Modulation of IL-6 and IL-8 Expression in Ovarian Cancer Cells by a Small Organic Compound

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This thesis titled
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Abstract

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Modulation of IL-6 and IL-8 Expression in Ovarian Cancer Cells by a Small Organic Compound

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Ovarian cancer ranks 5th in cancer-related deaths amongst women with 14,270 deaths in 2014 [1]. Its high mortality rate can be attributed to lack of treatment methods and the chemoresistant nature of ovarian cancer [2]. Various studies have linked high expression levels of interleukin-6 (IL-6) and interleukin-8 (IL-8) to poor prognosis in ovarian cancer. It was hypothesized that COB-141, a small organic molecule related to methimazole, could lower these expression levels. The secretion of IL-6 and IL-8 was assayed through enzyme linked immunosorbent assays (ELISAs) and the transcriptional expression was assayed through quantitative real time polymerase chain reactions (QRT-PCR) in three different ovarian cancer cell lines (CAOV-3, SKOV-3, and OVCAR-3). These techniques were used to find the compound concentration at which 50% of protein secretion or transcription was inhibited, also known as the IC₅₀ value. All IC₅₀ values shown are ± standard deviation of the trials. For IL-6 secretion, these values were determined to be 46.5 ± 19 µM, 30.8 ± 27 µM, and 7.65 ± 0.91 µM for the CAOV-3, SKOV-3, and OVCAR-3 cell lines respectively, and for IL-8 these values were determined to be 31.9 ± 12 µM and 15.1 ± 5.8 µM for the SKOV-3 and OVCAR-3 cell lines respectively. COB-141 induced IL-8 secretion in the CAOV-3 cell line so the IC₅₀ measure is not applicable. For IL-6 transcription, the IC₅₀ values were determined to be
34.0 ± 7.1 µM and 24.1 ± 7.4 µM for the SKOV-3 and OVCAR-3 cell lines respectively. Surprisingly, COB-141 had no effect on IL-6 transcription in the CAOV-3 cell line so the IC₅₀ measure is not applicable. For IL-8 transcription, the IC₅₀ value was found to be 8.45 ± 1.0 µM for the OVCAR-3 cell line. COB-141 had no effect on IL-8 transcription in the CAOV-3 and SKOV-3 cell lines so the IC₅₀ measure is not applicable. An MTS assay was used to determine how COB-141 affects the mitochondrial activity of the cells. The level at which half of the mitochondrial activity was reduced, also known as the TC₅₀ value, was determined. All TC₅₀ values shown are ± the standard deviation of the trials. The 24 hour TC₅₀ values from the MTS assay were found to be 217 ± 18 µM, 198 ± 25 µM, and 168 ± 85 µM for the CAOV-3, SKOV-3, and OVCAR-3 cell lines respectively. Cell proliferation was also measured using a BrdU assay and the level at which the compound reduced cell proliferation by 50%, also known as a TC₅₀ value, was determined. These 24 hour TC₅₀ values were found to be 731 ± 12 µM, 454 ± 59 µM, and 227 ± 120 µM for the CAOV-3, SKOV-3, and OVCAR-3 cell lines respectively.

A high performance liquid chromatography (HPLC) method to detect COB-141 in acetonitrile was also investigated. It was found that COB-141 could be detected at concentrations as low as 6.25 µM which is the lowest concentration that was used in the study. To summarize, COB-141 was able to modulate IL-6 and IL-8 expression in three ovarian cancer cells lines, and this warrants further investigation on the effects of COB-141 in vivo.
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1. Chapter 1: Introduction

1.1. Ovarian Cancer Statistics

In 2014, it was estimated that there were 21,980 new cases of ovarian cancer and 14,270 deaths resulting from ovarian cancer in the US [1]. Ovarian cancer ranks 5\textsuperscript{th} overall in cancer-related deaths amongst women (5\% of all cancer deaths in women) [1]. This is largely due to the lack of treatment methods and therapeutics for this disease, and the fact that many ovarian cancers develop chemoresistance after initial platinum-based therapies [2]. Ovarian cancer is most often seen in women 55-64 years of age with the median age at diagnosis being 63. The five year survival rate for patients with ovarian cancer is 44.2\% and this varies significantly depending on the stage of the diagnosis [3]. Stage I diagnosis of the localized disease results in a 90\% survival rate, but only 25\% of cancers are detected at this stage due to the absence of warning signs [4]. Common symptoms include abdominal pain and swelling which are associated with more common diseases and thus often result in misdiagnosis [4].

The high mortality rate and chemoresistant nature of ovarian cancer creates a large need for further research into treatment and therapeutics for ovarian cancer. There are studies that have linked high expression levels of IL-6 to poor prognosis in ovarian cancer and it is thought that IL-6 is a major promoter of tumor growth and progression [5]. Likewise, expression of IL-8 has been shown to correlate with metastatic potential, tumorigenicity, and angiogenesis \textit{in vivo} which is consistent with \textit{in vitro} studies which further supports IL-8 playing a large role in enhancing the tumor microenvironment [6].

\footnote{\ A portion of this thesis will likely be submitted for publication as a paper co-authored by Douglas Goetz, Kelly McCall, Stephen Bergmeier, Joshua Greenlee, Danielle Whelan, and Ian Armstrong}
The purpose of this study is to investigate the effects of a small organic compound on the expression of IL-6 and IL-8 in ovarian cancer cell lines. This compound is related to methimazole and has been modified to increase its efficacy.

1.2. Classifying Ovarian Cancer

Epithelial ovarian cancer (EOC) is the most common type of ovarian cancer and it is thought that these cancers arise through DNA mutations [7]. Some less common forms of ovarian cancer include borderline ovarian tumors, germ cell ovarian cancers, and stromal ovarian cancers [7]. There are four stages of ovarian cancer and they are as follows [8]:

- Stage I: Cancer limited to the ovaries
- Stage II: Cancer spread to the uterus or other organs in the pelvis
- Stage III: Cancer spread to the lymph nodes or lining tissues of the abdomen
- Stage IV: Cancer that has metastasized to distant sites [8]

As stated above, the five year survival rate is relatively good when ovarian cancer is found at an early stage, but the majority of epithelial ovarian cancers are found at stage 3 or later which means that the cancer has spread beyond the pelvis to the lymph nodes [9]. This is mainly due to lack of symptoms in early stage EOC [9].

As stated above, there are two other types of ovarian cancer that are not as prevalent or deadly as most epithelial ovarian cancers [10]. One type arises from germ cell tumors and the other from stromal tumors [10]. Most germ cell tumors are benign but some are cancerous and life threatening [10]. Ninety percent of patients with ovarian cancer arising from germ cell tumors can be cured [10]. Ovarian stromal tumors are quite
rare and develop from connective tissue cells that hold the ovary together or the cells that produce estrogen and progesterone [10]. Roughly 70 percent of stromal tumors are found as a stage I cancer with a five year survival rate of 95 percent [10][11].

1.3. Current Treatment Methods

Ovarian cancer is currently treated using surgery and/or chemotherapy [12]. For most cases, systemic chemotherapeutic drugs are used which are given either orally or intravenously (IV). The other method of chemotherapy is known as intraperitoneal (IP) chemotherapy where a drug is injected directly into the abdomen using a catheter [13]. Chemotherapy that is administered using the IP method can be considered systemic as well because the drugs are eventually absorbed into the bloodstream. For epithelial ovarian cancer, a combination of 2 or more drugs administered IV every 3 to 4 weeks is standard. This is typically a platinum compound such as cisplatin or carboplatin, coupled with a taxane such as paclitaxel (Taxol®) or docetaxel (Taxotere®) [13]. Three to six cycles of chemotherapy are standard. If the chemotherapy is effective and the cancer stays in remission for a period of 6-12 months, additional cycles of the same chemotherapy may be used [13]. Ovarian cancers often become chemoresistant [2] which is a reason why new methods of treatment are needed to raise the low survival rate that is associated with ovarian cancer.

Current methods of chemotherapy have temporary side effects that include nausea and vomiting, loss of hair, hand and foot rashes, mouth sores, and loss of appetite [13]. Chemotherapy can also damage blood-producing cells in the bone marrow, leading to patients with low blood counts who have an increased risk of infection, increased
bleeding or bruising in response to cuts, and fatigue [13]. These side effects are short term and will usually disappear when treatment stops. That said, some chemotherapy drugs may have permanent side effects. Cisplatin can cause permanent kidney damage, and cisplatin and taxanes can also cause nerve damage. Permanent bone marrow damage can also occur, which can later cause a bone marrow cancer [13].

Intraperitoneal chemotherapy (IP) involves injecting cisplatin and paclitaxel directly into the abdominal cavity via a catheter in addition to administering paclitaxel IV [13]. Administering chemotherapy this way concentrates the dose to the cancer cells in the abdominal cavity, and the bloodstream allows the drugs to reach outside of the cavity as well. Studies have also shown that women live longer on average when given IP treatment, but it is a more toxic treatment [13]. For this reason, women who undergo IP treatment must have healthy kidney function and be in overall good health for the doctor to consider IP treatment [13]. This is another reason that new therapeutics for ovarian cancer are needed.

1.4. IL-6

IL-6 is a pleotropic cytokine that has biological activities in pro-inflammatory inflammation, oncogenesis, regulation of the immune system, and hematopoiesis [14]. IL-6 is a glycosylated protein with a four-helix bundle structure and is 21-28 kDa in size [15]. Its functions have been determined by studying the receptor for IL-6 (IL-6R) and gp130 which is a common signal transducer for related cytokines [14]. It is mainly regarded as a pro-inflammatory cytokine, but also has many anti-inflammatory activities [15]. There is an IL-6 family of cytokines that includes IL-6, IL-11, ciliary inhibitory
factor (CNTF), cardiotropin-1 (CT-1), IL-27, IL-31, cardiotrophin-like related cytokine and stimulating neurotrophin-1/B-cell stimulating factor 3 (NNT-1), oncostatin M (OSM), and leukemia inhibitory factor (LIF) [15]. These are grouped together because, with the exception of IL-11, they all share a common receptor and signal transducer subunit [15]. Evidence suggests that IL-6 plays a part in the innate to acquired immunity transition, and there have been several observations of IL-6 playing a role in metabolic control [15].

