The Effect of Small Organic Compounds on Triple Negative Breast Cancer Cells

A thesis presented to
the faculty of
the Russ College of Engineering and Technology of Ohio University

In partial fulfillment
of the requirements for the degree
Master of Science

John D. O’Brien

August 2012

This thesis titled
The Effect of Small Organic Compounds on Triple Negative Breast Cancer Cells

by
JOHN D. O'BRIEN

has been approved for
the Department of Chemical & Biomolecular Engineering
and the Russ College of Engineering and Technology by

______________________________
Douglas J. Goetz
Professor of Chemical and Biomolecular Engineering

______________________________
Kelly D. McCall
Assistant Professor of Specialty Medicine

______________________________
Dennis Irwin
Dean, Russ College of Engineering and Technology
Abstract

O'BRIEN, JOHN D., M.S., August 2012, Biomedical Engineering

The Effect of Small Organic Compounds on Triple Negative Breast Cancer Cells

Directors of Thesis: Douglas J. Goetz and Kelly D. McCall

The ability of small organic compounds to inhibit the expression of a cytokine by triple negative breast cancer cells was explored. In addition, the ability of the cytokine to exert an autocrine effect on the cancer cells was probed.

Approved: _____________________________________________________________

Douglas J. Goetz
Professor of Chemical and Biomolecular Engineering

Approved: _____________________________________________________________

Kelly D. McCall
Assistant Professor of Specialty Medicine
Acknowledgments

First and foremost, I would like to thank my advisors, Dr. Douglas Goetz and Dr. Kelly McCall, for their encouragement and guidance over the last two years. The time spent in their laboratories, and the knowledge imparted to me, has allowed me to recognize my potential as a scientist. Secondly I would like to thank the Interthyr Corporation for the funding provided for my thesis work, as well as Dr. Stephen Bergmeir for supplying the compounds used in my research. I would also like to thank my thesis committee members, Drs. Fabian Benencia and Monica Burdick, both of whom have served as mentors in the classroom and laboratory alike. Next, I would like to thank Sudhir Deosarkar, for his mentorship and training throughout my thesis research. I would also like to thank Pooja Bhatt and Venktesh Shirure for their assistance and mentoring in the laboratory. Finally, I would like to thank Grady Carlson, Anuja Alipati, Chunyan Qi, and Tiantian Liu for their support and companionship throughout this most grueling process.
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>3</td>
</tr>
<tr>
<td>Acknowledgments</td>
<td>4</td>
</tr>
<tr>
<td>List of Figures</td>
<td>8</td>
</tr>
<tr>
<td>Chapter 1: Overview of Breast Cancer Characteristics</td>
<td>10</td>
</tr>
<tr>
<td>1.1 Cancer</td>
<td>11</td>
</tr>
<tr>
<td>Chapter 2: The Interleukin-6 (IL-6) Signaling Pathway</td>
<td>13</td>
</tr>
<tr>
<td>2.1 Interleukin-6 (IL-6)</td>
<td>13</td>
</tr>
<tr>
<td>2.2 Interleukin 6 Receptor (IL-6R) and gp130</td>
<td>13</td>
</tr>
<tr>
<td>2.3 Signal Transducer and Activation of Transcription 3 (STAT3)</td>
<td>15</td>
</tr>
<tr>
<td>Chapter 3: Non-Diseased Interleukin-6 Signaling</td>
<td>18</td>
</tr>
<tr>
<td>3.1 IL-6 Signaling in Immunity</td>
<td>18</td>
</tr>
<tr>
<td>3.2 IL-6 Signaling in Other Biological Functions</td>
<td>20</td>
</tr>
<tr>
<td>Chapter 4: Interleukin-6 Signaling in Diseased States</td>
<td>22</td>
</tr>
<tr>
<td>4.1 IL-6 Signaling in Autoimmune/Inflammatory Disorders</td>
<td>22</td>
</tr>
<tr>
<td>4.2 IL-6 Signaling in Cancer</td>
<td>23</td>
</tr>
<tr>
<td>Chapter 5: Role of Interleukin-6 in Breast Cancer</td>
<td>25</td>
</tr>
<tr>
<td>5.1 IL-6 Expression in Breast Cancer Cells</td>
<td>25</td>
</tr>
<tr>
<td>5.2 Expression of IL-6R and sIL-6R in Breast Cancer</td>
<td>26</td>
</tr>
<tr>
<td>5.3 Prognostic Role of IL-6 in Breast Cancer</td>
<td>26</td>
</tr>
<tr>
<td>5.4 Effect of IL-6 signaling on Breast Cancer</td>
<td>27</td>
</tr>
</tbody>
</table>
Chapter 6: Interleukin-6 as a Target for Treatment ..........................................................29

Chapter 7: Phenylmethimazole ......................................................................................31

Chapter 8: Specific Aims ...............................................................................................33

Chapter 9: Materials and Methodology ........................................................................35

  9.1 Cell Culture ........................................................................................................35

  9.2 Compound Cell Treatment .................................................................................35

  9.3 Quantitative Real Time Polymerase Chain Reaction (QRT-PCR) .................36

  9.4 ELISA Secreted Protein Analysis .....................................................................36

  9.5 MTS Assay ........................................................................................................37

  9.6 Bromodeoxyuridine (BrdU) Incorporation Proliferation Assay .......................38

  9.7 Flow Cytometry Assay (FACS) .......................................................................38

  9.8 Nuclear Protein Western Blot Analysis .............................................................40

  9.9 Statistics ............................................................................................................41

