-γ against viral, parasitic, and microbial infections (Esparza, Mannel, Ruppel, Falk, & Krammer, 1987;Green, Crawford, Hockmeyer, Meltzer, & Nacy, 1990; Wietzerbin, Gaudelet, Catinot, Chebath, & Falcoff, 1990). In a recent study, the combination of TNF-α and IFN-γ was used as inducer of depressive-like behavior in mice (O'Connor et al., 2009). TNF-α induces IL-6 production in undifferentiated THP-1 cells with modulation by IFN-γ (Sanceau et al., 1991). In this thesis, TNF-α and IFN-γ will be used to induce IL-6 expression.

Chapter 2: Hypothesis and Specific Aims

2.1 Hypothesis

COB-204 and COB-214 inhibit TNF-α- and IFN-γ-induced IL-6 expression in undifferentiated THP-1 cells and freshly isolated human monocytes.

2.2 Specific aims

The following specific aims will be completed to investigate the proposed hypothesis.

1) Determine whether the combination of TNF-α and IFN-γ induces IL-6 production in undifferentiated THP-1 cells and freshly isolated human monocytes.
2) Determine whether treatment with COB-204 or COB-214 decreases TNF-α- and IFN-γ-induced IL-6 expression in undifferentiated THP-1 cells.
3) Determine if treatment with COB-204 or COB-214 has an effect on the metabolic activity of normal vascular cells (in this case HUVEC) and undifferentiated THP-1 cells.
4) If TNF-α and IFN-γ can induce IL-6 in freshly isolated human monocytes, we will determine if treatment with COB-204 or COB-214 decreases TNF-α- and IFN-γ-induced IL-6 expression in freshly isolated monocytes and whether this treatment has an effect on the metabolic activity of this cell type.
5) If TNF-α and IFN-γ cannot induce IL-6 in freshly isolated human monocytes, flow cytometry may be conducted to determine if TNF-α and IFN-γ receptors are present on freshly isolated human monocytes.

Chapter 3: Materials and Methods

3.1 Culture of HUVECs and THP-1 cells

The culture of human umbilical vein endothelial cells (HUVECs) has been described previously by Nilesh Dagia and Douglas Goetz (Dagia & Goetz, 2003). Undifferentiated THP-1 cells were cultured in medium RPMI-1640 (#30-2001, ATCC, Manassas, VA) containing 10% non-heat inactivated FBS (#30-2020, ATCC, Manassas, VA) and 0.05 mM β-mercaptoethanol (#21985-023, Gibco, Grand Island, NY).

3.2 Isolation and culture of monocytes

Following the IRB approved protocol 08X073, human peripheral blood mononuclear cells (PBMCs) were isolated from the blood of healthy donors using Histopaque-1077 (#10771, Sigma, St. Louis, MO). Then monocytes were isolated from the PBMCs using a CD14 positive selection kit (#130-050-201, Miltenyi Biotec, San Diego, CA). Selected monocytes were allowed to settle in RPMI-1640 medium without FBS for two hours. After removing pre-culture medium, monocytes were cultured in RPMI-1640 containing 5% heat-inactivated FBS (#14-503F, Biowhittaker, Walkersville, MD) for two days prior to treatment.

3.3 Compound treatment

COB-204 and COB-214 stocks were stored in 200 mM 100% Dimethyl Sulfoxide (#DMSO-2650, Sigma, St. Louis, MO). Each was diluted into cell culture media to a DMSO concentration of 0.25%. Recombinant human (rh) TNF-α and rhIFN- γ (#285-IF-100, R&D Systems, Minneapolis, MN), both at a concentration of 180 ng/mL, were used to induce IL-6 production. A solution containing COB-204, COB-214, or 0.25% DMSO (carrier control) with the cytokines were added to the cells. To maintain the same cell

concentration used in each repeated experiment, cell counting was performed using a BIO-RAD Automated Cell Counter with counting slides (#145-0011, BIO-RAD, Hercules, CA) and Trypan Blue Solution (#T-8154, Sigma, St. Louis, MO). Cells were incubated at 5% CO_2 at 37°C for 24 hours.

3.4 QRT-PCR (Quantitative Real Time Polymerase Chain Reaction)

The PCR protocol followed that described by John O'Brien (O'Brien, 2012). THP-1 cells were cultured in 12-well plates (# 353043, BD Biosciences, San Jose, CA). Twenty four hours after the cell treatment, cell suspension were transferred to RNase-free microcentrifuge tubes (#07-200-210, Fisher Scientific, Morristown, NJ) and centrifuged into pellets at 1,200 rpm. Cell lysates were homogenized using QIAshredder microcentrifuge spin-column homogenizers (#79656, Qiagen, NV) and were purified using the RNeasy mini kit (#74106, Qiagen, NV) and the RNase-free DNase set (#79254, Qiagen, NV). The Nanodrop 2000 Spectrophotometer (Thermo Scientific, Pittsburgh, PA) was used for quantifying mRNA products. Six hundred-800 ng of each mRNA sample was used to make cDNA. The high capacity cDNA reverse transcription kit (#4368814, Applied Biosystems, Grand Island, NY) was used for cDNA synthesis. Taqman® gene expression assays (#186775206-1, Applied Biosystems, Grand Island, NY), Taqman® gene expression master mix (#4369016, Applied Biosystems, Grand Island, NY), and the StepOnePlus™ real-time PCR system (Applied Biosystems, Grand Island, NY) were used for PCR. HPRT-1 was used as the endogenous control. Fold change was calculated using the Delta Delta C(T) method relative to the 0.25% DMSO control.

3.5 ELISA secreted protein analysis

THP-1 cells were cultured in 96 well tissue culture vacuum plasma-treated plates. Cell supernatants were harvested after 24 hours of treatment, and were stored at -20°C before being used in the ELISA assay. The dilution factor for ELISA was 1:5 or 1:10. Non-tissue culture treated 96-well plates (#12-565-213, Fisher Scientific, Morristown, NJ), BD OptEIA Human IL-6 ELISA set (#555220, BD Biosciences, San Jose, CA) and Reagent set B (#550534, BD Biosciences, San Jose, CA) were used in the ELISA. IL-6 standards were prepared using a kit-provided stock. Plates were read at dual wavelengths of 450 nm and 570 nm.

3.6 MTS assay

Cell titer 96 aqueous non-radioactive cell proliferation kits (#G5421, Promega, Madison, WI) were purchased. MTS solution was prepared according to the kit instructions. Cells with 100 µL medium in each well were treated with 20 µL MTS solution. Subsequently, the plate was incubated at 37ºC for one hour in humidified 5% CO_2 atmosphere. The plate was read at a wavelength of 490 nm. A control without cells was required for later analysis. The control group contained only medium, from which an absorbance is obtained at 490 nm. This medium absorbance is deducted from the absorbance of all sample groups to eliminate the influence of the medium absorbency.