1.5. IL-6 Signaling

The first step in the classical IL-6 signaling cascade is the binding of IL-6 to the IL-6 receptor molecule, IL-6R. This IL-6/IL-6R complex associates with the gp130 protein and induces its dimerization [16], which leads to the activation of additional pathways [17]. The first pathway results from the IL-6/IL-6R ligation of gp130 leading to the induction of Janus tyrosine kinases that include (JAK1, JAK2, and TYK2) [18] [17]. The JAK residues then serve as docking sites for downstream molecules. The better known molecules that engage in this docking include STAT1, STAT2, STAT3, SHP-2, and PI3K [19], which induce transcriptional activation of cell differentiation, inflammation, or cell survival depending on the context [17]. The rat sarcoma (Ras) pathway is also activated by the activation of the Janus kinases which helps in regulating cell proliferation, apoptosis, and differentiation [17]. The last pathway activated by activation of the Janus kinases is the PI3K pathway which plays a role in cell proliferation by acting on multiple substrates [17]. In certain cases, cells lack the membrane bound IL-6 receptor, yet these pathways still exist in these cells due to the
trans signaling mechanism [20]. In IL-6 trans signaling, IL-6 associates with a soluble IL-6 receptor, sIL-6R, which then associates with the gp130 proteins and activates the pathways described above [20]. Figure 1 below taken from *Clinical significance of interleukin (IL)-6 in cancer metastasis to bone: potential of anti-IL-6 therapies* highlights the classical and trans signaling pathways of IL-6 [21].

![Classical and trans IL-6 signaling](image)

*Figure 1. Classical and trans IL-6 signaling*
1.6. IL-6 as a Target in Ovarian Cancer

Recently, there has been an increasing focus on the role that inflammatory cytokines play in ovarian cancer [22] and specifically how they affect the tumor microenvironment. There is evidence that IL-6 plays a significant role in promoting tumor initiation, growth, and metastasis [18]. Because of this, IL-6 has emerged as an important therapeutic target in treating ovarian cancer [17]. Results from a tissue array study from 221 patients with ovarian cancer was completed and the intensity of IL-6 staining directly correlated with poor patient prognosis [23]. Research on IL-6 has made it clear that its effects extend beyond direct effects on cancer cell growth and extend into effects on the microenvironment by suppressing aspects of the adaptive immune system [18]. A recent study on the tumor microenvironment indicates that IL-6 is a major factor controlling tumor growth by acting on various stromal constituents [18], and it has been shown to activate STAT3 in its signaling cascade which is well known to support tumor cell survival and proliferation [24]. IL-6R and gp130 have also been shown to play an important role in cancer and inflammatory responses [25]. Thus it may be possible that inhibition of IL-6, and perhaps other cytokines as well, may alter the tumor microenvironment enough to give it growth-inhibiting characteristics rather than growth-stimulating.

Treatment of platinum-resistant ovarian cancer with an anti-IL-6 antibody, siltuximab, has yielded measurable clinical effects [23]. This treatment inhibited IL-6 signaling and reduced both cytokine and chemokine production in ovarian cancer cells [23]. It was also found that siltuximab was able to inhibit IL-6 signaling and tumor
growth in xenografts from patients with ovarian cancer [23]. In patients treated for 6 months with siltuximab, there was a decline in plasma levels of IL-6-regulated VEGF, CXCL12, CCL2, and IL-8 [23]. It is also noteworthy to point out that the gene expression levels of the factors that were reduced by siltuximab correlated with high IL-6 gene expression in microarray analysis of biopsies from ovarian cancer [23]. While these results are encouraging, a recent clinical study targeting castration-resistant prostate cancer patients who were treated with siltuximab had issues regarding what the authors referred to as “the formation of antibody/analyte complexes” between siltuximab and IL-6 which actually led to high expression levels of IL-6 [26]. It could not be determined if this was a result of these complexes or from increased secretion due to inhibition of the IL-6 signaling pathway [26], but in either case this result shows that significant problems can arise with the use of biologics as therapeutics.

JAK, a key kinase in the IL-6 signaling pathway [27], has also been thought to be a suitable target for cancer prevention. Ruxolitinib is a small organic molecule that inhibits this kinase and is currently used in the treatment of myelofibrosis [28]. With this being said, a JAK inhibitor was developed (AZD 1480) for patients with solid tumors but was discontinued due to limiting toxicities and lack of clinical activity [19] [29]. Regardless of the approach that is used, it is known that cancers can often develop resistance to a given molecular therapeutic [30].

These observations show that IL-6 and its signaling pathway intermediates are an important therapeutic target for cancer, including various ovarian cancers, and motivates
further identification and development of compounds that modulate IL-6 expression and activity.

1.7. IL-8

IL-8 belongs to a family of cytokines that are structurally related with similarities to platelet factor 4 [31]. IL-8 was identified in 1987 as a new type of cytokine involved in neutrophil activation [31]. Today, its 3D structure is known as well as many of its biological functions and the properties of its receptors [31]. IL-8 gene transcription encodes for a processed protein that is 72 amino acids in macrophages and monocytes, and 77 amino acids in immune cells [6]. It is a member of the CXC family of chemokines and its primary function is to mediate the migration and activation of neutrophils to tissue from circulating blood [32]. IL-8 is a proinflammatory cytokine whose expression is regulated by a number of different stimuli. These stimuli include chemical stresses, environmental stresses, inflammatory signals, and various steroid hormones that include androgens, estrogen, and dexamethasone [6]. In vivo, IL-8 has been shown to produce large neutrophil accumulation at the injection site [31].

1.8. IL-8 Signaling

IL-8 signaling is mediated by two cell surface G-protein receptors (CXCR1 and CXCR2) when IL-8 binds [6]. IL-8 induces a conformational change, and a signal is transmitted across the membrane which promotes coupling to heterotrimeric G proteins [6]. This is done by exposing different epitopes on the carboxy terminal tail and intracellular loops of the receptor [6]. CXCR1 and CXCR2 have a diverse range of signaling pathways that can be attributed to the versatility of the Gβγ subunits of G
protein coupled receptors [6]. Figure 2 below, taken from *The Interleukin-8 Pathway in Cancer*, illustrates the main known signaling pathways for which IL-8 is involved [6]. These include activation of phosphatidyl-inositol-3-kinase (PI3-K) or phospholipase C (PLC) which promote various signaling cascades [6]. Pictured are the Akt, PKC, calcium mobilization, and MAPK signaling cascades [6]. These different cascades are known to regulate various transcription factors and promote translation of proteins that are also pictured in Figure 2 [6]. In addition to these responses, IL-8 signaling can activate RhoGTPase or other tyrosine kinases which regulate the interaction of elements of the cell cytoskeleton with the surrounding extracellular environment [6].
IL-8 is also thought to play a large role in regulating the tumor microenvironment. It is known that IL-8 secretion from cancer cells enhances cancer cell survival by increasing cell proliferation via autocrine signaling [6]. IL-8 also promotes angiogenesis and neutrophil recruitment to the tumor site, and it can induce tumor-associated macrophages to secrete various growth factors that lead to increased cell proliferation and invasion of cancer cells in the tissue surrounding the tumor [6]. Therefore, inhibiting the expression of IL-8 may reduce tumor growth. Figure 3 below taken from The Interleukin-
*Pathway in Cancer* summarizes its role and alludes to the profound effects that IL-8 has in regulating tumor growth [6].

1.9. IL-8 as a Target in Ovarian Cancer

Increased IL-8 signaling has been shown in cancer cells, neutrophils, and tumor associated macrophages, which suggests that IL-8 plays a large role in regulating the tumor microenvironment [6]. IL-8 expression in the tumor itself is a regulator of neutrophil recruitment in the tumor microenvironment. Expression of IL-8 correlates with metastatic potential, tumorigenicity, and angiogenesis in vivo which is consistent with in vitro studies which further supports IL-8 playing a large role in support of the tumor microenvironment [6]. The potential also exists that targeting IL-8 may sensitize cancer cells to known chemotherapies and other treatments. This may be a result of the fact that numerous chemotherapies such as paclitaxel, decarbazine, and adriamycin induce IL-8 expression in cancer cells [6].

Another study investigated the effects of IL-8 on two cell lines, one that did not express IL-8 and one that overexpressed IL-8. The non-expressing cell line, A2780, was transfected with a gene coding for IL-8, and the increase in IL-8 expression led to proliferation, growth, and angiogenic potential. The overexpressing cell line, SKOV-3, was transfected with a plasmid that encoded for antisense IL-8, thus inhibiting IL-8 expression and decreasing its effects in the A2780 cell line and also caused apoptosis which was seen by an increase in caspase-3-activation [33].

Contrary to the previous studies that are mentioned, a study that focused on IL-8 induction by paclitaxel showed that increasing IL-8 expression can reduce tumorigenicity in ovarian cancer [34]. This was done by injecting mice with both IL-8 expressing human ovarian cancer cell lines and human cell lines that did not express IL-8. Tumor formation
in the IL-8 expressing cell lines either did not grow at all, or grew at a very slow rate. In contrast, cell lines that did not express IL-8 formed tumors at a relatively fast rate [34]. These studies motivate investigation into the effect that a particular therapeutic has on IL-8 secretion and transcription in ovarian cancer cells.

1.10. COB-141

Methimazole has been used clinically in the treatment of Grave’s disease, and it was found in a previous study that a phenyl derivative of methimazole, phenylmethimazole, displayed increase activity [35]. COB-141 is related to phenylmethimazole and has previously been shown to reduce levels of IL-6 in triple negative breast cancer (TNBC) cells [36]. It was identified as a highly active compound in this regard from an initial screening in TNBC cells [36], and thus it was chosen as the starting point for our study on reducing the basal expression of IL-6 in ovarian cancer cells. The structure of COB-141 and methimazole are shown in Figure 4 below. This compound was synthesized in Dr. Stephen Bergmeier’s lab at Ohio University.
A previous study found that in the MDA-MB-231 TNBC cell line, COB-141 had an IC$_{50}$ for IL-6 transcription of 8.3 µM and an IC$_{50}$ of 43µM for IL-6 secretion [36]. Recently, myself in collaboration with other Goetz lab students have studied two other TNBC cell lines (HS578T and MDA-MB-468) and found that IL-6 transcription and secretion were both inhibited by COB-141. This result led us to suspect that COB-141 might have an inhibitory effect on IL-6 in ovarian cancer cells as well.