Chapter 10: Experimental Results ..................................................................................42

  10.1 MDA-MB-231 cells express high levels of interleukin 6 (IL-6) and Toll-like
       receptor 3 (TLR3) ..............................................................................................42

  10.2 C10 and COB-141 decrease IL-6 expression in MDA-MB-231 cells ..........44

  10.3 IL-6 half maximal inhibition concentrations (IC_{50}) of C10 in MDA-MB-231
       cells ......................................................................................................................45

  10.4 IL-6 half maximal inhibition concentrations (IC_{50}) of COB-141 in MDA-
       MB-231 cells ........................................................................................................47

  10.5 Interleukin-6 does not affect phosphorylation of STAT3 ............................49

  10.5.1 Treatment of MDA-MB-231 cells with exogenous IL-6 does not affect
       STAT3 phosphorylation ....................................................................................49
10.5.2 Sequestration of IL-6 does not change the phosphorylation state of STAT3 in MDA-MB231..................................................................................................................51

10.6 MDA-MB-231 cells lack expression of surface bound Interleukin 6 receptor α (IL-6Rα)......................................................................................................................53

10.7 MDA-MB-231 cells express surface bound gp130 (a.k.a. IL-6ST/IL-6Rβ).55

10.8 Effect of C10 and COB-141 on growth/proliferation of MDA-MB-231 cells ........................................................................................................................................57

10.9 C10 and COB-141 show limited effects on the growth of MDA-MB-231 cells ........................................................................................................................................60

Chapter 11: Discussion of Results........................................................................................................62

Chapter 12: Future Aims................................................................................................................................65

12.1 In vitro study of the paracrine effects of MDA-MB-231 cell-produced IL-6 on cells commonly found in the tissue microenvironment........................................65

12.2 In vivo study of the effects of C10 and COB-141 on the 4T1 triple negative mouse mammary epithelial cancer model........................................................................66

References...........................................................................................................................................67
## List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>11</td>
</tr>
<tr>
<td>2.</td>
<td>16</td>
</tr>
<tr>
<td>3.</td>
<td>43</td>
</tr>
<tr>
<td>4.</td>
<td>44</td>
</tr>
<tr>
<td>5.</td>
<td>45</td>
</tr>
<tr>
<td>6.</td>
<td>46</td>
</tr>
<tr>
<td>7.</td>
<td>47</td>
</tr>
<tr>
<td>8.</td>
<td>48</td>
</tr>
<tr>
<td>9.</td>
<td>49</td>
</tr>
<tr>
<td>10.</td>
<td>51</td>
</tr>
<tr>
<td>11.</td>
<td>52</td>
</tr>
<tr>
<td>12.</td>
<td>54</td>
</tr>
<tr>
<td>13.</td>
<td>56</td>
</tr>
<tr>
<td>14.</td>
<td>58</td>
</tr>
</tbody>
</table>
15. COB-141 decreases proliferation of MDA-MB-231 cells at higher concentrations.


17. COB-141 at high dosage causes a decrease in MDA-MB-231 cell viability.
Chapter 1: Overview of Breast Cancer Characteristics

Breast cancer can be defined as cancer that forms in the localized tissue of the breast. Breast cancer is more prevalent in women than in men. This prevalence is thought to occur due to the functional mammary gland in mature female mammals, as opposed to the testosterone-limited male anatomy. Currently breast cancer is the third leading cause of cancer death in the United States, and the second leading cause of cancer deaths in American women (Society, 2011). According to the American Cancer Society (ACS), an estimated 300,000 women will be diagnosed with some form of breast cancer in 2012 (Society, 2011). Of these cases, approximately 230,000 will be diagnosed as invasive, compared to the 70,000 cases characterized as in situ (Siegel, Naishadham, & Jemal, 2012; Society, 2011). It is estimated that among those women currently diagnosed with breast cancer, approximately 40,000 will die in 2012 (Siegel et al., 2012; Society, 2011).

The majority of breast cancer cases are diagnosed as adenocarcinomas, defined as malignant tumors of a glandular origin, occurring in either the ducts or lobules of the mammary gland (Wiseman & Werb, 2002). More often than not, these malignant tissues are characterized as invasive and aggressive, increasing the potential for a negative prognosis (Society, 2011). While there are recorded cases of sarcoma—cancer originating from mesenchymal cells (i.e. muscle, fat, and blood vessels)—breast cancer, sarcomas are rare. Triple-negative breast cancer (TNBC), a form of breast cancer characterized by a lack of estrogen receptor (ER), progesterone receptor (PR), and low to nil expression of human epidermal growth factor-2 receptors (HER2), represents an estimated 15% of diagnosed breast cancers (Figure 1) (Gucalp & Traina, 2011). The lack
of these receptors rules out the use of exogenous hormone treatment, a successful therapy in the treatment of other forms of breast cancer. While current chemotherapy and antibody treatments may be used for treatment, success rates are diminished due to the lack of specific targeted therapies. Taking into account the invasive and aggressive nature of TNBC, it is essential that targeted therapies are found.