3.7 Flow Cytometric Assay

Flow cytometry protocol published by Dr. Monica Burdick (Burdick, Chu, Godar, & Sackstein, 2006) was followed. Mouse anti-human CD120a antibody (#MCA1340T, AbD Serotec, Raleigh, NC) was used to detect TNF receptor 1. Mouse anti-human CD120b antibody (#MAB2261, R&D Systems, Minneapolis, MN) was used to detect

TNF receptor 2. Mouse anti- human CD119 antibody (#MCA1450T, AbD Serotec, Raleigh, NC) was used to detect the receptor of IFN-γ. Mouse IgG2a isotype control ((#MCA929, AbD Serotec, Raleigh, NC) and goat anti-mouse IgG fluorescein antibody (#F11021, Life Technologies, Grand Island, NY) were purchased. Freshly isolated monocytes from human peripheral blood and THP-1 cells were obtained and suspended at 10^7 /mL in 0.1% BSA/DPBS without Ca^{2+} or Mg^{2+}. 20 μL of cell suspension were deposited in micro-centrifuge tubes and centrifuged into pellets. Cells were treated with primary antibodies of CD120a, CD120b, CD119, or mouse IgG2a isotype control and incubated for 30 min on ice. Subsequently, the cells were centrifuged, the supernatants discarded, and the pellets washed with 0.1% BSA/DPBS without Ca^{2+} or Mg^{2+}. The cells were treated with an FITC-conjugated secondary antibody (#F11021, Life Technologies, Grand Island, NY) and incubated for 30 min in the dark on ice. Cells were washed twice with 0.1% BSA/DPBS without Ca^{2+} or Mg^{2+} and then washed with DPBS without Ca^{2+} or Mg^{2+}. Cells were suspended in 100 μL DPBS without Ca^{2+} or Mg^{2+} and transferred to individual flow cytometry tubes. Cells were analyzed in a BD FACSDiva software. For each sample, 10,000 events were recorded.

Chapter 4: Results

4.1 MTS results for HUVECs treated with COB-204 and COB-214

Once a drug is administered, it will enter the blood stream. Thus, the vasculature is exposed to the drug. In order to investigate the effect of the compounds on the metabolic activity of vascular cells, an MTS assay was performed with cultured endothelial cells. Endothelial cells are the cells that line the blood vessel walls. Human umbilical vein endothelial cells (HUVECs) are an established *in vitro* model for vascular endothelial cells, and were thus chosen as the model for a vascular cell. HUVECs were treated with COB-204 or COB-214 at a series of concentrations to generate a dose-response curve, from which a TC_{50} value could be determined. TC_{50} is the compound concentration at which the MTS signal is decreased by 50% relative to carrier (DMSO) control treated cells. Concentrations of COB-204 used were 3.1, 6.3, 12.5, 25, and 50 μM. Figure 3 indicates that the metabolic activity of HUVECs did not appear to be affected by COB-204 concentration. ANOVA revealed that the percentage metabolic activity was not a function of COB-204 concentration. The reason why concentrations higher than 50 μM were not used was that COB-204 precipitates at concentrations above 50 μM.

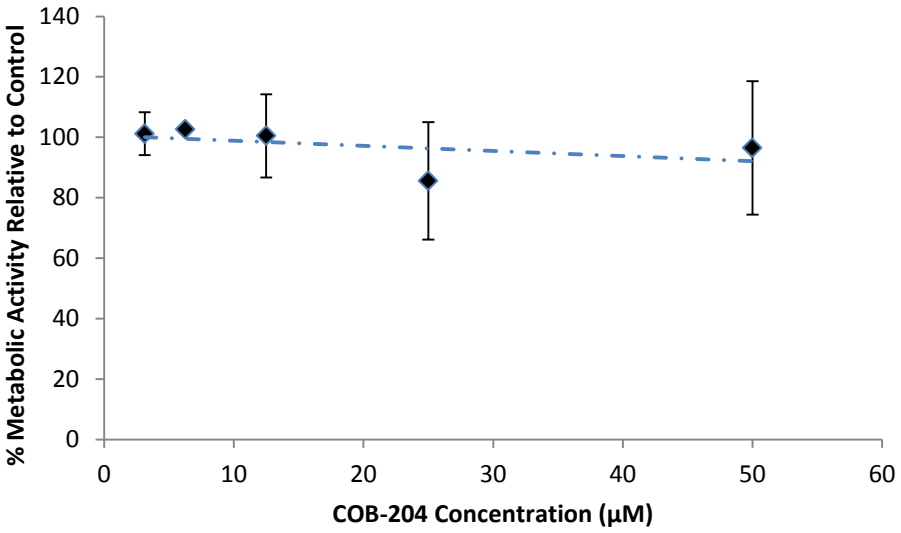

Figure 3. Effect of 24-hour COB-204 treatment on the metabolic activity of HUVECs. HUVECs were treated with increasing COB-204 concentrations. ANOVA indicated that % metabolic activity is not a function of COB-204 concentration. Data are average ±SD of n=3.

Concentrations of COB-214 used were 31.3, 62.5, 125, 250, and 500 µM. Figure 4 indicates that metabolic activity of HUVECs decreases as COB-214 concentration increases. In Figure 4, the best correlation was achieved using an exponential regression. This analysis yielded a TC_{50} value of 712.7µM.

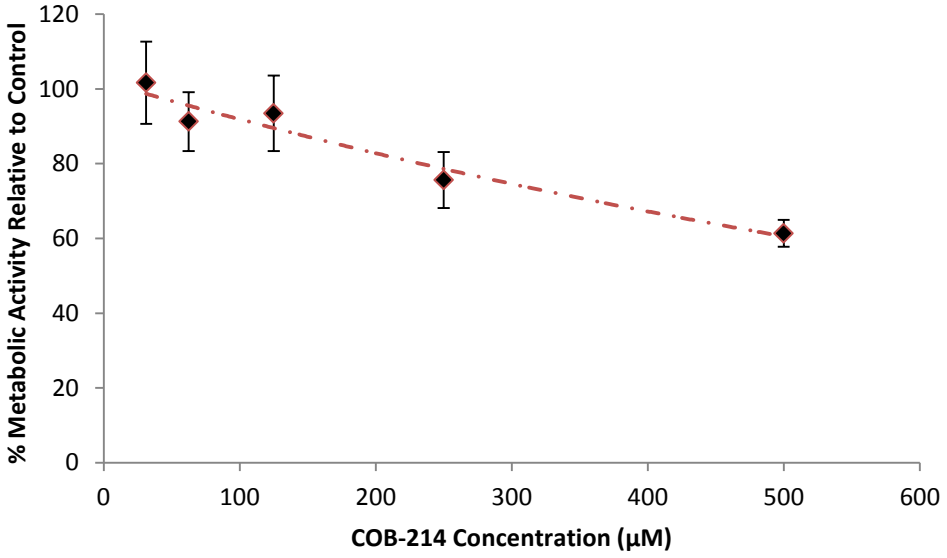

Figure 4. Effect of 24-hour COB-214 treatment on the metabolic activity of HUVECs. To calculate the TC_{50} value, HUVECs were treated with increasing COB-214 concentrations. The calculated mean TC_{50} based on an exponential regression $y=101.97e^{-0.001x}$ ($R^2=0.9604$) is 712.7µM. Data are average ±SD of n=3.