1.11. IL-6 and IL-8 Modulation via COB-141

There are several ways that COB-141 might modulate IL-6 and IL-8 activity in the ovarian cancer cell lines, including the following. It might act as a transcription factor or modulate the activity of transcription factors that control the level of IL-6 or IL-8 mRNA that is transcribed. It might modulate the translation of IL-6 or IL-8 mRNA into protein. It might prevent secretion of folded IL-6 or IL-8 protein from the cell. Figure 5
shows a simplified view of IL-6 being transcribed, translated, and secreted from the cell. The three blue arrows represent the steps that could potentially be modulated by COB-141 activity, and thus either inhibit or enhance these actions. Figure 5 shows the simplified view for IL-6, but this is true for IL-8 as well.

Figure 5. Simplified view of IL-6 transcription, translation, and secretion in a cell.

Based on previous work by John O’Brien [36], we suspect that COB-141 acts by modulating transcription of the IL-6 or IL-8 gene. This means that it is acting as a
transcription factor, or acts on a transcription factor of IL-6 or IL-8. Figure 6 below taken from NF-κB, chemokine gene transcription and tumour growth shows transcription factors associated with IL-6 and IL-8 transcription [37].

Figure 6. Transcription factors associated with IL-6 and IL-8 transcription. Please note that CXCL8 is an alternate name for IL-8. Figure adapted from A. Richmond, “NF-κB, chemokine gene transcription and tumour growth,” Nat. Rev. Immunol., vol. 2, no. 9, pp. 664–674, Sep. 2002. Used with permission.

1.12. Pharmacokinetics

A future aim of this project involves investigating the pharmacokinetics of COB-141 in a murine model. Pharmacokinetics can be defined as the mathematical description of the rates of adsorption, distribution, metabolism, and excretion of a compound in the body [38][39]. An important parameter in pharmacokinetics is the area under the curve
(AUC) which represents the compound’s total exposure over a given time. The curve from which this is found is the concentration of compound in the bloodstream plotted over time.

Other factors are also taken into account when determining the pharmacokinetics of a compound such as whether the model should be linear or nonlinear, the variability between patients, variability in a single patient, and interactions between other compounds or drugs that are administered to the patient [39]. Knowing the pharmacokinetics of a compound helps in designing its applications and saves time during in vivo testing.

Most drugs are given in fixed doses at specified intervals to maximize efficacy. The dosing regimen is largely dependent on the half-life of the compound which can be altered by modifying the structure of the compound, as well as administering enzyme inhibitors to prolong the effects of the compound [38]. Identifying the factors related to drug metabolism and pharmacokinetics (DMPK) early in the process of drug discovery is important because it saves costs and time later in the process of bringing the compound to market [38].

1.13. Development of an Assay to Detect COB-141 in Mouse Serum

Our lab sought to develop an assay that allows quantification of COB-141 in murine serum. Dr. Stephen Bergmeier has a reverse phase HPLC system in his lab that was used for testing. Similar equipment has been used to determine the pharmacokinetic parameters of an everninomicin, a new compound that has good activity against bacterial strains that are gram-positive [40].
HPLC is a form of column chromatography that operates at pressures up to 400 atm. These high pressures allow for smaller particle size in the column, which increases the overall surface area, allowing for more interactions between the compounds of interest and the stationary phase. This in turn allows for a better separation [41].

The two common forms of HPLC are normal phase HPLC and reverse phase HPLC. Normal phase HPLC utilizes a polar stationary phase and a non-polar mobile phase. Polar compounds elute slower than non-polar compounds due to the polar-polar interactions of the compounds and the stationary phase [41].

Reverse phase HPLC is more common than normal phase HPLC and it involves the use of a non-polar stationary phase and a polar mobile phase. This causes non-polar compounds to elute more slowly than polar compounds [41]. Below is a simplified schematic of an HPLC system [41].
Sample injection is an automated process, and retention times are the point of interest in compound detection. The retention time is the time from which the compound is injected in the column to the time that it is detected [41]. A number of different factors affect the retention time of a compound. These include the pressure in the system, the material and size of the particles that make up the stationary phase, the composition of the mobile phase, and the temperature of the column [41]. These factors need to be controlled when using retention times to identify compounds [41]. An attempt was made to develop an HPLC assay that will allow quantification of COB-141 in murine serum.

Figure 7. Schematic of HPLC system.
2. Chapter 2: Hypothesis and Specific Aims

2.1. Hypothesis

It was hypothesized that COB-141 will have an inhibitory effect on transcription and secretion of IL-6 and IL-8 in ovarian cancer cells with an IC$_{50}$ between 10 µM and 50 µM. Further, it was hypothesized that this inhibitory effect is caused by the compound disrupting signaling pathways, and not from cytotoxicity.

2.2. Specific Aims

Our first aim was to determine if COB-141 has an inhibitory effect on IL-6 and IL-8 transcription and/or secretion in various ovarian cancer cell lines. IL-6 and IL-8 enzyme linked immunosorbent assays (ELISAs) were used for detecting IL-6 and IL-8 levels in three ovarian cancer cell lines (CAOV-3, SKOV-3, and OVCAR-3). The CAOV-3 cell line is a human epithelial ovarian cancer cell line that was derived from ovarian tissue from a 54 year old female with adenocarcinoma [42]. The SKOV-3 cell line is also a human epithelial ovarian cancer cell line that was derived from ovarian ascites of a 64 year old female with adenocarcinoma [43]. Lastly, the OVCAR-3 cell line is a human epithelial ovarian cancer cell line derived from the ovaries of a 60 year old female with adenocarcinoma [44].

Our second aim was to lay the groundwork for testing the effect of COB-141 in vivo. As a first measure towards achieving this goal, an attempt was made to generate an assay to quantify the level of COB-141 in murine serum. The assay is based on HPLC. Detecting COB-141 via HPLC was the first step in determining the pharmacokinetic parameters which will help us understand how the compound works, and will also serve
as a standard way for our lab to measure the pharmacokinetic parameters of other compounds that we work with.
3. Chapter 3: Materials and Methods

3.1. Culture of CAOV-3, SKOV-3, and OVCAR3 Cell Lines

All cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and they were cultured in their respective mediums which were also obtained from ATCC. CAOV-3 cells were cultured in ATCC-formulated Dulbecco’s Modified Eagle’s Medium (30-2002, ATCC) and 10% fetal bovine serum (FBS) [42]. SKOV-3 cells were cultured in ATCC-formulated McCoy’s 5a Medium Modified (30-2007, ATCC) and 10% FBS [43]. OVCAR-3 cells were cultured in ATCC-formulated RPMI-1640 Medium (30-2001, ATCC) and 20% FBS. [44]. All cell lines had 5 mL of penicillin streptomycin added (17-602E, Lonza, Allendale, NJ) and all FBS was non-heat inactivated (30-2020, ATCC). Trypsin-Versene Mixture (17-161E, Lonza, Allendale, NJ) was used to remove the cells from tissue culture plate surfaces for subculturing.

3.2. Cytokine Arrays

Human Inflammatory Cytokines Multi-Analyte ELISAArray™ Kits (336161, MEH-004A, QIAGEN, Valencia, CA) were utilized to test the effect of COB-141 on secretion of IL-6 and other pro-inflammatory and anti-inflammatory cytokines including IL1α, IL1β, IL2, IL4, IL8, IL10, IL12, IL17A, IFN-γ, TNF-α, and GM-CSF. The pro-inflammatory cytokines from this list include IL1α [45], IL2 [46], IL-8 [6], IL-12 [47], IL17A [48], and IFN-γ [49], GM-CSF[50], IL1β, and TNF-α [51]. The anti-inflammatory cytokines include IL-4, and IL-10 [51]. IL-6 can be classified as either a pro or anti-inflammatory cytokine [51].
The cells grown in a 96 well plate were treated at approximately 80% confluency with 100 µL of media containing various concentrations of COB-141, all at a concentration of 0.25% Dimethyl Sulfoxide (DMSO) (D2650, Sigma, St. Louis, MO). DMSO was the drug vehicle. COB-141 was stored at a concentration of 200 mM in 100% DMSO, and was diluted with the appropriate cell culture medium. DMSO was then added to control cells so that all treatments had a concentration of 0.25% DMSO by volume. A treatment of 0.25% DMSO, with no COB-141, was used as a vehicle control.

Before applying the treatments, the old media was removed and the wells were washed three times with 200 µL of “wash media”, which is comprised of the appropriate growth media without FBS added. This washing is done to remove any dead cells or unwanted cellular byproducts in the wells that could skew the data.

The concepts for the cytokine array is the same as a standard ELISA, and the protocol used can be found in the Multi-Analyte ELISArray Handbook [52].

3.3. IL-6 ELISA Using Cell Culture Supernatant

An enzyme-linked immunosorbent assay (ELISA) is a test that uses antibodies and relative absorbance values to quantify amounts of proteins. The ELISA kit that was used is a Human IL-6 ELISA obtained from BD Biosciences (555220, BD Biosciences, San Diego, CA), along with reagent set B (550534, BD Biosciences, San Jose, CA). CAOV-3, SKOV-3, and OVCAR-3 cells were cultured in 96 well tissue culture plates (3599, Corning Incorporated, Corning, NY). The cells were treated at approximately 80% confluency with various concentrations of COB-141 in cell growth medium, all at a concentration of 0.25% DMSO. DMSO was the drug vehicle. COB-141 was stored at a
concentration of 200 mM in 100% DMSO, and was diluted with the appropriate cell culture medium. DMSO was added to the media prior to addition to the cells so that all treatments had a concentration of 0.25% DMSO by volume. A treatment of 0.25% DMSO, with no COB-141, was used as a vehicle control.