![Figure 1. Percentage of breast cancer diagnosis type.](image)

Of the cases of breast cancer diagnosed in 2012, 77% will be classified as invasive in nature. 15% of the total population will be triple-negative (Siegel et al., 2012; Society, 2011).

### 1.1 Cancer

Cancer can be described as a disease that arises from the culmination of multiple mutations in the genome of normal cells, resulting in a change of function of the cell. Due to the rapid proliferation of cancer cells, the probability of mutation occurrence and accumulation is increased over a short period of time. This may explain why cancers often present themselves later in the average life, as mutations could aggregate in a particular cell type. As discussed in Hanahan and Weinberg's “Hallmarks of Cancer”
(Hanahan & Weinberg, 2000), the mutated functions tend to be dominantly expressed and are seen as a preferable evolutionary changes for the cell. In this manner, it is suggested that all cancers exhibit six key characteristics that are gained in the progression from normal tissue to that of a malignant tissue. This allows a cancerous tissue to exist relatively unregulated by the body’s physiological immune responses (Hanahan & Weinberg, 2000, 2011). Hanahan’s characteristics are summarized: an invasive nature mediated through metastasis, self regulated production of growth factors, evasion of apoptosis, unregulated proliferation, down regulation of anti-growth stimuli, and continual angiogenesis (Hanahan & Weinberg, 2000). Further discussion shows that each of these characteristics is not necessarily autonomous, and may require each other to exist. For example, the lack of self-produced growth factors or a normal production of anti-growth stimuli, could limit the overall ability of a malignant tissue to be invasive, evade apoptosis, proliferate, or stimulate angiogenesis. The lack of any of these gained functions may decrease the formation of mutations that could pose a viable threat to the body. Hypotheses such as these have been driving research toward the involvement of cellular signaling in cancer and disease (Hanahan & Weinberg, 2011; Hodge, Hurt, & Farrar, 2005).
Chapter 2: The Interleukin-6 (IL-6) Signaling Pathway

2.1 Interleukin-6 (IL-6)

A vast amount of research has been completed over the past three decades to characterize Interleukin-6 (IL-6). Also previously designated as Interferon β2 or B-cell stimulatory factor-2, IL-6 has been described as a glycosylated pleiotropic inflammatory cytokine, primarily produced and secreted by immune cell types such as macrophages, monocytes, and T cells at localized sites of inflammation. Transcription of IL-6 is mediated by coordination between the two transcription factors nuclear factor κB (NF-κB) and CAAT/enhancer -binding protein β (C/EBPβ) to activate the IL-6 promoter (Dendorfer, Oettgen, & Libermann, 1994). This cascade is generally induced by exogenous stimuli including signaling from other innate pro-inflammatory cytokines like Interleukin-1 (IL-1) and tumor necrosis factor (TNF)-α, and interferons (IFN); toll-like receptor (TLR) signaling via DNA and RNA virus infiltration, as well as bacterial endotoxin, and lipopolysaccharide (LPS) (Hong, Angelo, & Kurzrock, 2007; Naugler & Karin, 2008).

2.2 Interleukin 6 Receptor (IL-6R) and gp130

Classical IL-6 signaling (Figure 2) is initiated by the binding of IL-6 to a membrane bound receptor complex consisting of the ligand binding interleukin-6 receptor (IL-6Ra) and the glycoprotein 130 (gp130) signal transducer subunit. The ligand binding IL-6Ra is a type 1 cytokine receptor specific to IL-6. In order to transduce any signal, IL-6Ra must be coupled with a tyrosine phosphorylated gp130 homodimer, as
it is incapable of signaling by itself (Heinrich et al., 2003; Hong et al., 2007; Scheller, Chalaris, Schmidt-Arras, & Rose-John, 2011). The IL-6Rα protein is only expressed on select cell types, mainly neutrophils, monocytes, hepatocytes, and lymphocytes. Despite this particular expression, gp130 was found to be intrinsically expressed on most cells (Kishimoto, 2010; Knupfer & Preiss, 2010; Scheller et al., 2011). Interestingly, several studies have confirmed the active shedding of functional IL-6Rα to produce a soluble receptor (sIL-6Rα) by expression of an alternatively spliced mRNA of the IL-6R protein. This alternatively spliced molecule lacks both the cytoplasmic and trans-membrane portion of the receptor, however continues to retain its biological function (Mullberg et al., 1993). These soluble receptors have been detected in the circulation during inflammatory events, as well as several cancers (Scheller et al., 2011). This event allows for trans-signaling (Figure 2), where secreted IL-6 can bind with the sIL-6Rα, and ultimately allow signaling with the gp130 trans-membrane protein on stromal and malignant cells. Recent studies have suggested that this trans-signaling event is more prominent than the aforementioned classical signaling in coordinating the events of the IL-6 signaling pathway. This has been exhibited in neural cells (Marz, Otten, & Rose-John, 1999), as well as several stem cells and progenitor cells (Audet, Miller, Rose-John, Piret, & Eaves, 2001; Humphrey et al., 2004; Islam, Gong, Rose-John, & Heese, 2009; Yeoh et al., 2007) [reviewed in (Scheller et al., 2011)].
2.3 Signal Transducer and Activation of Transcription 3 (STAT3)