4.2 MTS results for undifferentiated THP-1 cells treated with COB-204 and COB-214

Any effect of the compounds on IL-6 expression in THP-1 cells could be due to a general "toxic" effect on the cells. To investigate this possibility, MTS assay was performed on undifferentiated THP-1 cells to evaluate the TC_{50} values of the compounds. Concentrations of COB-204 used were 5, 10, 20, 40, and 50 µM. Figure 5 indicates that the metabolic activity of undifferentiated THP-1 cells decreases as COB-204 concentration increases. The reason why concentrations higher than 50 µM were not used was that COB-204 precipitates at a concentration above 50 µM. In Figure 5, the calculated mean TC_{50} based on a linear regression is 79.8 µM.

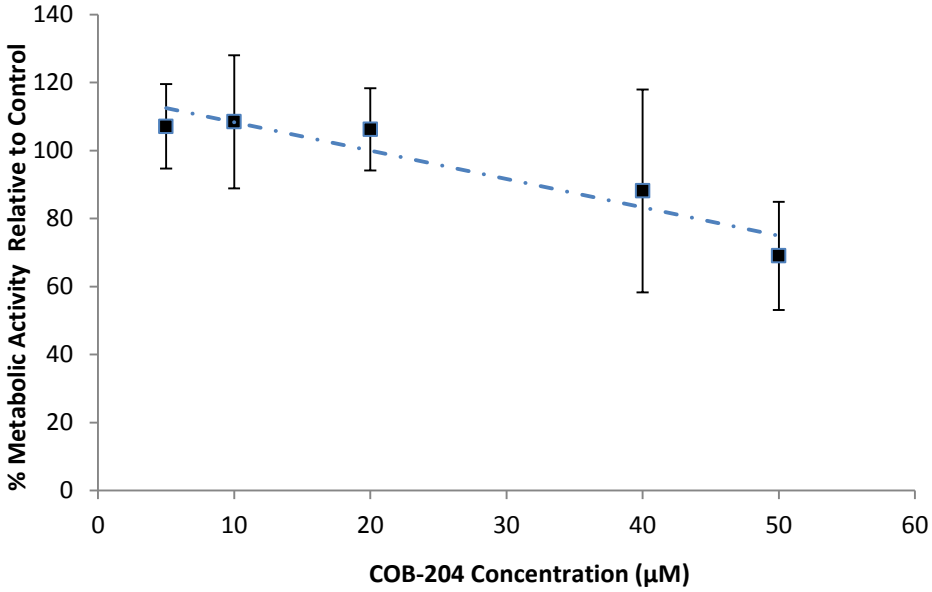

Figure 5. Effect of 24-hour COB-204 treatment on the metabolic activity of undifferentiated THP-1 cells. To calculate the TC_{50} value, undifferentiated THP-1 cells were treated with increasing COB-204 concentrations. The calculated mean TC_{50} based on a linear regression $y = -0.8352x + 116.66$ (R^2=0.8921) is 79.8 µM. Data are average ±SD of n=3.

Concentrations of COB-214 used were 10, 20, 40, 50, 100, 200, 400, and 500 µM. Figure 6 indicates that cell metabolic activity of undifferentiated THP-1 cells decreases as COB-214 concentration increases. In Figure 6, the calculated mean TC_{50} based on a logarithmic regression is 312.2 µM.

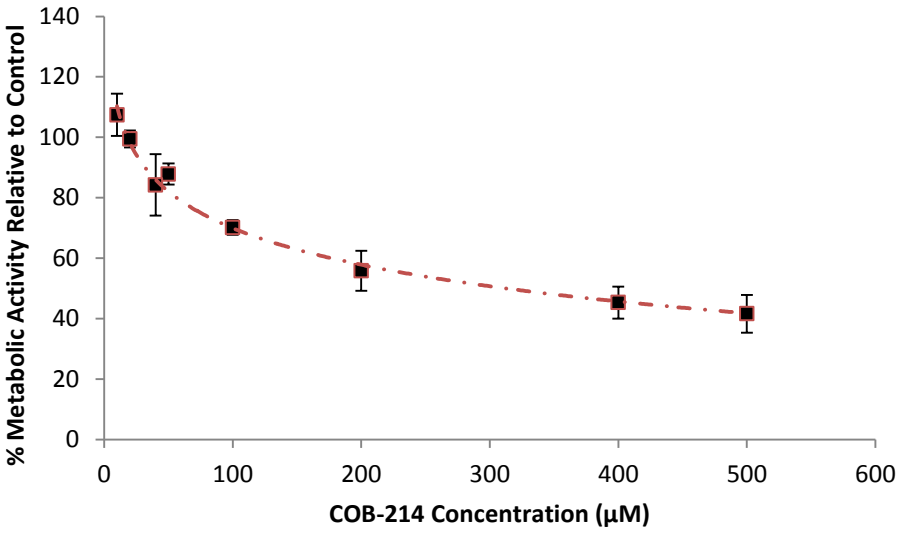

Figure 6. Effect of 24-hour COB-214 treatment on the metabolic activity of undifferentiated THP-1 cells. To calculate the TC_{50} value, undifferentiated THP-1 cells were treated with increasing COB-214 concentrations. The calculated mean TC_{50} based on a logarithmic regression $y = -17.52 \ln(x) + 150.63$ (R^2=0.9883) is 312.2 µM. Data are average ±SD of n=3.

4.3 PCR results for TNF-α and IFN-γ stimulated undifferentiated THP-1 cells in the presence of COB-204 and COB-214

QRT-PCR was performed to determine the effect of COB-204 and COB-214 on IL-6 mRNA production induced by TNF-α (180 ng/ml) and IFN-γ (180 ng/ml) in undifferentiated THP-1 cells. COB-204 concentrations of 2.5, 5, 10, 20, and 40 µM were used. Figure 7 indicates that inhibition of IL-6 mRNA increases in THP-1 cells as COB-204 concentration increases. In Figure 8, a logarithmic regression curve reveals that the IC_{50} for COB-204 is 9.4 µM. IC_{50} is the compound concentration at which IL-6 production is inhibited by 50% relative to carrier (DMSO) control treated cells, because DMSO has been used as the solvent for the compounds. In Figure 7, the DMSO control group is the second lane where COB-204 concentration is zero.