Before applying the treatments, the old media was removed and the wells were washed three times with 200 µL of “wash media”, which is comprised of the appropriate growth media without FBS added. This washing is done to remove any dead cells or unwanted cellular byproducts in the wells that could skew the data. For ELISA assays, cells were retreated after 6 hours to account for any IL-6 mRNA that may have already been produced prior to treatment. This retreatment consisted of removing the previous treatment, washing three times with 200 µL of wash media, and adding 100 µL of the appropriate treatment. Supernatant was collected 24 hours after the initial treatment and was either stored at -80°C or used immediately.

To bring the absorbance values of the ELISA within the range of the standard curve, a dilution factor was used for each cell line. Cell supernatant was diluted with assay diluent that is provided in reagent set B (550534, BD Biosciences, San Jose, CA) of the ELISA kit. These dilution factors were 1:30, 1:10, and 1:2 for the CAOV-3, SKOV-3, and OVCAR-3 cell lines respectively. Maxisorp non-sterile 96 well plates (442404, Thermo Scientific, Waltham, MA) were used for the ELISA assays and the plates were read at wavelengths of 450 nm and 570 nm as specified in the protocol [53].
3.4. IL-8 ELISA Using Protein from Cell Culture Supernatant

It was found that the three cell lines expressed IL-8 as well, so the effect of COB-141 on IL-8 secretion was tested via an ELISA. The ELISA kit that was used is a Human IL-8 ELISA obtained from BD Biosciences (555244, BD Biosciences, San Diego, CA), along with reagent set B (550534, BD Biosciences, San Jose, CA). CAOV-3, SKOV-3, and OVCAR-3 cells were cultured in 96 well tissue culture plates (3599, Corning Incorporated). The cells were treated at approximately 80% confluency with various concentrations of COB-141 in cell growth medium, all with a concentration of 0.25% DMSO. DMSO was the drug vehicle. COB-141 was stored at a concentration of 200 mM in 100% DMSO, and was diluted with the appropriate cell culture medium. DMSO was added to the media prior to addition to the cells so that all treatments had a concentration of 0.25% DMSO by volume. A treatment of 0.25% DMSO, with no COB-141, was used as a vehicle control.

Before applying the treatments, the old media was removed and the wells were washed three times with 200 µL of “wash media”, which is comprised of the appropriate growth media without FBS added. This washing is done to remove any dead cells or unwanted cellular byproducts in the wells that could skew the data. For ELISA assays, cells were retreated after 6 hours to account for any IL-8 mRNA that may have already been produced prior to treatment. This retreatment consisted of removing the previous treatment, washing three times with 200 µL of wash media, and adding 100 µL of the appropriate treatment. Supernatant was collected 24 hours after the initial treatment and was either stored at -80°C or used immediately.
To bring the absorbance values of the ELISA within the range of the standard curve, a dilution factor was used for each cell line. Cell supernatant was diluted with assay diluent that is provided in reagent set B (550534, BD Biosciences, San Jose, CA) of the ELISA kit. These dilution factors were 1:30, 1:10, and 1:2 for the CAOV-3, SKOV-3, and OVCAR-3 cell lines respectively. Maxisorp non-sterile 96 well plates (442404, Thermo Scientific, Waltham, MA) were used for the ELISA assays and the plates were read at wavelengths of 450 nm and 570 nm as specified in the protocol [54].

3.5. Cell Proliferation Assay using MTS

The effect of COB-141 on metabolic activity was also tested using a CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay (G5421, Promega, Madison, WI). The cells were treated at approximately 80% confluency with various concentrations of COB-141 in cell growth medium, all at a concentration of 0.25% DMSO. DMSO was the drug vehicle. COB-141 was stored at a concentration of 200 mM in 100% DMSO, and was diluted with the appropriate cell culture medium. DMSO was added to the media prior to addition to the cells so that all treatments had a concentration of 0.25% DMSO by volume. A treatment of 0.25% DMSO, with no COB-141, was used as a vehicle control. Before applying the treatments, the old media was removed and the wells were washed three times with 200 µL of “wash media”, which is comprised of the appropriate growth media without FBS added. This washing is done to remove any dead cells or unwanted cellular byproducts in the wells that could skew the data. For the MTS assay, 100 µL COB-141 in medium was added and the cells were allowed to incubate for 24, 48, or 72 hours. After the incubation, 20 µL of MTS solution was added. The cells were
allowed to incubate for 1 to 4 hours and the absorbance of supernatant in each well was determined by reading the plate at 490nm [55]. The absorbance value was then adjusted by subtracting the absorbance value of wells with media and MTS, but no cells.

### 3.6. QRT-PCR (RNA Extraction, cDNA Synthesis, and Quantitative Real Time Polymerase Chain Reaction) for IL-6 and IL-8

Transcriptional effects of COB-141 were studied using Quantitative Real Time Polymerase Chain Reaction assays (QRT-PCR). The cells were treated at approximately 80% confluency with various concentrations of COB-141 in cell growth medium, all at a concentration of 0.25% DMSO. DMSO was the drug vehicle. COB-141 was stored at a concentration of 200 mM in 100% DMSO, and was diluted with the appropriate cell culture medium. DMSO was added to the media prior to addition to the cells so that all treatments had a concentration of 0.25% DMSO by volume. A treatment of 0.25% DMSO, with no COB-141, was used as a vehicle control. Before applying the treatments, the old media was removed and the wells were washed three times with 1 mL of “wash media”, which is comprised of the appropriate growth media without FBS added. This washing is done to remove any dead cells or unwanted cellular byproducts in the wells that could skew the data.

The CAOV-3, SKOV-3, and OVCAR-3 cell lines were plated in Falcon 6 well plates (353046, Corning, NY) prior to extracting RNA. RNA was extracted from the ovarian cancer cell lines 24 hours after treatment with COB-141 using an RNeasy Mini Kit from Qiagen (74106, QIAGEN, Valencia, CA). QIAshredder columns was used to lyse the cells (79656, QIAGEN, Valencia, CA). RNase free DNase sets were used in the
RNA extraction process (79254, QIAGEN, Valencia, CA). The RNaseasy® Mini Handbook protocol for “Purification of Total RNA from Animal Cells using Spin Technology” was used. A noteworthy deviation from the protocol occurs before step 3a. The 6 well plates were washed three times with approximately 1mL PBS without calcium or magnesium (17-516F, Lonza, Allendale, NJ), and then 350 µL of buffer RLT (79216, QIAGEN, Valencia, CA) was added to each well. The wells were then scraped using a cell scraper (08-100-240, Fisher Scientific, Waltham, MA), and the contents were transferred to a QIAshredder column as stated in step 3a of the protocol. All optional steps in the protocol were performed, and 30 µL of RNase free water was used to elute the RNA. A NanoVue Plus Spectrophotometer (28-9569-62, GE Healthcare Life Sciences, Marlborough, MA) was used to quantify the RNA which was then used for cDNA synthesis. 1µg of RNA was the standard amount used for cDNA synthesis, but some trials used as low as 0.6 µg of RNA because of lower yields.

CDNA was synthesized from the extracted RNA using a High Capacity cDNA Reverse Transcription Kit with RNase inhibitor (4374966, Applied Biosystems, Grand Island, NY).

Taqman gene expression assays (4331182, Life Technologies, Grand Island, NY), Taqman gene expression master mix (4369016, Life Technologies, Grand Island, NY) and the StepOnePlus™ real-time PCR system (4376600, Life Technologies, Grand Island, NY) were used for QRT-PCR. HPRT was used as the housekeeping control gene (4448490, Life Technologies, Grand Island, NY). The delta C_T method was used to find the IC50 value relative to a 0.25% DMSO control.
3.7. **BrdU Assay to Measure Cell Viability**

Cell proliferation was measured using a BrdU Cell Proliferation ELISA Kit (ab126556, abcam, Cambridge, MA). The cells were treated at approximately 80% confluency with various concentrations of COB-141 in cell growth medium at a concentration of 0.25% DMSO and 20 µL of BrdU solution. DMSO was the drug vehicle. COB-141 was stored at a concentration of 200 mM in 100% DMSO, and was diluted with the appropriate cell culture medium. DMSO was added to the media prior to addition to the cells so that all treatments had a concentration of 0.25% DMSO by volume. A treatment of 0.25% DMSO, with no COB-141, was used as a vehicle control. Before applying the treatments, the old media was removed and the wells were washed three times with 1 mL of “wash media”, which is comprised of the appropriate growth media without FBS added. This washing is done to remove any dead cells or unwanted cellular byproducts in the wells that could skew the data. The BrdU ELISA was performed as specified in the protocol [56].

3.8. **Groundwork for in vivo Testing**

A high performance liquid chromatography (HPLC) system was used to lay the groundwork for in vivo testing. A mobile phase of 80% degassed acetonitrile (A998-1, Fisher Scientific, Waltham, MA) and 20% degassed water was used in an isocratic method to find the elution time of COB-141. The system was equipped with a Prominence DGU-20A5 degasser, a Shimadzu LC-10AT liquid chromatograph, a Shimadzu SPD-10A UV-VIS Detector, and a Shimadzu ELSD-LT detector.
4. Chapter 4: Results

4.1. Cytokine Array Results

Cytokine arrays were used to investigate the effects of COB-141 on important pro-inflammatory and anti-inflammatory cytokines. This test was similar to an ELISA in the sense that absorbance values correlate with cytokine expression, although no standards are used. This makes the test semi-quantitative because the values of cytokine expression are not known, but can be visualized relative to control values and various treatments. For this reason, it was used as a screening technique to find what cytokines we should investigate further. It was found that IL-8 was highly expressed in all three cell lines in addition to IL-6. Granulocyte macrophage colony stimulating factor (GM-CSF) was also expressed in the cell lines, but the relative amounts were not high enough to warrant further testing via ELISA and QRT-PCR. Treatments of COB-141 ranged from 12.5 µM to 100 µM for the CAOV-3 cell line and from 6.25 µM to 100 µM in the SKOV-3 and OVCAR-3 cell lines.

Figures 8-10 below show the results of the cytokine array for each cell line. The y-axis is the absorbance of the well measured at 450 nm and the x-axis is the concentration of COB-141 that was used as treatment.