Interaction of the heterodimer IL-6/IL-6Rα complex, or alternatively the IL-6/sIL-6R complex, results in Janus kinase (JAK) activation, which subsequently phosphorylates tyrosine residues on the cytoplasmic domain of the gp130 homodimer (Goldberg & Schwertfeger, 2010). This step is necessary, since neither the membrane bound IL-6Rα nor gp130 contain kinase activity on their cytoplasmic domains (Ihle, 1995; Stahl et al., 1994). Rather, the JAK family proteins are thought to be in contact with the cytoplasmic tyrosine residues of the gp130 protein (Stahl et al., 1994). The resultant phosphorylation of gp130 allows for the sequestering and tyrosine phosphorylation of signal transducers and activators of transcription (STAT) proteins. Studies have shown that IL-6 signaling most commonly activates STAT3. These studies have also shown that IL-6 triggers STAT1 activation as well (Heinrich, Behrmann, Muller-Newen, Schaper, & Graeve, 1998; Zhong, Wen, & Jr, 1994) [reviewed in (Heinrich et al., 2003)].
STAT3 phosphorylation by the IL-6R/gp130 complex occurs at the Tyr$^{705}$ residue (Kaptein, Paillard, & Saunders, 1996). Upon phosphorylation, STAT3 proteins form a homodimer configuration and are actively translocated into the nucleus (Bromberg & Darnell, 2000). Once in the nucleus, STAT3 interacts and coordinates with several nuclear transcription factors to promote target gene expression in facilitating a major portion of the IL-6 induced signal transduction response. Removal of STAT3 reveals a total lack of IL-6 signaling, thus demonstrating the necessity of STAT3 in the IL-6 pathway [reviewed in (Calo et al., 2003)].
STAT3 has also found to be phosphorylated at another residue during IL-6 signaling. The Ser\textsuperscript{727} residue has been shown to be phosphorylated in several cancers where STAT3 is constitutively phosphorylated, including endometrial and cervical cancers (C. L. Chen et al., 2007), as well as myeloid leukemia (Schuringa, Wierenga, Kruijer, & Vellenga, 2000). The phosphorylation of STAT3 at the Ser727 residue has been found in ER\textalpha(-) breast cancer cells, and based on recent studies is thought to play a significant role in breast cancer pathogenesis (Yeh et al., 2006). This phosphorylation is thought to be signaled by IL-6 mediated activation of protein kinase C\textdelta (PKC\textdelta) after nuclear translocation, acting as an inhibitor of STAT3 DNA binding (Jain, Zhang, Kee, Li, & Cao, 1999). Several other IL-6 signaling events are also believed to have involvement in the phosphorylation of Ser\textsuperscript{727} on STAT3. However, the mediators from initial IL-6 signaling to activation of these kinase events are not yet known.
Chapter 3: Non-Diseased Interleukin-6 Signaling

3.1 IL-6 Signaling in Immunity

Through the rigorous research that has been performed over the last several decades, IL-6 has been linked to several aspects of normal biological function. Paramount is the role that IL-6 plays in late innate immunity and the transition to adaptive immunity. Studies performed by Hurst (Hurst et al., 2001) and Jones (Jones, 2005) have shown that IL-6 produced from neutrophil and local tissues in an acute inflammatory response mediates the down regulation of neutrophil chemokines (CXCL1, IL-8), while up-regulating the expression of monocytic chemokines (CCL2, CXCL5, CXCL6). These actions also coordinate with studies showing that IL-6 trans-signaling on endothelial cells mediates the expression of intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, and E-selectin (Q. Chen et al., 2006), as well as L-selectin expression on lymphocytes (Q. Chen et al., 2004). These functions facilitate active recruitment of cells essential to acquired immunity.

In T cell mediation, IL-6 trans-signaling has been shown to be necessary for T cell recruitment (McLoughlin et al., 2005). Similarly, IL-6 trans-signaling may inhibit T cell apoptosis through STAT3 induced Bcl-2, as shown in a study performed by Curnow (Curnow et al., 2004) in patients with uveitis, a disorder chronicled by a prolonged inflammation of the interior eye. IL-6 signaling has also been linked to the differentiation of both B- and T-cell lymphocyte lineages (Kopf et al., 1994). Recent studies have also shown that IL-6 may play an important role in the formation and continued maintenance of T_{H}17 cells, recently considered to be a factor in the onset of
autoimmune disorders (Bettelli et al., 2006; Ghoreschi et al., 2010) [reviewed in (Scheller et al., 2011)].