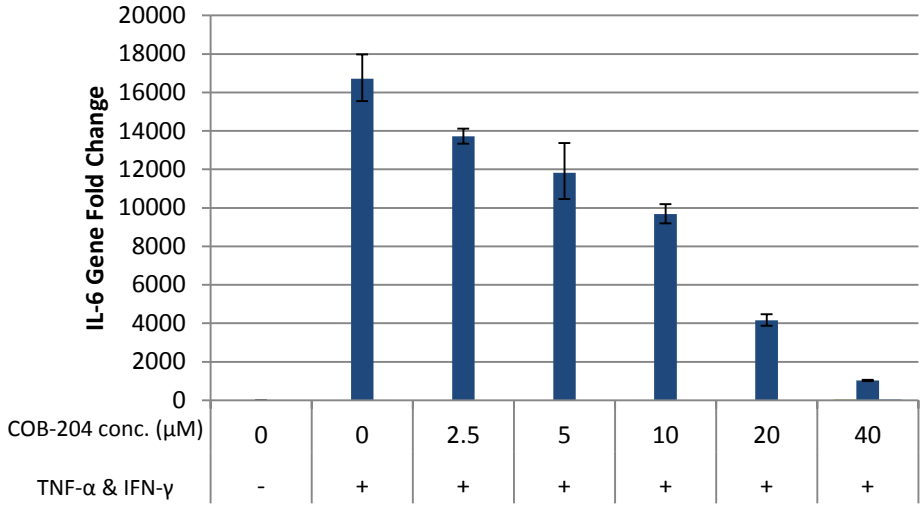

Figure 7. Fold change of 24-hour TNF-α- and IFN-γ-induced IL-6 mRNA in undifferentiated THP-1 cells incubated with COB-204. Undifferentiated THP-1 cells were treated with increasing COB-204 concentrations: 2.5, 5, 10, 20, and 40 µM (24 hours) to determine compound effects on IL-6 mRNA expression in response to TNF-α and IFN-γ treatment (n=2). Group DMSO is the control group treated with only TNF-α and IFN-γ (second lane).

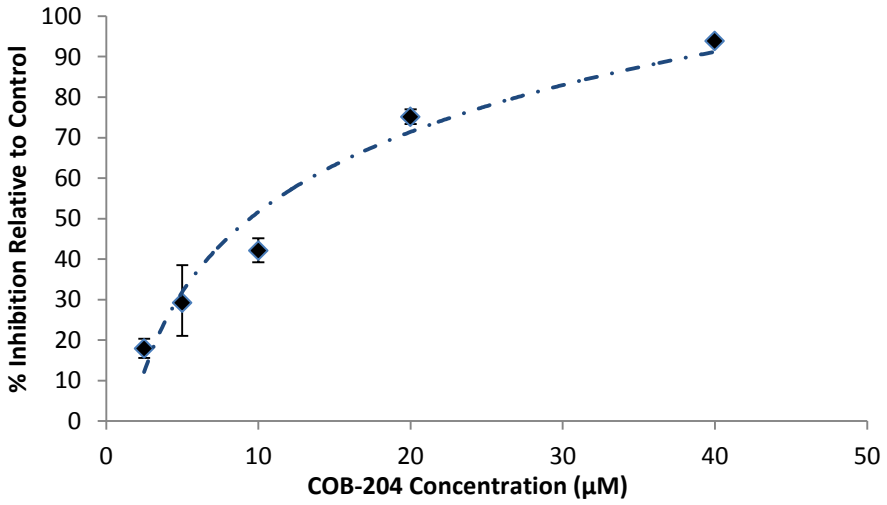

Figure 8. IC_{50} value of 24-hour TNF-α- and IFN-γ-induced IL-6 mRNA in undifferentiated THP-1 cells incubated with COB-204. Undifferentiated THP-1 cells were treated with a range of COB-204 concentrations to determine the compound effect on IL-6 mRNA expression in response to TNF-α and IFN-γ treatment. For IL-6 inhibition, the calculated IC_{50} based on a logarithmic regression $y=28.523\ln(x)-14.023$ ($R^2=0.9625$) is 9.4 µM. Data are average ±SD of n=2.

In the COB-214 PCR, different concentrations of COB-214 were used: 2.5, 5, 10, 20, and 40 µM. Figure 9 indicates that inhibition of IL-6 mRNA increases in undifferentiated THP-1 cells as COB-214 concentration increases. In Figure 10, a logarithmic regression curve reveals that the IC_{50} for COB-214 is 13.6 µM. TNF-α (180 ng/ml) and IFN-γ (180 ng/ml) were used as the inducers to activate IL-6 mRNA production.

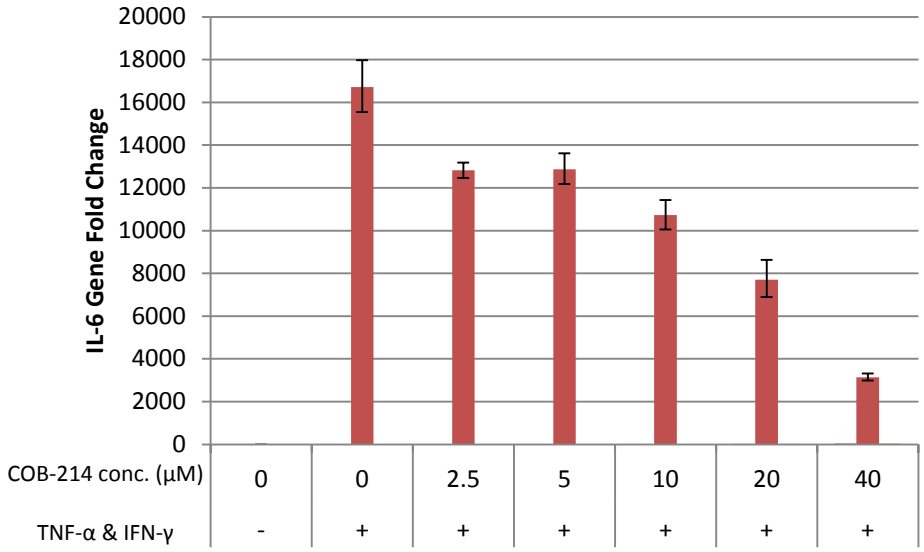

Figure 9. Fold change of 24-hour TNF-α- and IFN-γ-induced IL-6 mRNA in undifferentiated THP-1 cells incubated with COB-214. Undifferentiated THP-1 cells were treated with a range of COB-214 concentrations: 2.5, 5, 10, 20, and 40 µM. (24 hours) to determine compound effects on IL-6 mRNA expression in response to TNF-α and IFN-γ treatment (n=2). Group DMSO is the control group treated with only TNF-α and IFN-γ (second lane).