Figure 8 shows the secretion levels of a panel of cytokines in the CAOV-3 cell line. This cell line had high IL-6 and IL-8 secretion values relative to the other cytokines, and COB-141 appeared to have an inhibitory effect on IL-6 secretion. COB-141 appeared to have limited to no effect on IL-8 secretion. Figure 8 also shows that the CAOV-3 cell line has low secretion levels of GM-CSF, and COB-141 inhibited GM-CSF secretion.
Figure 8. CAOV-3 cytokine secretion as a function of COB-141 concentration. This figure shows that the CAOV-3 cell line expresses high levels of IL-8 and IL-6, with moderate expression levels of GM-CSF. Cells were treated with COB-141 for 24 hours which decreased IL-6 and GM-CSF secretion, but had limited to no effect on IL-8 secretion. The data presented is representative of one trial (n=1).

Figure 9 shows the secretion levels of a panel of cytokines in the SKOV-3 cell line. This cell line had high IL-6 and IL-8 secretion values relative to the other cytokines, and COB-141 appeared to have an inhibitory effect on IL-6 secretion. COB-141 did not appear to have any effect on IL-8 secretion. Figure 9 also shows that the SKOV-3 cell line has low secretion levels of GM-CSF, and COB-141 inhibited GM-CSF secretion.
Figure 9. SKOV-3 cytokine secretion as a function of COB-141 concentration. This figure shows that the SKOV-3 cell line expresses high levels of IL-8 and IL-6, with moderate expression levels of GM-CSF. Cells were treated with COB-141 for 24 hours which decreased IL-6 and GM-CSF secretion, but had no effect on IL-8 secretion. The data presented is representative of one trial (n=1).

Figure 10 shows the secretion levels of a panel of cytokines in the OVCAR-3 cell line. This cell line had high IL-8 secretion values relative to the other cytokines, but much lower secretion values than the CAOV-3 or SKOV-3 cell lines. Levels of IL-6 secretion were low, but COB-141 appeared to have an inhibitory effect on IL-6 secretion. COB-141 also had an inhibitory effect on IL-8 secretion.
Figure 10. OVCAR-3 cytokine secretion as a function of COB-141 concentration. This figure shows that the OVCAR-3 cell line expresses IL-8, and expresses low levels of IL-6. Cells were treated with COB-141 for 24 hours which decreased IL-6 and IL-8 secretion. The data presented is representative of one trial (n=1).

4.2. IL-6 ELISA Assays

ELISA tests were performed to further investigate the effects of COB-141 on IL-6 secretion. Treatments of COB-141 ranged from 12.5 µM to 100 µM for the CAOV-3 and SKOV-3 cell lines and from 6.25 µM to 100 µM in the OVCAR-3 cell line. Three trials of the same experiment were performed for each cell line. For each trial, regressions were performed in Excel and the model was varied to maximize the R² value. The IC₅₀ value was found for each trial using this regression curve, and the three IC₅₀ values were averaged to find the reported IC₅₀ value for the cell line. The IC₅₀ values were found to be 46.5 ± 19 µM, 30.8 ± 27 µM, and 7.65 ± 0.91 µM for the CAOV-3, SKOV-3, and
OVCAR-3 cell lines respectively. All ± values listed in the paper are the standard deviation values for the trials unless otherwise specified.

Figures 11-13 show the results of the IL-6 ELISA for each cell line. Each figure is the average of three separate trials, and the samples for each trial were done in triplicate. The regression curves shown on the figures are examples of what was done to find the IC$_{50}$ value for the cell line, but were not used directly to find the IC$_{50}$ values. As stated above, the reported IC$_{50}$ values were found by taking the average of the IC$_{50}$ values for each individual trial so that a standard deviation could be determined. Note that the curves do not extend to the y-axis where the concentration of COB-141 equals zero because this was set to be 100% for each figure.

As part of the experimental design for the ELISA experiments, cells were treated with 0.25% DMSO and separately with only media and no DMSO. 0.25% DMSO was used as the negative control for comparison with cells treated with compounds since this was the vehicle for the compounds. If the results with 0.25% DMSO appeared to be similar to the results with media alone, no comment is made regarding these two treatment groups. If this were not the case for a given experiment, a comment has been added indicating that the results with 0.25% DMSO was disparate from the media alone control.

Figure 11 shows the secretion levels of IL-6 in the CAOV-3 cell line. COB-141 had an inhibitory effect on IL-6 secretion with an IC$_{50}$ of 46.5 ± 19 µM. This tells us that COB-141 disrupts some mechanism upstream or directly on IL-6 secretion. This could mean it is acting on a transcription factor that prevents IL-6 mRNA from being produced,
that it is disrupting the translation of IL-6 mRNA into protein, that it is acting in some other fashion to prevent secretion of IL-6 from the cell, or that it is cytotoxic at high concentrations.

Figure 11. Effect of COB-141 on IL-6 secretion by CAOV-3 cells. The IC₅₀ was determined to be 46.5 ± 19 µM by averaging the IC₅₀ values from each individual trial. The regression equation \( y = -30.79 \ln(x) + 171.99 \) shown on the graph is an example of how an IC₅₀ value can be calculated. The data presented is an average from 3 separate trials (n=3) ± SEM. The model shown had an \( R^2 \) value of 0.9258.

Figure 12 shows the secretion levels of IL-6 in the SKOV-3 cell line. COB-141 had an inhibitory effect on IL-6 secretion with an IC₅₀ of 30.8 ± 27 µM. This tells us that COB-141 disrupts some mechanism upstream or directly on IL-6 secretion. This could mean it is acting on a transcription factor that prevents IL-6 mRNA from being produced, that it is disrupting the translation of IL-6 mRNA into protein, that it is acting in some
other fashion to prevent secretion of IL-6 from the cell, or that it is cytotoxic at high concentrations.

**Figure 12.** Effect of COB-141 on IL-6 secretion by SKOV-3 cells. The IC₅₀ was determined to be 30.8 ± 27 µM by averaging the IC₅₀ values from each individual trial. The regression equation \( y = -35.13\ln(x) + 178.98 \) shown on the graph is an example of how an IC₅₀ value can be calculated. The data presented is an average from 3 separate trials (n=3) ± SEM. The model shown had an \( R^2 \) value of 0.9947.

Figure 13 shows the secretion levels of IL-6 in the OVCAR-3 cell line. COB-141 had an inhibitory effect on IL-6 secretion with an IC₅₀ of 7.65 ± 0.91 µM. This tells us that COB-141 disrupts some mechanism upstream or directly on IL-6 secretion. This could mean it is acting on a transcription factor that prevents IL-6 mRNA from being produced, that it is disrupting the translation of IL-6 mRNA into protein, that it is acting in some other fashion to prevent secretion of IL-6 from the cell, or that it is cytotoxic at high concentrations. Note that the 0.25% DMSO control did appear to have an effect on IL-6 secretion relative to cells treated with only media and no DMSO. There was a
reduction in secretion of approximately 50% between cells treated with only 0.25% DMSO in media and cells treated with only media.

Figure 13. Effect of COB-141 on IL-6 secretion by OVCAR-3 cells. The IC50 was determined to be 7.65 ± 0.91 µM by averaging the IC50 values from each individual trial. The regression equation \( y = 543.09x^{-1.153} \) shown on the graph is an example of how an IC50 value can be calculated. The data presented is an average from 3 separate trials (n=3) ± SEM. The model shown had an R^2 value of 0.9627.

4.3. IL-8 ELISA Assays

ELISA tests were performed to further investigate the effects of COB-141 on IL-8 secretion. Treatments of COB-141 ranged from 6.25 µM to 100 µM for all three cell lines. Three trials of the same experiment were performed for each cell line. For each trial, regressions were performed in Excel and the model was varied to maximize the R^2 value. The IC50 value was found for each trial using this regression curve, and the three IC50 values were averaged to find the reported IC50 value for the cell line. The IC50 values were found to be 31.9 ± 12 µM and 15.1 ± 5.8 µM for the SKOV-3, and OVCAR-3 cell
lines respectively. It was not applicable in the CAOV-3 cell line because COB-141 induced IL-8 secretion.

Figures 14-16 show the results of the IL-8 ELISA for each cell line. Each figure is the average of three separate trials, and the samples for each trial were done in triplicate. The regression curves shown on the figures are examples of what was done to find the IC\textsubscript{50} value for the cell line, but were not used directly to find the IC\textsubscript{50} values. As stated above, the reported IC\textsubscript{50} values were found by taking the average of the IC\textsubscript{50} values for each individual trial so that a standard deviation could be determined. Note that the curves do not extend to the y-axis where the concentration of COB-141 equals zero because this was set to be 100% for each figure.

As part of the experimental design for the ELISA experiments, cells were treated with 0.25% DMSO and separately with only media and no DMSO. 0.25% DMSO was used as the negative control for comparison with cells treated with compounds since this was the vehicle for the compounds. If the results with 0.25% DMSO appeared to be similar to the results with media alone, no comment is made regarding these two treatment groups. If this were not the case for a given experiment, a comment has been added indicating that the results with 0.25% DMSO was disparate from the media alone control.

Figure 14 shows the secretion levels of IL-8 in the CAOV-3 cell line. COB-141 induced IL-8 secretion so an IC\textsubscript{50} value is not applicable. This tells us that COB-141 affects some mechanism upstream or directly on IL-8 secretion. This could mean it is acting on a transcription factor that promotes transcription of IL-8 mRNA, that it is
promoting the translation of IL-8 mRNA into protein, or that it is acting in some other fashion to induce secretion of IL-8 from the cell.

Figure 14. Effect of COB-141 on IL-8 secretion by CAOV-3 cells. This figure shows the 24 hour effect of COB-141, for which no IC₅₀ can be calculated because IL-8 secretion was induced. The data shown is an average from 3 separate trials (n=3) ± SEM.

![Graph showing the effect of COB-141 on IL-8 secretion by CAOV-3 cells.](image)

Figure 15 shows the secretion levels of IL-8 in the SKOV-3 cell line. COB-141 had an inhibitory effect on IL-8 secretion with an IC₅₀ of 31.9 ± 12 µM. This tells us that COB-141 disrupts some mechanism upstream or directly on IL-8 secretion. This could mean it is acting on a transcription factor that prevents IL-8 mRNA from being produced, that it is disrupting the translation of IL-8 mRNA into protein, that it is acting in some other fashion to prevent secretion of IL-8 from the cell, or that it is cytotoxic at high concentrations.
Figure 15. Effect of COB-141 on IL-8 secretion by SKOV-3 cells. The IC\textsubscript{50} was determined to be 31.9 ± 12 µM by averaging the IC\textsubscript{50} values from each individual trial. The regression equation $y = 299.44x^{-0.517}$ shown on the graph is an example of how an IC\textsubscript{50} value can be calculated. The data presented is an average from 3 separate trials (n=3) ± SEM. The model shown had an $R^2$ value of 0.8835.