While IL-6 signaling is heavily involved in the local immune response, research has also shown involvement in the systematic acute phase response (APR) (Heinrich, Castell, & Andus, 1990). Described as more of an innate response, APR acts in part as a protection for the body against itself. In more persistent infections, the over production of pro-inflammatory molecules combined with the debris and refuse left behind during an active immune response, would begin to cause additional harm. This is often seen as the link between autoimmune and malignant diseases and chronic inflammation (Hanahan & Weinberg, 2011). Therefore, acute phase proteins (APP) are secreted from the liver to curb the effects of these stimulants and protect against systematic tissue damage. IL-6 has been shown to be one of the dominant effectors through STAT3 phosphorylation (Castell et al., 1988). Interestingly, a similar albeit local response has been shown to occur in the involution of milk-producing cells post birth. This process allows for the extensive apoptosis of cells, combined with localized tissue remodeling, without excessive inflammation. Like the acute phase response, proteins are secreted through a required IL-6/STAT3 mediated gene promotion of APP (Pensa, Watson, & Poli, 2009). This has also been linked to a possible causality for breast cancer, due to the convenient involvement of those factors necessary for tumor progression and success (Stein et al., 2004; Z. K. Sun, Yao, Liu, Tang, & Yan, 2006) [reviewed in (Pensa et al., 2009)].
3.2 IL-6 Signaling in Other Biological Functions

Aside from the determined role of IL-6/STAT3 signaling in and among the immune response, research over the years has continued to affirm the importance of IL-6/STAT3 signaling in several non-immune cell related biologic functions. Recent studies chronicled by Pedersen (Pedersen & Febbraio, 2008) have coined the term "myokine" for IL-6, due to the discovery that IL-6 is produced by muscle contraction during exercise. This inherent role in metabolism was also shown in studies where a lack of IL-6 in mice, lead to an increase in glucose intolerance, as well as insulin resistance (Matthews et al., 2010). To further explain the role of IL-6 in metabolism, studies have shown that mice with transgenic IL-6 production show a particularly smaller size of growth when compared to wild type mice, leading to the notion that classical IL-6 signaling in hepatocytes is necessary for the regulation of insulin sensitivity and the formation of adipose tissue (Peters et al., 2000).

Research also suggests that IL-6 may play an important role in osteoclast production. Studies have shown that trans-signaling events with sIL-6R induces osteoclast production, revealing that IL-6 is important in bone homeostasis (Poli et al., 1994; Tamura et al., 1993) [reviewed in (Scheller et al., 2011)]. Research has also shown that IL-6 signaling is relevant in the central nervous system, and along with other cytokines can activate glial cells (John, Lee, & Brosnan, 2003) [reviewed in (Hong et al., 2007)]. It is interesting to note that a great many of the roles that IL-6 plays in normal cellular function have been initially characterized in studies relevant to disease of particular tissues. Many of the functions overlap or are correlated to the immune
response, meaning that while the signaling may be presented as unique, the mechanisms are inherently inflammatory in nature.
Chapter 4: Interleukin-6 Signaling in Diseased States

Since IL-6 has been shown to play a significant part in a wide range of biological functions, it is no surprise that it may also play an important role in disease through abnormal signaling. This concept is what also makes many disorders so hard to treat. If a main mediator of the abnormal or diseased state is also involved in normal function, how will treatment aimed at this mediator affect the body systematically?

4.1 IL-6 Signaling in Autoimmune/Inflammatory Disorders

Since IL-6 is paramount in the proper function of both the innate and adaptive immune systems, any change of its function, whether it is over expression or a blockade of expression, causes adverse immune effects in the body (Neurath & Finotto, 2011). IL-6 has been extensively studied with regard to the role that it plays in autoimmune disorders. While most all disorders are characterized by a combined effect of several inflammatory cytokines and chemokines, it may only take one signaling event to prevent the cascade. Due to the direct pleiotropic regulation of inflammation by IL-6, almost all disorders characterized by chronic inflammation have been studied with regards to IL-6 signaling (Kallen, 2002; Naugler & Karin, 2008; Neurath & Finotto, 2011).

Rheumatoid arthritis (RA), a disease characterized by the chronic inflammation of joints, has been shown to express high levels of IL-6 in patient serum and synovium (Naugler & Karin, 2008). This expression is coupled with the presence of TH17 cells, described above as a known downstream signaling event of IL-6. Addition of anti-IL-6R antibody was effective at eliminating early onset TH17 cell production (Fujimoto et al.,
It has been shown that both classical and trans-signaling may play an important role in the progression of RA (Neurath & Finotto, 2011; Nowell et al., 2003).

Inflammatory bowel diseases (IBD), specifically Crohn's disease (CD) and ulcerative colitis (UC) have also been linked to IL-6 signaling (Mudter & Neurath, 2007b). As with RA, an increased presence of differentiated T cells was established in IBD. This is thought to be the cause of increased and sustained IL-6 production in the local tissue environment (Mudter & Neurath, 2007a; Yen et al., 2006). Recent researchers believe that IL-6 serum levels may be an effective way of determining the activity of IBD, as well as a promising marker for relapse prognosis (Umehara, Kudo, Nakaoka, Kawasaki, & Shiomi, 2006). Like in RA, the presence of sIL-6R has shown increases in the clinical activity of IBDs (Hosokawa et al., 1999).