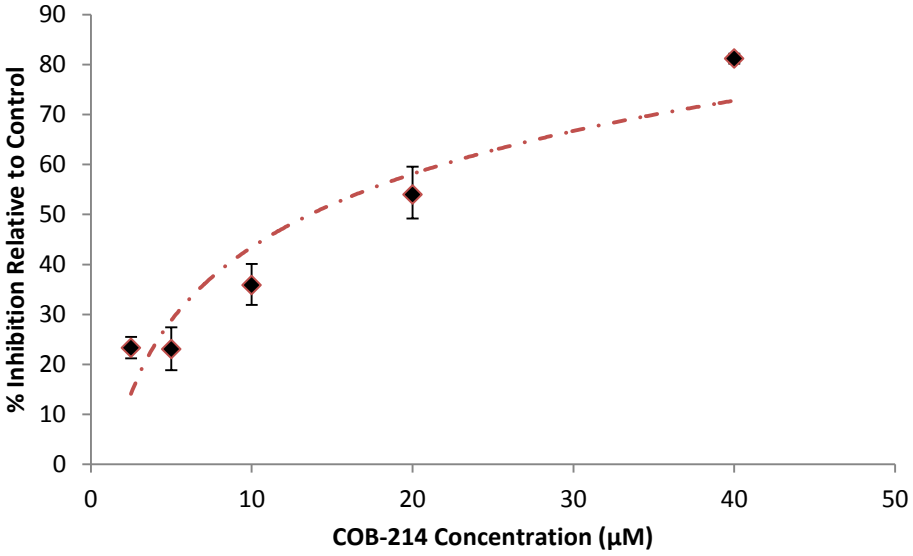

Figure 10. IC_{50} value of 24-hour TNF-α- and IFN-γ-induced IL-6 mRNA in undifferentiated THP-1 cells incubated with COB-214. Undifferentiated THP-1 cells were treated with a range of COB-214 concentrations to determine the compound effect on IL-6 protein expression in response to TNF-α and IFN-γ treatment. For IL-6 inhibition, the calculated IC_{50} based on a logarithmic regression $y=21.169\ln(x)-5.2879$ ($R^2=0.891$) is 13.6 µM. Data are average ±SD of n=2.

4.4 ELISA results for TNF-α and IFN-γ stimulated undifferentiated THP-1 cells in the presence of COB-204 and COB-214

Because COB-204 was effective at significantly decreasing TNF-α and IFN-γ induction of IL-6 mRNA in THP-1 cells, a range of compound concentrations were applied to investigate IL-6 protein expression in THP-1 cells. The effect of COB-204 on TNF-α and IFN-γ induced IL-6 protein expression in the culture supernatant of THP-1 were tested via ELISA. COB-204 treatment concentrations were 2.5, 5, 10, 15, 20, and 50 µM. The supernatant of each sample was saved and diluted at a dilution factor of 1:10. Figure 11 indicates that, as was seen at the mRNA level, inhibition of IL-6 protein increases in THP-1 cells as COB-204 concentration increases. The optimal correlation

was achieved using a logarithmic regression. This analysis yielded an IC_{50} value of 5.8 µM.

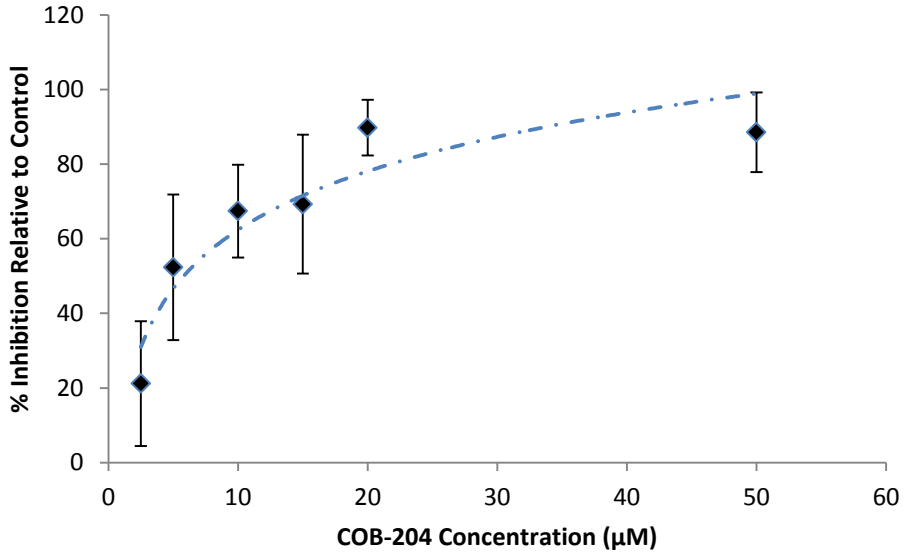

Figure 11. IC_{50} value of 24-hour TNF-α- and IFN-γ-induced IL-6 protein in undifferentiated THP-1 cells incubated with COB-204. Undifferentiated THP-1 cells were treated with a range of COB-204 concentrations to determine compound effect on IL-6 protein expression in response to TNF-α and IFN-γ treatment. For IL-6 inhibition, the calculated IC_{50} based on a logarithmic regression $y=22.711\ln(x)+10.093$ ($R^2=0.8775$) is 5.8 µM. Data are average ±SD of n=3.

The effect of COB-214 on TNF-α- and IFN-γ-induced IL-6 protein expression was investigated. Concentrations of COB-214 were 2.5, 5, 10, 20, 40, and 50 µM. The supernatant of each sample was saved and diluted at a dilution factor of 1:10. Figure 12 indicates that, as was seen at the mRNA level, inhibition of IL-6 protein increases in undifferentiated THP-1 cells as COB-214 concentration increases. The optimal correlation was achieved using a logarithmic regression. This analysis yielded an IC_{50} value of 10.5 µM.

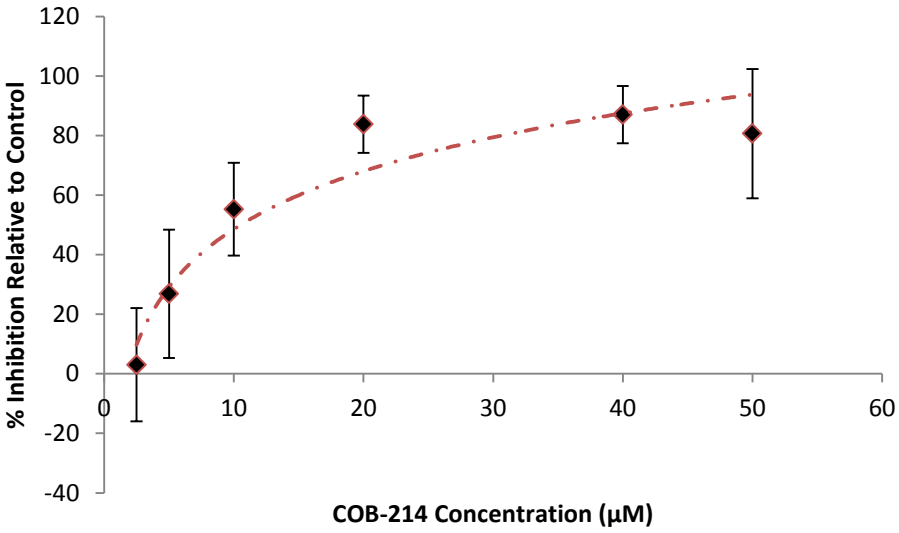

Figure 12. IC_{50} value of 24-hour TNF-α- and IFN-γ-induced IL-6 protein in undifferentiated THP-1 cells incubated with COB-214. Undifferentiated THP-1 cells were treated with a range of COB-214 concentrations to determine compound effects on IL-6 protein expression in response to TNF-α and IFN-γ treatment. For IL-6 inhibition, the calculated IC_{50} based on a logarithmic regression $y=28.062\ln(x)-16.051$ ($R^2=0.9145$) is 10.5 µM. Data are average ±SD of n=3.