Figure 16 shows the secretion levels of IL-8 in the OVCAR-3 cell line. COB-141 had an inhibitory effect on IL-8 secretion with an IC\textsubscript{50} of 15.1 ± 5.8 µM. This tells us that COB-141 disrupts some mechanism upstream or directly on IL-8 secretion. This could mean it is acting on a transcription factor that prevents IL-8 mRNA from being produced, that it is disrupting the translation of IL-8 mRNA into protein, that it is acting in some other fashion to prevent secretion of IL-8 from the cell, or that it is cytotoxic at high concentrations. Note that the 0.25% DMSO control did appear to have an inhibitory effect on IL-8 secretion relative to cells treated with only media and no DMSO. There was a reduction in secretion of approximately 60% between cells treated with only 0.25% DMSO in media and cells treated with only media.
The IC₅₀ was determined to be 15.1 ± 5.8 µM by averaging the IC₅₀ values from each individual trial. The regression equation $y = 66.196e^{-0.023x}$ shown on the graph is an example of how an IC₅₀ value can be calculated. The data presented is an average from 3 separate trials (n=3) ± SEM. The model shown had an $R^2$ value of 0.9795.

**4.4. IL-6 QRT-PCR**

Quantitative real time polymerase chain reaction assays were used to investigate the effects of COB-141 on IL-6 transcription. Treatments of COB-141 ranged from 6.25 µM to 100 µM for all three cell lines. Three trials of the same experiment were performed for each cell line. For each trial, regressions were performed in Excel and the model was varied to maximize the $R^2$ value. The IC₅₀ value was found for each trial using this regression curve, and the three IC₅₀ values were averaged to find the reported IC₅₀ value for the cell line. The IC₅₀ values were found to be 34.0 ± 7.1 µM and 24.1 ± 7.4 µM for the SKOV-3, and OVCAR-3 cell lines respectively. It was not applicable in the CAOV-3 cell line because COB-141 had no effect on IL-6 transcription.
Figures 17-19 show the results of the IL-6 QRT-PCR assays for each cell line. Each figure is the average of three separate trials, and the samples for each trial were done in triplicate. The regression curves shown on the figures are examples of what was done to find the IC$_{50}$ value for the cell line, but were not used directly to find the IC$_{50}$ values. As stated above, the reported IC$_{50}$ values were found by taking the average of the IC$_{50}$ values for each individual trial so that a standard deviation could be determined. Note that the curves do not extend to the y-axis where the concentration of COB-141 equals zero because this was set to be 100% for each figure.

As part of the experimental design for the QRT-PCR experiments, cells were treated with 0.25% DMSO and separately with only media and no DMSO. 0.25% DMSO was used as the negative control for comparison with cells treated with compounds since this was the vehicle for the compounds. If the results with 0.25% DMSO appeared to be similar to the results with media alone, no comment is made regarding these two treatment groups. If this were not the case for a given experiment, a comment has been added indicating that the results with 0.25% DMSO was disparate from the media alone control.

Figure 17 shows the levels of IL-6 transcription in the CAOV-3 cell line. COB-141 did not have an effect on IL-6 transcription in the experimental range, so an IC$_{50}$ value is not applicable. Knowing that COB-141 did have an effect on IL-6 secretion, but not transcription, shows that COB-141 acts after transcription. This means it could be inhibiting translation or secretion, or it could be promoting protein degradation.
Figure 17. Effect of COB-141 on IL-6 transcription by CAOV-3 cells. This figure shows the 24 hour effect of COB-141. An IC50 value cannot be calculated because the compound did not inhibit IL-6 transcription. The data presented is an average from 3 separate trials (n=3) ± SEM. The model shown had an $R^2$ value of 0.5412.

\[ y = 0.0095x^2 - 1.1446x + 119.56 \]
\[ R^2 = 0.5412 \]

Figure 18 shows the levels of IL-6 transcription levels in the SKOV-3 cell line. COB-141 had an inhibitory effect on IL-6 transcription with an IC50 of 34.0 ± 7.1 µM. This tells us that COB-141 disrupts some mechanism upstream or directly on IL-6 mRNA transcription. This could mean it is acting on a transcription factor and prevents IL-6 mRNA from being produced, or that it disrupts IL-6 mRNA transcription via some other mechanism.
Figure 18. Effect of COB-141 on IL-6 transcription by SKOV-3 cells. The transcriptional IC$_{50}$ was determined to be 34.0 ± 7.1 µM by averaging the IC$_{50}$ values from each individual trial. The regression equation $y = -21.36\ln(x) + 124.56$ shown on the graph is an example of how an IC$_{50}$ value can be calculated. The data presented is an average from 3 separate trials (n=3) ± SEM. The model shown had an $R^2$ value of 0.9973.

Figure 19 shows the levels of IL-6 transcription levels in the OVCAR-3 cell line. COB-141 had an inhibitory effect on IL-6 transcription with an IC$_{50}$ of 24.1 ± 7.4 µM. This tells us that COB-141 disrupts some mechanism upstream or directly on IL-6 mRNA transcription. This could mean it is acting on a transcription factor and prevents IL-6 mRNA from being produced, or that it disrupts IL-6 mRNA transcription via some other mechanism. Note that the 0.25% DMSO control did appear to have an inhibitory effect on IL-6 transcription relative to cells treated with only media and no DMSO. There was a reduction in transcription of approximately 30% between cells treated with only 0.25% DMSO in media and cells treated with only media.
Figure 19. Effect of COB-141 on IL-6 transcription by OVCAR-3 cells. The transcriptional IC$_{50}$ was determined to be 24.1 ± 7.4 µM by averaging the IC$_{50}$ values from each individual trial. The regression equation $y = 144.47x^{-0.349}$ shown on the graph is an example of how an IC$_{50}$ value can be calculated. The data presented is an average from 3 separate trials (n=3) ± SEM. The model shown had an R$^2$ value of 0.9823.

4.5. IL-8 QRT-PCR

Quantitative real time polymerase chain reaction assays were used to investigate the effects of COB-141 on IL-8 transcription. Treatments of COB-141 ranged from 6.25 µM to 100 µM for all three cell lines. Three trials of the same experiment were performed for each cell line. For each trial, regressions were performed in Excel and the model was varied to maximize the R$^2$ value. The IC$_{50}$ value was found for each trial using this regression curve, and the three IC$_{50}$ values were averaged to find the reported IC$_{50}$ value for the cell line. The IC$_{50}$ for the OVCAR-3 cell line was found to be 8.45 ± 1.0 µM. It was not applicable in the CAOV-3 or SKOV-3 cell lines because COB-141 had no effect on IL-8 transcription.
Figures 20-22 show the results of the IL-8 QRT-PCR assays for each cell line. Each figure is the average of three separate trials, and the samples for each trial were done in triplicate. The regression curves shown on the figures are examples of what was done to find the IC$_{50}$ value for the cell line, but were not used directly to find the IC$_{50}$ values. As stated above, the reported IC$_{50}$ values were found by taking the average of the IC$_{50}$ values for each individual trial so that a standard deviation could be determined. Note that the curves do not extend to the y-axis where the concentration of COB-141 equals zero because this was set to be 100% for each figure.

As part of the experimental design for the QRT-PCR experiments, cells were treated with 0.25% DMSO and separately with only media and no DMSO. 0.25% DMSO was used as the negative control for comparison with cells treated with compounds since this was the vehicle for the compounds. If the results with 0.25% DMSO appeared to be similar to the results with media alone, no comment is made regarding these two treatment groups. If this were not the case for a given experiment, a comment has been added indicating that the results with 0.25% DMSO was disparate from the media alone control.

Figure 20 shows the levels of IL-8 transcription in the CAOV-3 cell line. COB-141 did not have an effect on IL-8 transcription so an IC$_{50}$ value is not applicable. Knowing that COB-141 induced IL-8 secretion gives us insight into what mechanism COB-141 acts in this cell line. It appears that it acts after transcription, potentially inducing translation or secretion. Note that the 0.25% DMSO control did appear to have an inhibitory effect on IL-8 transcription relative to cells treated with only media and no
DMSO. There was a reduction in transcription of approximately 40% between cells treated with only 0.25% DMSO in media and cells treated with only media.

![Graph showing the effect of COB-141 on IL-8 transcription by CAOV-3 cells.](image)

**Figure 20.** Effect of COB-141 on IL-8 transcription by CAOV-3 cells. This figure shows the 24 hour effect of COB-141. An IC₅₀ value cannot be calculated because the compound did not inhibit IL-6 transcription. The data is an average from 3 separate trials (n=3) ± SEM. The model had an R² value of 0.7791.

Figure 21 shows the levels of IL-8 transcription in the SKOV-3 cell line. COB-141 had a small inhibitory effect on IL-8 transcription, but an IC₅₀ value could not be calculated based on the regressions that were used. Knowing that COB-141 did have a significant effect on IL-8 secretion, but not transcription gives us insight into what mechanism COB-141 acts in for this cell line. It appears that it acts after transcription, potentially inhibiting translation or secretion.
Figure 21. Effect of COB-141 on IL-8 transcription by SKOV-3 cells. This figure shows the 24-hour effect of COB-141. An IC$_{50}$ value cannot be calculated because the compound only had a small inhibitory effect on IL-8 transcription. The data is an average from 3 separate trials (n=3) ± SEM. The model shown had an $R^2$ value of 0.9385.