4.2 IL-6 Signaling in Cancer

As previously described, one of the main attributes of cancer, is that its genetic makeup is nearly identical to that of a normal tissue. By exploring this fact, more research has become available linking the mechanisms of cancer survival, to an abnormal signaling event at the cellular level. Because of its inherent relevance in the mediation of inflammation, angiogenesis, and anti-apoptotic signaling, a significant amount of research has been done in an attempt to understand IL-6 signaling in cancer. As discussed in a review by Hong et al. (Hong et al., 2007), IL-6 has been implicated in multiple cancer types. Results have shown that IL-6 signaling can initiate stimulatory growth in glioma (Tchirkov et al., 2007), leukemia (Emilie et al., 1993), lymphoma
(Seymour, Talpaz, Cabanillas, Wetzler, & Kurzrock, 1995), multiple myeloma (Ludwig, Nachbaur, Fritz, Krainer, & Huber, 1991), ovarian (Watson, Sensintaffar, Berek, & Martinezmaza, 1990), and prostate (Giri, Ozen, & Ittmann, 2001) cancers. However, results have also shown IL-6 to act as a tumor growth inhibitor in lung (Bihl et al., 1998), melanoma (W. H. Sun, Kreisle, Phillips, & Ershler, 1992), and leukemia (Koistinen, Saily, Poromaa, & Savolainen, 1997) cancers. Contrary to these results, the McCall lab has published data showing IL-6 treatment as a stimulus of melanoma cells in vitro. Their published results suggest that phosphorylation of STAT3 at the Serine 727 residue occurs in M93-047 melanoma cells after IL-6 stimulation (Schwartz et al., 2009). One important correlation can be drawn however, that regardless of the inhibitory or stimulatory effect of IL-6, inflammation and its regulation seems to play an important role in cancer mediation. Similar results can also be found when discussing different types of breast cancer. (Bihl et al., 1998; Emilie et al., 1993; Giri et al., 2001; Hosokawa et al., 1999; Ludwig et al., 1991; Seymour et al., 1995; Tchirkov et al., 2007; Umehara et al., 2006; Watson et al., 1990) [reviewed in (Hong et al., 2007)].
Chapter 5: Role of Interleukin-6 in Breast Cancer

5.1 IL-6 Expression in Breast Cancer Cells

Breast cancer is a generic term that describes a malignancy of a tissue originating in the breast. Because of this, there are multiple types of breast cancers, each type characterized by unique gene expression. Several methods have been used for grouping specific types of breast cancer cells for effective analysis. The two most often used groupings are by phenotype (i.e. luminal, basal, basal-like) and hormone receptor gene expression (i.e. ER+, ER-, Triple negative, etc.) (Kao et al., 2009; Neve et al., 2006). The effect and expression of IL-6, is therefore different in unique breast cancer cell lines. Recent studies have shown that the triple negative breast cancer (TNBC) cell line MDA-MB-231 has high basal expression of IL-6 (Voss et al., 2011), while ER+ breast cancer cell line MCF-7 have been shown to have little to no expression of IL-6 (Chiu, Sgagias, & Cowan, 1996). It is interesting to note that when phenotypes are compared, MCF-7 cancer cells are a less invasive luminal cancer cell line (Perou et al., 2000) [reviewed in (Neve et al., 2006)], whereas MDA-MB-231 are a classic model of a post-epithelial to mesenchymal transition (EMT) cell line, often exhibiting a more aggressive and invasive nature (Chin et al., 2006) [reviewed in (Kao et al., 2009)]. However, independent of IL-6 autocrine signaling in breast cancer, the presence of IL-6 in the local microenvironment could illicit paracrine signaling in the surrounding tissues. Signaling of this manner may lead to further paracrine signaling, and the possibility of continual upregulation of IL-6. This ability may also involve tandem signaling with other cytokines and regulatory molecules.
5.2 Expression of IL-6R and sIL-6R in Breast Cancer

The true mediator of IL-6 signaling is the gene expression status of membrane bound IL-6R, or expression of, or paracrine presence of, sIL-6R. Previous research has shown that IL-6R is not present on endothelial tissues (Romano et al., 1997). The presence of membrane bound IL-6R on epithelial breast cancer remains controversial. A study performed by Chiu describes the expression of membrane bound IL-6R on the surface of ER(-) (i.e. MDA-MB-231) breast cancer cells, but not on ER(+) (i.e. MCF-7) breast cancer cells. The same study claimed that sIL-6R was secreted by MCF-7 cells, but was not secreted by MDA-MB-231 (Chiu et al., 1996). These results regarding the secretion of sIL-6R in ER(+) and ER(-) breast cancer cells were also shown in research performed by Singh et al. (Singh et al., 1995).

5.3 Prognostic Role of IL-6 in Breast Cancer

Several studies have been performed in an attempt to link the presence of IL-6 in the local tissue environment, as well as blood serum levels of IL-6, to a possible prognosis for the disease. In studies performed by Salgado (Salgado et al., 2003) and Zhang (Zhang & Adachi, 1999), high levels of IL-6 have been shown to correlate with poor survival rates in patients with metastatic breast cancer. These levels of IL-6 in metastatic breast cancer patients have been compared to local (non-metastatic) breast cancer patients with lower levels of IL-6. Non-metastatic cancers, exhibiting lower levels of IL-6 exhibited a higher survival rate (Benoy et al., 2002). In another comparison, high
levels of IL-6 most often correlated with a clinical stage of cancer progression (Bozcuk et al., 2004).