4.5 ELISA results for freshly isolated human monocytes in the presence of a combination of TNF-α and IFN-γ

To determine whether a combination of TNF-α and IFN-γ can induce IL-6 production in freshly isolated monocytes from human peripheral blood. Freshly isolated monocytes from human peripheral blood and undifferentiated THP-1 cells were treated with TNF-α (180 ng/ml) and IFN-γ (180 ng/ml) for 24 hours. Supernatants were harvested for ELISA. IL-6 protein production was determined via ELISA (Figure 13). The combination of TNF-α and IFN-γ induced high IL-6 protein expression in undifferentiated THP-1 cells. However, IL-6 protein expression was not induced in freshly isolated human monocytes treated with TNF-α and IFN-γ.

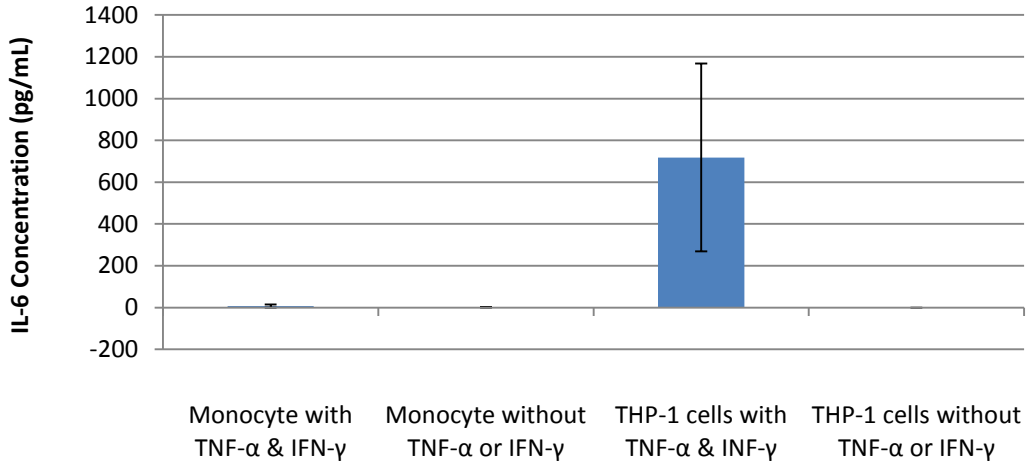

Figure 13. IL-6 protein production in freshly isolated human monocytes and undifferentiated THP-1 cells treated with TNF-α and IFN-γ for 24 hours. The combination of TNF-α and IFN-γ only induced IL-6 production in undifferentiated THP-1 cells. Data are average ±SD of n=3.

4.6 Flow cytometric analysis of TNF-α and IFN-γ receptor expression in freshly isolated human monocytes and undifferentiated THP-1 cells

To determine whether TNF-α and IFN-γ receptors are expressed on freshly isolated human monocytes and undifferentiated THP-1 cells, antibodies to CD120a (TNF receptor 1), CD120b (TNF receptor 2), and CD119 (IFN-γ receptor) were used. However, as shown below, this attempt was confounded by the high binding of the isotype control to the isolated human monocytes and undifferentiated THP-1 cells. In Figure 14, the orange line represents monocytes without antibody treatment, the black dashed line represents monocytes treated with only secondary antibody, and the black line represents monocytes treated with IgG2a isotype negative control and secondary antibody. The black line will be used in Figure 15 as the negative control.

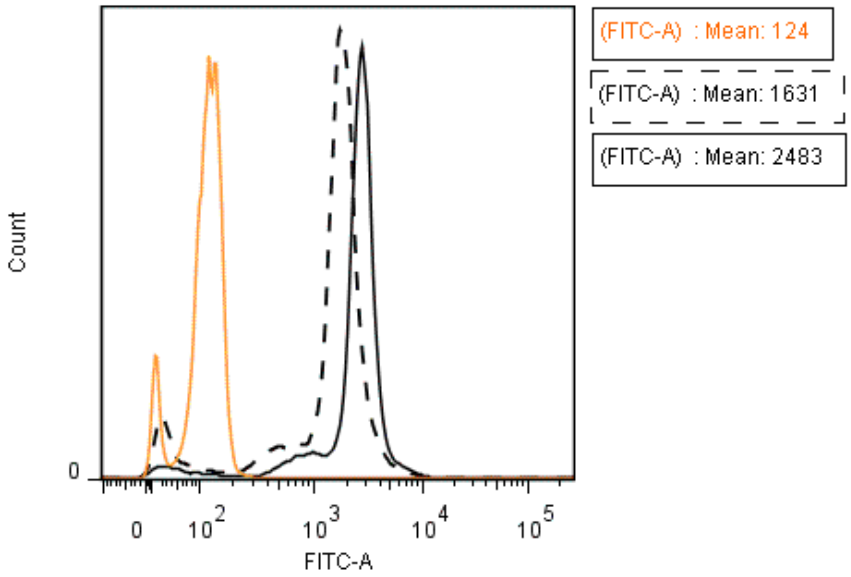

Figure 14. Negative controls for flow cytometric analysis of freshly isolated human monocytes. Orange line: No treatment. Black dashed line: Secondary antibody only. Black line: IgG2a isotype control and secondary antibody treatment.

In Figure 15, the red line represents monocytes treated with primary CD120a (TNF receptor 1) antibody and secondary antibody, the blue line represents monocytes treated with primary CD120b (TNF receptor 2) antibody and secondary antibody, and the orange line represents monocytes treated with primary CD119 (IFN-γ receptor) antibody and secondary antibody. This result suggests that no TNF receptor 1or TNF receptor 2 is present on freshly isolated human monocytes. The result also suggests that there may be a low level of expression of the IFN-γ receptor. However, the isotype control was quite high relative to no treatment or secondary treatment only (Figure 14), making it difficult to draw firm conclusions from the data.