Figure 22 shows the levels of IL-8 transcription levels in the OVCAR-3 cell line. COB-141 had an inhibitory effect on IL-8 transcription with an IC$_{50}$ of 8.45 ± 1.0 µM. This tells us that COB-141 disrupts some mechanism upstream or directly on IL-8 mRNA transcription. This could mean it is acting on a transcription factor and prevents IL-8 mRNA from being produced, or that it disrupts IL-8 mRNA transcription via some other mechanism. Note that the 0.25% DMSO control did appear to have an inhibitory effect on IL-8 transcription relative to cells treated with only media and no DMSO. There was a reduction in transcription of approximately 40% between cells treated with only 0.25% DMSO in media and cells treated with only media.
Figure 22. Effect of COB-141 on IL-8 transcription by OVCAR-3 cells. The transcriptional IC\textsubscript{50} was determined to be 8.45 ± 1.0 µM by averaging the IC\textsubscript{50} values from each individual trial. The regression equation $y = 270.11x^{-0.788}$ shown on the graph is an example of how an IC\textsubscript{50} value can be calculated. The data presented is an average from 3 separate trials (n=3) ± SEM. The model shown had an $R^2$ value of 0.9872.

4.6. MTS Assays

We investigated the effect of COB-141 on cell metabolic activity by performing an MTS assay. In this assay, MTS solution is added to the cells, which is converted to formazan via dehydrogenase enzymes in viable cells, and the amount of formazan can be found by measuring the absorbance. The MTS assay is an indirect measure of cell viability because the test actually measures cell mitochondrial enzyme activity, which can be correlated to cell viability. This provides insight into whether the effect of COB-141 is due to destroying the viability of the cells, or if it is acting via a different mechanism that inhibits IL-6 and IL-8 transcription and secretion. Treatments of COB-141 ranged from 12.5 µM to 400 µM for all three cell lines, and the cell metabolic activity was measured at 24, 48, and 72 hours after the initial treatment. Three trials of the same experiment
were performed for each cell line. For each trial, regressions were performed in Excel and the model was varied to maximize the $R^2$ value. The TC$_{50}$ value was found for each trial using this regression curve, and the three TC$_{50}$ values were averaged to find the reported TC$_{50}$ value for the cell line.

Figures 23-31 show the results of the MTS assays for each cell line. Each figure is the average of three separate trials, and the samples for each trial were done in triplicate. The regression curves shown on the figures are examples of what was done to find the TC$_{50}$ value for the cell line, but were not used directly to find the TC$_{50}$ values. As stated above, the reported TC$_{50}$ values were found by taking the average of the TC$_{50}$ values for each individual trial so that a standard deviation could be determined. Note that the curves do not extend to the y-axis where the concentration of COB-141 equals zero because this was set to be 100% for each figure.

Figures 23-25 show the levels of metabolic activity in the CAOV-3 cell line at 24, 48, and 72 hours after treatment. As COB-141 concentration increased, metabolic activity of the cells decreased. The TC$_{50}$ values were found to be $217 \pm 18 \mu M$, $229 \pm 19 \mu M$, and $192 \pm 65 \mu M$ at 24, 48, and 72 hours respectively. Interestingly, the TC$_{50}$ value did not appear to be a strong function of incubation time. This tells us that COB-141 does cause a decrease in cell metabolic activity in the CAOV-3 cell line, but only at concentrations much higher than what were tested via ELISA and QRT-PCR. This means that the modulation of IL-6 and IL-8 secretion and transcription from COB-141 in the CAOV-3 cell line is likely not a result of damaging cell viability.
Figure 23. CAOV-3 metabolic activity relative to 0.25% DMSO after 24 hour treatment with COB-141.

The TC\textsubscript{50} was calculated to be 217 ± 18 µM by averaging the TC\textsubscript{50} values from each individual trial. The regression equation $y = 6E-05x^2 - 0.2219x + 94.886$ shown on the graph is an example of how a TC\textsubscript{50} value can be calculated. The data presented is an average from 3 separate trials (n=3) ± SEM. The model shown had an R\textsuperscript{2} value of 0.9727.
Figure 24. *CAOV-3 metabolic activity relative to 0.25% DMSO after 48 hour treatment with COB-141.*

The TC50 was calculated to be 229 ± 19 µM by averaging the TC50 values from each individual trial. The regression equation $y = -9E-05x^2 - 0.1633x + 91.956$ shown on the graph is an example of how a TC50 value can be calculated. The data presented is an average from 3 separate trials (n=3) ± SEM. The model shown had an $R^2$ value of 0.982.
Figure 25. CAOV-3 metabolic activity relative to 0.25% DMSO after 72 hour treatment with COB-141. The TC50 was calculated to be 192 ± 65 µM by averaging the TC50 values from each individual trial. The regression equation $y = 0.0002x^2 - 0.3119x + 103.43$ shown on the graph is an example of how a TC50 value can be calculated. The data presented is an average from 3 separate trials (n=3) ± SEM. The model shown had an $R^2$ value of 0.994.

Figures 26-28 show the levels of metabolic activity in the SKOV-3 cell line at 24, 48, and 72 hours after treatment. As COB-141 concentration increased, metabolic activity of the cells decreased. The TC50 values were found to be 198 ± 25 µM, 198 ± 3.2 µM, and 168 ± 7.3 µM at 24, 48, and 72 hours respectively. Again, the effect on viability did not appear to be a strong function of incubation time. This tells us that COB-141 does cause a decrease in cell metabolic activity in the SKOV-3 cell line, but only at concentrations much higher than what were tested via ELISA and QRT-PCR. This means that the modulation of IL-6 and IL-8 secretion and transcription from COB-141 in the SKOV-3 cell line is likely not a result of destroying cell viability.
Figure 26. SKOV-3 metabolic activity relative to 0.25% DMSO after 24 hour treatment with COB-141.

The TC$_{50}$ was calculated to be 198 ± 25 µM by averaging the TC$_{50}$ values from each individual trial. The regression equation $y = 102.35e^{-0.004x}$ shown on the graph is an example of how a TC$_{50}$ value can be calculated. The data presented is an average from 3 separate trials (n=3) ± SEM. The model shown had an $R^2$ value of 0.9783.
Figure 27. SKOV-3 metabolic activity relative to 0.25% DMSO after 48 hour treatment with COB-141.

The TC\textsubscript{50} was calculated to be 198 ± 3.2 µM by averaging the TC\textsubscript{50} values from each individual trial. The regression equation $y = 110.68e^{-0.004x}$ shown on the graph is an example of how a TC\textsubscript{50} value can be calculated. The data presented is an average from 3 separate trials (n=3) ± SEM. The model shown had an R\textsuperscript{2} value of 0.9724.
Figure 28. SKOV-3 metabolic activity relative to 0.25% DMSO after 72 hour treatment with COB-141. The TC$_{50}$ was calculated to be 168 ± 7.3 µM by averaging the TC$_{50}$ values from each individual trial. The regression equation $y = 122.26e^{-0.005x}$ shown on the graph is an example of how a TC$_{50}$ value can be calculated. The data presented is an average from 3 separate trials (n=3) ± SEM. The model shown had an R$^2$ value of 0.9717.

Figures 29-31 show the levels of metabolic activity in the OVCAR-3 cell line at 24, 48, and 72 hours after treatment. As COB-141 concentration increased, metabolic activity of the cells decreased. The TC$_{50}$ values were found to be 168 ± 85 µM, 130. ± 3.3µM, and 72.3 ± 18 µM at 24, 48, and 72 hours respectively. In this case, the effect on viability did appear to be a function of incubation time. This tells us that COB-141 does cause a decrease in cell metabolic activity in the OVCAR-3 cell line, but only at concentrations much higher than what were tested via ELISA and QRT-PCR. This means that the modulation of IL-6 and IL-8 secretion and transcription from COB-141 in the OVCAR-3 cell line is likely not a result of destroying cell viability.
Figure 29. OVCAR-3 metabolic activity relative to 0.25% DMSO after 24 hour treatment with COB-141.

The TC50 was calculated to be 168 ± 85 µM by averaging the TC50 values from each individual trial. The regression equation $y = 92.234e^{-0.004x}$ shown on the graph is an example of how a TC50 value can be calculated. The data presented is an average from 3 separate trials (n=3) ± SEM. The model shown had an $R^2$ value of 0.9902.
Figure 30. OVCAR-3 metabolic activity relative to 0.25% DMSO after 48 hour treatment with COB-141.
The TC50 was calculated to be 130. ± 3.3 µM by averaging the TC50 values from each individual trial. The regression equation $y = 109.29e^{-0.006x}$ shown on the graph is an example of how a TC50 value can be calculated. The data presented is an average from 3 separate trials ($n=3$) ± SEM. The model shown had an $R^2$ value of 0.9881.
Figure 31. OVCAR-3 metabolic activity relative to 0.25% DMSO after 72 hour treatment with COB-141. The TC$_{50}$ was calculated to be 72.3 ± 18 µM by averaging the TC$_{50}$ values from each individual trial. The regression equation $y = 89.46e^{-0.007x}$ shown on the graph is an example of how a TC$_{50}$ value can be calculated. The data presented is an average from 3 separate trials (n=3) ± SEM. The model shown had an $R^2$ value of 0.991.

4.7. BrdU Assays

A BrdU assay was performed on each cell line to further measure the effect of COB-141 on cell viability. Treatments of COB-141 ranged from 12.5 µM to 400 µM for all three cell lines. Three trials of the same experiment were performed for each cell line. For each trial, regressions were performed in Excel and the model was varied to maximize the $R^2$ value. The TC$_{50}$ value was found for each trial using this regression curve, and the three TC$_{50}$ values were averaged to find the reported TC$_{50}$ value for the cell line.

Figures 32-34 show the results of the BrdU assays for each cell line. Each figure is the average of three separate trials, and the samples for each trial were done in
triplicate. The regression curves shown on the figures are examples of what was done to find the TC₅₀ value for the cell line, but were not used directly to find the TC₅₀ values. As stated above, the reported TC₅₀ values were found by taking the average of the TC₅₀ values for each individual trial so that a standard deviation could be determined. Note that the curves do not extend to the y-axis where the concentration of COB-141 equals zero because this was set to be 100% for each figure.