5.4 Effect of IL-6 signaling on Breast Cancer

The effect of IL-6 signaling on breast cancer cells *in vitro* has been shown to be dependent on the type of breast cancer cells receiving the signal. In terms of effects on growth and proliferation, studies have shown that IL-6 has no effect on MDA-MB-231 cells (Asgeirsson, Olafsdottir, Jonasson, & Ogmundsdottir, 1998). A similar study also reported that the growth of MCF-7 breast cancer cells were also not affected by IL-6 stimulation (Honma et al., 2002). However, Chiu (Chiu et al., 1996) reported that IL-6 signaling in ERα(+) breast cancer cell lines (MCF-7, ZR-75-1) caused cellular apoptosis. It should be noted that this same study also recorded results that support the lack of an effect on MDA-MB-231 cell growth. Similar studies have suggested that IL-6 signaling may also be responsible for drug resistance in both MCF-7 and MDA-MB-231 breast cancer cell lines (Conze et al., 2001; Haverty, Harmey, Redmond, & BouchierHayes, 1997).

The proangiogenic signaling of IL-6 has also been studied. Angiogenic factors such as matrix metaloproteases (MMP) and vascular endothelial growth factor (VEGF) are direct products of STAT3 signaling (Yu, Kortylewski, & Pardoll, 2007).

Recent studies have shown that STAT3 knockdown may cause inhibition of the growth and size of those breast cancers that usually express constitutive p-STAT3. The same studies have shown that a removal of available IL-6 also causes a decrease in
STAT3 mRNA and p-STAT3 activation in MDA-MB-231 (Berishaj et al., 2007; Bromberg, 2000). These results suggest that IL-6 is necessary for invasive breast cancer growth and proliferation.
Chapter 6: Interleukin-6 as a Target for Treatment

The targeting of IL-6 and the IL-6/STAT3 signaling pathway has been explored and implemented in several other diseases. Foremost of these treatments is Tocilizumab/Actemra, a humanized anti-IL-6Rα antibody used to treat arthritis and Castleman’s disease in Japan (Emery et al., 2008). Initially a Japanese product, Actemra is licensed in North America by Roche and has been approved by the Food and Drug Administration (FDA) for treatment of rheumatoid arthritis (Genovese et al., 2008; Smolen et al., 2008) [reviewed in (Ara & DeClerck, 2010)]. Recently, toculizumab has been examined in pre-clinical cancer trials, as well as animal models for oral squamous cell carcinoma in vivo (Shinriki et al., 2009). Results reported by Shinriki show that mice injected with OSCC tumor cells, show a decrease in tumor vascularization and STAT3 phosphorylation after treatment with toculizumab (Shinriki et al., 2009).

Non-antibody small molecule treatments for the IL-6/STAT3 signaling pathway are also being explored. However, the majority of these molecules have only been tested in preclinical applications of cancer. INCB20 is a small molecule inhibitor of the JAK family of kinases, and has shown potential for the treatment of myeloma in vitro and in vivo mouse models (Burger et al., 2009). Another molecule, 531-201 from the National Cancer Institute library inhibits the DNA binding potential of STAT3 in MDA-MB-231 in vitro mouse studies (Siddiquee et al., 2007). A recent FDA approved thalidomide derivative, Lenalidomide (Revlimid, Celgene, NJ), has shown inhibition of IL-6 in melanoma and myeloid cell lines (Lu et al., 2009). Because of the potential for IL-6
signal inhibition, these compounds are currently being tested both *in vitro* and *in vivo* in multiple diseases and cancers.
Chapter 7: Phenylmethimazole

Recent research has shown that phenylmethimazole (C10), a derivative of methimazole (MMI), a drug used for the treatment of autoimmune Grave’s disease, may show promise for the treatment of cancer (McCall et al., 2007; Schwartz et al., 2009). Currently, C10 and its derivatives are being included in studies for the treatment of several types of cancer and several autoimmune disorders. Initial research has shown evidence that C10 is successful at inhibiting the TLR3 signaling pathway in thyrocytes, a finding with relevance to Hashimoto's thyroiditis (Harii et al., 2005), as well as in papillary thyroid cancer (PTC) (McCall et al., 2007). More recent studies have shown that C10 treatment of pancreatic and melanoma cancers caused a decrease in growth and migration/proliferation (Schwartz et al., 2009). This was again paired with a reduction in TLR3 signaling (Schwartz et al., 2009). Because of the decrease in TLR3, a decrease of the subsequent TLR3 activation of NF-κB and IRF3 transcription factors also lead to a reduction of inflammatory cytokines, including IL-6. Interestingly, these studies also revealed that C10 effectively blocked IL-6 induced phospho-STAT3\textsuperscript{Tyr705} and phospho-STAT3\textsuperscript{Ser727} in pancreatic and melanoma cells \textit{in vitro} (McCall et al., 2007; Schwartz et al., 2009).

These findings have led to the hypothesis that C10 may be effective at treating other types of cancers similar in expression to the previously studied pancreatic and melanoma cancer types. In particular, several invasive breast cancers have been characterized by basally high levels of IL-6. MDA-MB-231—a triple negative breast cancer cell line—is a prime of example of this. Based on the knowledge that C10 has
been successful at decreasing levels of TLR3-induced cytokines (i.e. IL-6), it is prudent
to examine the effects of C10 on this cell line. A characterization of any subsequent
effects on growth may produce a link between IL-6 over expression and its prospective
necessity to the overall progression and health of the cancer cells. Secondarily, a
chemical derivative of C10, COB-141, may exhibit similar potential for inhibition in
cancer cells. A parallel study with COB-141 could reveal a compound with an even
higher potential for the treatment of cancer than the already established C10.
Chapter 8: Specific Aims

**Hypothesis:** Phenylmethimazole (C10) and a closely related derivative, C-141, will cause a decrease of basally high interleukin-6 production, along with a decrease in growth in MDA-MB-231 triple negative breast cancer cells.