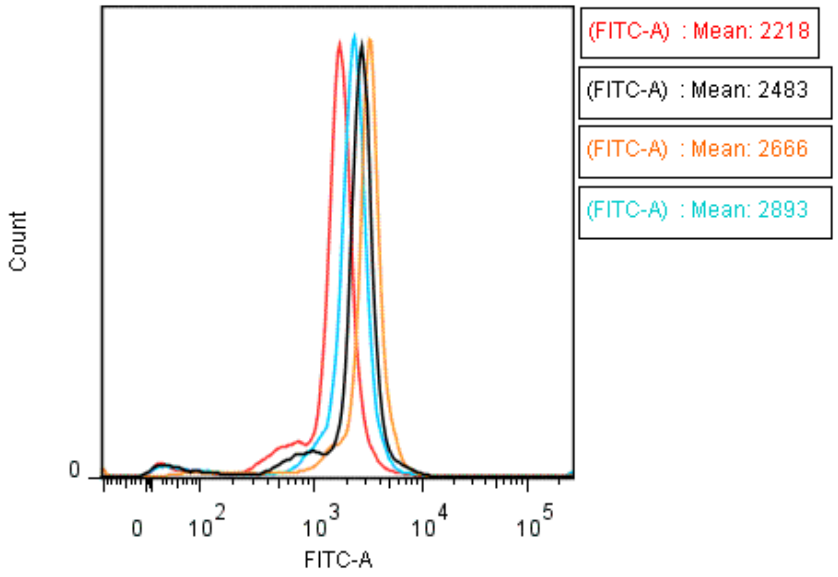

Figure 15. Flow cytometric results of CD120a (TNF receptor 1), CD120b (TNF receptor 2), and CD119 (IFN-γ receptor) expression on freshly isolated human monocytes. Red line: CD120a primary antibody and secondary antibody treatment. Blue line: CD120b primary antibody and secondary antibody treatment. Orange line: CD119 primary antibody and secondary antibody treatment. Black line (the negative control): IgG2a isotype control and secondary antibody treatment.

An attempt was made to use undifferentiated THP-1 cells as a positive control for the freshly isolated human monocytes flow cytometric analysis. However, as shown below, this attempt was confounded by the high binding of the isotype control to the undifferentiated THP-1 cells. In Figure 16, the orange line represents THP-1 cells without antibody treatment, the black dashed line represents THP-1 cells treated with only secondary antibody, and the black line represents THP-1 cells treated with IgG2a isotype negative control and secondary antibody. The black line will be used in Figure 17 as the negative control.

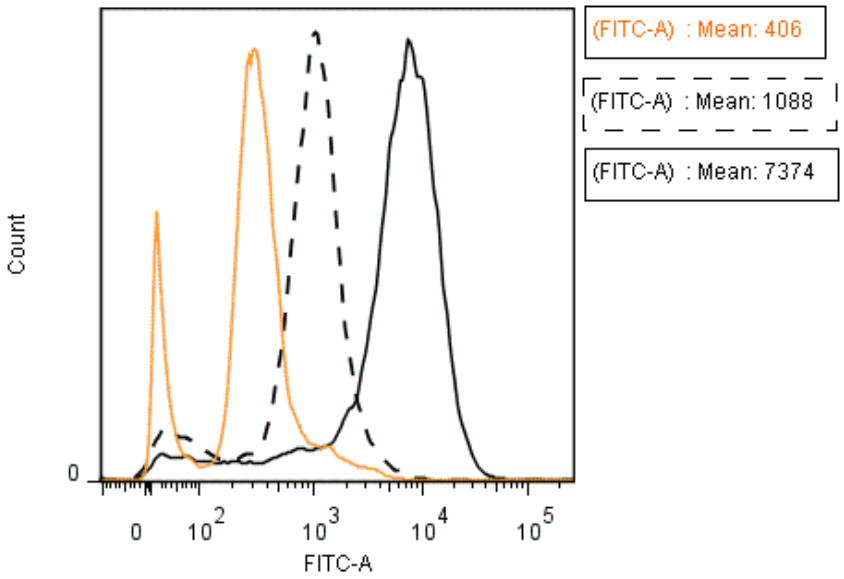

Figure 16. Negative controls for flow cytometric analysis of undifferentiated THP-1 cells. Orange line: No treatment. Black dashed line: Secondary antibody treatment only. Black line: IgG2a negative isotype control and secondary antibody treatment.

In Figure 17, the red line represents THP-1 cells treated with primary CD120a (TNF receptor 1) antibody and secondary antibody, the blue line represents THP-1 cells treated with primary CD120b (TNF receptor 2) antibody and secondary antibody, and the orange line represents THP-1 cells treated with primary CD119 (IFN-γ receptor) antibody and secondary antibody. This figure indicates that no TNF receptor 1, TNF receptor 2, or IFN-γ receptor was expressed on undifferentiated THP-1 cells. However, the isotype control was quite high relative to no treatment or secondary treatment only (Figure 16), making it difficult to draw firm conclusions from the data.

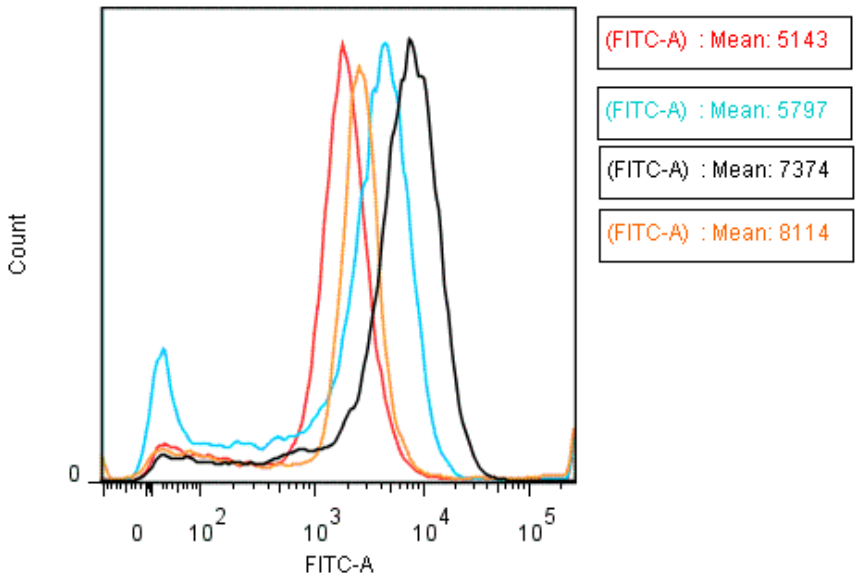

Figure 17. Flow cytometric results of CD120a (TNF receptor 1), CD120b (TNF receptor 2), and CD119 (IFN-γ receptor) expression on undifferentiated THP-1 cells. Red line: CD120a primary antibody and secondary antibody treatment. Blue line: CD120b primary antibody and secondary antibody treatment. Orange line: CD119 primary antibody and secondary antibody treatment. Black line (the negative control): IgG2a isotype control and secondary antibody treatment.

Chapter 5: Discussion

In previous human breast cancer research conducted by John O'Brien, compounds synthesized at Ohio University were shown to inhibit IL-6 protein production in the human breast cancer cell line MDA-MB-231 (O'Brien, 2012). Based on this finding, the present study has been conducted to determine if compounds, such as COB-204 and COB-214 that were generated in the same study, may be effective at inhibiting cytokine induced IL-6 in undifferentiated THP-1 cells and freshly isolated monocytes from human peripheral blood.