Figure 32 shows the effect of COB-141 on cell proliferation in the CAOV-3 cell line. As COB-141 concentration increased, proliferation of the cells decreased. The TC₅₀ value was found to be 731 ± 12 µM. This tells us that COB-141 does cause a decrease in cell proliferation in the CAOV-3 cell line, but only at concentrations much higher than what were tested via ELISA and QRT-PCR. This means that in Figures 11 and 14 where IL-6 and IL-8 secretion was modulated in the CAOV-3 cell line by COB-141, it was likely not due to an effect on proliferation.
Figure 32. CAOV-3 cell proliferation relative to 0.25% DMSO after 24 hour treatment with COB-141. The TC$_{50}$ was calculated to be 731 ± 12 µM by averaging the TC$_{50}$ values from each individual trial. The regression equation $y = 104.19e^{-0.001x}$ shown on the graph is an example of how a TC$_{50}$ value can be calculated. The data presented is an average from 3 separate trials (n=3) ± SEM. The model shown had an R$^2$ value of 0.9051.

Figure 33 shows the effect of COB-141 on cell proliferation in the SKOV-3 cell line. As COB-141 concentration increased, proliferation of the cells decreased. The TC$_{50}$ value was found to be 454 ± 59 µM. This tells us that COB-141 does cause a decrease in cell proliferation in the SKOV-3 cell line, but only at concentrations much higher than what were tested via ELISA and QRT-PCR. This means that in Figures 12, 15, and 18 where IL-6 and IL-8 secretion and transcription were modulated in the SKOV-3 cell line by COB-141, it was likely not due to an effect on proliferation.
**Figure 33.** SKOV-3 cell proliferation relative to 0.25% DMSO after 24 hour treatment with COB-141.

The TC$_{50}$ was calculated to be 454 ± 59 µM by averaging the TC$_{50}$ values from each individual trial. The regression equation $y = -0.0003x^2 + 0.0065x + 100.72$ shown on the graph is an example of how a TC$_{50}$ value can be calculated. The data presented is an average from 3 separate trials (n=3) ± SEM. The model shown had an R$^2$ value of 0.8002.

Figure 34 shows the effect of COB-141 on cell proliferation in the OVCAR-3 cell line. As COB-141 concentration increased, proliferation of the cells decreased. The TC$_{50}$ value was found to be 227 ± 120 µM. This tells us that COB-141 does cause a decrease in cell proliferation in the CAOV-3 cell line, but only at concentrations much higher than what were tested via ELISA and QRT-PCR. This means that in Figures 13, 16, 19, and 22 where IL-6 and IL-8 secretion and transcription were modulated in the OVCAR-3 cell line by COB-141, it was likely not due to an effect on proliferation.
Figure 34. OVCAR-3 cell proliferation relative to 0.25% DMSO after 24 hour treatment with COB-141. The TC₅₀ was calculated to be 227 ± 120 µM by averaging the TC₅₀ values from each individual trial. The regression equation $y = 119.62e^{-0.004x}$ shown on the graph is an example of how a TC₅₀ value can be calculated. The data presented is an average from 3 separate trials (n=3) ± SEM. The model shown had an $R^2$ value of 0.8628.

4.8. Therapeutic Index

The therapeutic index is an overall measure of the efficacy of a compound. It is defined as the TC₅₀/IC₅₀ and a high value is desired. This allows us to compare different compounds and different cell lines. The IC₅₀ value for secretion and not transcription was used. This is because the larger scope of our study relates to the tumor microenvironment, and secretion is a more direct measure of cytokines acting in the tumor microenvironment. With our study, we found the TC₅₀ using two different methods so there are two therapeutic indices for each cell line. For the MTS therapeutic index, the 24 hour TC₅₀ was used. Therapeutic indices for IL-8 in the CAOV-3 cell line were not applicable because COB-141 induced secretion.
4.8.1. IL-6 Therapeutic Indices

*Table 1. Therapeutic Indices for IL-6*

<table>
<thead>
<tr>
<th></th>
<th>CAOV-3</th>
<th>SKOV-3</th>
<th>OVCAR-3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MTS</strong></td>
<td>4.67 ± 1.9</td>
<td>6.43 ± 5.7</td>
<td>22.0 ± 11</td>
</tr>
<tr>
<td><strong>BrdU</strong></td>
<td>15.72 ± 6.4</td>
<td>14.7 ± 13</td>
<td>29.7 ± 16</td>
</tr>
</tbody>
</table>

4.8.2. IL-8 Therapeutic Indices

*Table 2. Therapeutic Indices for IL-8*

<table>
<thead>
<tr>
<th></th>
<th>CAOV-3</th>
<th>SKOV-3</th>
<th>OVCAR-3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MTS</strong></td>
<td>N/A</td>
<td>6.21 ± 2.5</td>
<td>11.1 ± 7.1</td>
</tr>
<tr>
<td><strong>BrdU</strong></td>
<td>N/A</td>
<td>14.2 ± 5.7</td>
<td>15.0 ± 9.8</td>
</tr>
</tbody>
</table>

4.9. HPLC Assay Results

High performance liquid chromatography (HPLC) was used to detect COB-141 in acetonitrile. In order to generate a standard curve and to verify COB-141 could be detected via HPLC, concentrations of 100 µM, 50 µM, 25 µM, 12.5 µM, and 6.25 µM COB-141 in acetonitrile were made and run through the HPLC described in the materials and methods section. The mobile phase composition was 20% water in acetonitrile (80% acetonitrile/20% water). Figures 35-39 are the computer readouts that show the area of each peak. These are the values that were used to generate the standard curve in Figure 40. Note that we are only interested in the peak with a retention time of 2.90 minutes. The
other peaks are some sort of contamination that are not relevant to generation of the external standard.

*Figure 35.* HPLC readout for 100 μM COB-141 in acetonitrile.
Figure 36. HPLC readout for 50 µM COB-141 in acetonitrile

Figure 37. HPLC readout for 25 µM COB-141 in acetonitrile
Figure 38. HPLC readout for 12.5 µM COB-141 in acetonitrile

Figure 39. HPLC readout for 6.25 µM COB-141 in acetonitrile
Figure 40 below shows the standard curve that was generated using the peak areas for each concentration of COB-141. This will, in theory, allow us to quantify unknown concentrations of COB-141 by using the area of the peak that is produced by the HPLC system.

\[ y = 1811.6x + 2627.8 \]
\[ R^2 = 0.9977 \]

*Figure 40. External Standard to Determine COB-141 Concentration from Peak Area*
5. Chapter 5: Discussion

An ELISA study showed that COB-141 reduced IL-6 and IL-8 secretion in the SKOV-3 and OVCAR-3 cell lines. In the CAOV-3 cell line, COB-141 reduced IL-6 secretion, but induced IL-8 secretion. The IC$_{50}$ values for IL-6 secretion were found to be $46.5 \pm 19$ µM, $30.8 \pm 27$ µM, and $7.65 \pm 0.91$ µM for the CAOV-3, SKOV-3, and OVCAR-3 cell lines respectively, and for IL-8 these values were determined to be $31.9 \pm 12$ µM and $15.1 \pm 5.8$ µM for the SKOV-3 and OVCAR-3 cell lines. It was very interesting that COB-141 induced IL-8 secretion in the CAOV-3 cell lines and the reason for this is not clear. COB-141 did not have an effect on the transcription of IL-6 or IL-8 in the CAOV-3 cell line.

QRT-PCR was used to investigate the effects that COB-141 had on IL-6 and IL-8 transcription. The IC$_{50}$ values for IL-6 transcription were $34.0 \pm 7.1$ µM and $24.1 \pm 7.4$ µM for the SKOV-3 and OVCAR-3 cell lines respectively. COB-141 had no effect on IL-6 transcription in the CAOV-3 cell line so the IC$_{50}$ measure is not applicable. For IL-8 transcription, the IC$_{50}$ value was found to be $8.45 \pm 1.0$ µM for the OVCAR-3 cell line. COB-141 had no effect on IL-8 transcription in the CAOV-3 and SKOV-3 cell lines so the IC$_{50}$ measure is not applicable.

Cell metabolism was measured via an MTS assay. MTS was applied to the treated wells, which was converted to formazan via dehydrogenase enzymes in viable cells, and the amount of formazan was measured in a plate reader at 490 nm [55]. This is essentially a measure of the mitochondrial activity of the cells, which can be inferred to be proportionate to the number of viable cells. Cell proliferation was measured as well via a
BrdU cell proliferation ELISA. This test works as BrdU is incorporated into the DNA of proliferating cells which can be measured. This is done through antibody binding to the BrdU, and then by an anti-BrdU detection antibody [56].

It was determined that COB-141 does modulate IL-6 and IL-8 secretion and transcription in most instances for the three cell lines, and it is not toxic to the cells at tested concentrations (below 100µM). Regarding IL-6, it appears that COB-141 is acting at the transcriptional level in the OVCAR-3 and SKOV-3 cell lines, and at the translational level or level of secretion in the CAOV-3 cell line. Regarding IL-8, COB-141 appears to act at the transcriptional level in the OVCAR-3 cell lines, and at the translational level or level of secretion in the CAOV-3 and SKOV-3 cell lines, inducing secretion in the CAOV-3 cell line and inhibiting it in the SKOV-3 cell line. The compound does not prevent cell proliferation at the concentrations that we tested because BrdU was incorporated into the DNA of proliferating cells at the same levels as untreated cells. The results of this study warrant further investigation of COB-141 to see its effect on the tumor microenvironment.

The groundwork for creating an HPLC method to detect COB-141 in mouse serum was completed. The first step was to verify if the compound could be detected in acetonitrile alone before attempting to detect in mouse serum. This was performed on the HPLC in Dr. Begmeier’s lab at Ohio University. Through trial and error methods, the best run conditions for compound detection were determined, and an external standard curve was generated with concentrations of COB-141 varying from 100 µM to 6.25 µM.
6. **Chapter 6: Future Aims**

The future aim of this project is to pursue *in vivo* studies in mice. Once COB-141 can be detected in mouse serum, the pharmacokinetic parameters of the compound can be measured. With these parameters known, the effects of the compound on a mouse model with ovarian cancer can be studied, and if the compound causes the tumor microenvironment to transform to growth inhibiting, then tumor size should decrease. If this is the case, the compound can begin clinical studies in humans with a phase 0 clinical trial. Phase 0 is an pharmacokinetic investigation of how a drug acts in the human body [57]. If the drug passes this stage, then the goal is to make it through Phase I, II, III, and IV clinical trials. This is the long term aim for COB-141.
References