In QRT-PCR analysis, it was revealed that undifferentiated THP-1 cells express limited, if any, IL-6 mRNA without induction. However, a combination of TNF-α and IFN-γ successfully induced IL-6 production in this cell type. Therefore, the combination of cytokines TNF-α and IFN-γ was used to induce IL-6 production in undifferentiated THP-1 cells. Compounds COB-204 and COB-214 were used in this model to determine the compounds' effects on induced IL-6 production. According to the QRT-PCR results, induced undifferentiated THP-1 cells exhibited a significant decrease in IL-6 gene expression with COB-204 (Figure 7 and 8) and COB-214 (Figure 9 and 10) treatments. Meanwhile, according to the ELISA results, induced undifferentiated THP-1 cells also exhibited a significant decrease in IL-6 protein expression with COB-204 (Figure 11) and COB-214 (Figure 12) treatments. The IC_{50} values for inhibiting IL-6 production were calculated. The IC_{50} values for compound COB-204 were 9.4 μM at the gene expression level and 5.8 μM at the protein expression level for 24-hour TNF-α- and IFN-γ-induced IL-6 in undifferentiated THP-1 cells. The IC_{50} values for compound COB-214 were 13.6 μM at the gene expression level and 10.5 μM at the protein expression level for 24-hour

TNF-α- and IFN-γ-induced IL-6 in undifferentiated THP-1 cells. These results were consistent with the hypothesis that COB-204 and COB-214 can inhibit both IL-6 gene expression and protein production in TNF-α and IFN-γ induced undifferentiated THP-1 cells.

In order to investigate the effect of the compounds on the metabolic activity of undifferentiated THP-1 cells, MTS assay was performed. The MTS assay is often used for a quantification of cell metabolic activity, with the absorbance signal reflecting the relative cell mitochondrial enzyme activity. According to the results, although a decrease in cell metabolic activity was found while compound concentration goes up, COB-204 and COB-214 do not appear to affect cell metabolic activity as dramatically as affecting IL-6 expression. The TC_{50} values were 79.8 μM and 312.2 μM respectively for undifferentiated THP-1 cells treated for 24 hours with COB-204 and COB-214.

The therapeutic index (TI) value is the TC_{50} value divided by the IC_{50} value, referring to the ratio of the drug dose that causes half metabolic activity effects divided by the dose that leads to half inhibition effects. Using undifferentiated THP-1 TC_{50} values generated in MTS, the TI values for compound COB-204 were 8.5 at the gene expression level and 13.8 at the protein expression level for 24-hour TNF-α- and IFN-γ-induced IL-6 in undifferentiated THP-1 cells; the TI values for compound COB-214 were 23.0 at the gene expression level and 29.7 at the protein expression level for 24-hour TNF-α- and IFN-γ-induced IL-6 in undifferentiated THP-1 cells. TI values can be also generated using HUVEC MTS TC_{50} values divided by undifferentiated THP-1 IC_{50} values. The TI values for compound COB-204 could not be determined in this manner since we were unable to determine the TC_{50} for COB-204 on HUVECs (Figure 3). The TI values for

compound COB-214 were 52.4 at the gene expression level and 67.9 at the protein expression level. Since COB-214 resulted in higher TI values compared to COB-204, it may have a better safety profile compared to COB-204.

We determined whether a combination of TNF-α and IFN-γ can induce IL-6 production in freshly isolated monocytes from human peripheral blood. Freshly isolated human monocytes were treated with TNF-α and IFN-γ for 24 hours. According to the IL-6 ELISA results, the combination of TNF-α and IFN-γ did not induce IL-6 production in freshly isolated monocytes from human peripheral blood. Hence, flow cytometric experiments were conducted to detect TNF-α and IFN-γ receptor expression on freshly isolated human monocytes. The results suggested that little, if any, TNF receptor 1, TNF receptor 2, and perhaps a small amount of IFN-γ receptor were present (Figure 15). However, due to the high signal of IgG2a isotype control binding to the monocytes, it was difficult to draw a firm conclusion regarding the expression of these receptors.

Chapter 6: Future Aims

6.1 *In vitro* studies of COB-204 and COB-214 metabolic activities of various cell types

Since COB-204 and COB-214 exhibit low effects on the metabolic activity of HUVECs (Figure 3 and 4), it would be of interest to investigate the effect of the compounds on the metabolic activity of additional cell types, such as fibroblasts and hepatocytes. Fibroblasts are cells that synthesize the framework of tissues, such as elastic fibers and collagens. They play important roles in connecting tissues and wound healing (Gabbiani, Ryan, & Majne, 1971). Hepatocytes are the main cells in the liver. They play important roles in metabolizing drugs and detoxification (J. Liu et al., 1998). These cell types are good models for determining the compounds' effects of potential drugs on healthy tissues and cells. Therefore, it would be of interest to determine the TC_{50} values for the compounds using these cell types.

6.2 *In vitro* studies on defining IL-6 inducers in various cell models

Different IL-6 inducers work on different cell types. Since a combination of TNF-α and IFN-γ induces IL-6 production in undifferentiated THP-1 cells and appears to have no effect on freshly isolated monocytes from human peripheral blood, it is important to determine specific inducers for human monocytes (Folkard et al., 2014). For instance, it would be insightful to treat differentiated monocytes with TNF-α and IFN-γ to determine if IL-6 would be induced in differentiated monocytes. If IL-6 production were to be increased, COB-204 and COB-214 could be used to determine the compounds' effects on inhibiting IL-6 expression. A series of these studies will help to identify additional *in*

vitro models that can create IL-6 hyper expression, so that the effect of COB-204 and COB-214 on more cell types can be studied.

6.3 Find a suitable IgG isotype control or determine the expression of TNF receptor 1, TNF receptor 2, and IFN-γ receptor on undifferentiated THP-1 cells and freshly isolated human monocytes using PCR method

It was difficult to interpret the flow cytometric analysis due to the high signal of secondary antibody and IgG2a isotype control binding to the cells (Figure 14 and 16). The monocytes were isolated using magnetic microbeads conjugated to mouse IgG2a antibodies. If the IgG2a antibody remains bound to the monocytes after isolation, the bound IgG2a can combine with the secondary antibody and give a signal in flow cytometric analysis. In addition, if the IgG2a used in the flow cytometric analysis binds to the Fc receptor on the monocytes, high negative control signal would occur. Therefore, it would be of interest to choose other antibodies to probe for the presence of the receptors. In addition, it would be insightful to investigate TNF receptor 1, TNF receptor 2, and IFN-γ receptor expression using PCR to potentially achieve a more conclusive result. Finally, note that a small orange peak occurred on the left side (Figure 17). This is potentially a small amount of dead cells and could be explored via gating analysis.

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