The following specific aims will be examined experimentally in order to test the validity of the given hypothesis.

**A. Determine whether treatment with either C10 or C-141 decreases the production of IL-6 by MDA-MB-231 cells.**

As previous research has revealed, C10 has shown a positive effect in the reduction of IL-6 in other cancer cell lines (McCall et al., 2007; Schwartz et al., 2009). This aim will test whether or not there is a disruption of IL-6 at either the transcriptional or translational levels in MDA-MB-231 breast cancer cells after treatment with C10 or COB-141. Cells will be treated with each drug at a predetermined concentrations used in previous research. Transcription will be tested via QRT-PCR, and translation by an ELISA of MDA-MB-231 conditioned media. Expression of mRNA quantity and concentration of secreted protein will be compared to untreated and DMSO-carrier control samples. Both time and dose dependent studies will be run separately to determine the decrease in IL-6 production that either compound has on MDA-MB-231 cells. Using the results from the dose dependent study, a half-maximal inhibitory concentration (IC$_{50}$) of C10 and C-141 can be calculated for IL-6 reduction.
B. Determine whether treatment with either C10 or C-141 has an effect on the viability and growth of MDA-MB-231 cells.

This aim will analyze any effect that the compounds may have on the overall health of the cancer cells. This will be accomplished by the use of a live cell MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay to determine relative viability of the cells post treatment. A bromodeoxyuridine (BrdU) incorporation assay will be used to determine any effect on cell proliferation and growth post treatment. Independent dose and time response studies will be utilized to determine any prolonged effects.

C. Characterize the role of the IL-6 autocrine signaling pathway on the cellular viability and growth of MDA-MB-231 cells.

Previous research from multiple groups has resulted in an indecisive conclusion as to whether or not IL-6 does in fact act in an autocrine manner in MDA-MB-231 triple negative cells. Since the cells do express a rather high basal level of IL-6, this aim will experimentally test for the presence gp130, and IL-6 receptor to determine if the IL-6 signaling pathway is intact in MDA-MB-231 cells. This will be accomplished through QRT-PCR. FACS analysis will be used to evaluate the presence of functional membrane bound IL-6 receptor and the gp130 signal transduction molecule. As a known effect of IL-6 stimulation, western blot analysis for phosphorylated STAT3 will be performed after exogenous treatments with recombinant human IL-6. To determine the effect of endogenously produced IL-6 on MDA-MB-231 cells, a set of IL-6 antibody blocking studies will be performed, followed by reintroduction of rhIL-6.
Chapter 9: Materials and Methodology

9.1 Cell Culture

The triple-negative breast cancer cell line (MDA-MB-231), ERα+ breast cancer cell line (MCF-7), and pancreatic adenocarcinoma cell line (PANC-1) were purchased from the American Type Tissue Center (ATTC) (Manassus, VA). All three cell lines were cultured in Dulbecco’s Modified Eagle Medium (DMEM) with high glucose purchased from Thermo Fisher Scientific (Waltham, MA), 10% heat inactivated fetal bovine serum (FBS) purchased from Lonza (Walkersville, MD), and 1% penicillin/streptomycin (Lonza) (Berishaj et al., 2007). Human whole blood was drawn in compliance with IRB approval. Peripheral blood mononuclear cells (PBMC) were isolated through ficoll density gradient centrifugation using Histopaque 1077 purchased from Sigma Aldrich (St. Louis, MO) and were immediately used in the experiments.

9.2 Compound Cell Treatment

All treatments of cultured cells were done in prepared growth culture media. Phenylmethimazole (C10) and COB-141 were diluted in 0.25% Dimethyl Sulfoxide (DMSO) (Lonza) as a carrier to maintain compound solubility. Cells were incubated at 5% CO₂ at 37°C. For studies longer than one day, culture media containing the respective drugs cells was replaced every 24 hours. At this time, treated media was completely aspirated and replaced with freshly prepared treatment media to ensure continuous and consistent exposure to the compound.
9.3 Quantitative Real Time Polymerase Chain Reaction (QRT-PCR)

All cell lines were cultured in 6-well tissue culture vacuum plasma-treated plates (BD Biosciences). Cells underwent treatment and were lysed directly on the growth surface. Lysates were homogenized using QIAshredder microcentrifuge spin-column homogenizers and were purified using the RNeasy Mini Kit, both purchased from Qiagen (Valencia, CA) (Schwartz et al., 2009). Genomic DNA was eliminated using the RNase-free DNase Set (Qiagen) (Schwartz et al., 2009). Purified mRNA samples were quantified using the Nanodrop 2000 Micro-volume UV-Vis Spectrophotometer (Thermo Scientific). cDNA was subsequently synthesized from 1000 ng of each sample with the High capacity cDNA Reverse Transcription Kit purchased from Applied Biosystems (now Life Technologies, Carlsbad, CA) using an Eppendorf Mastercycler gradient. Real-time PCR was performed using Taqman® Gene Expression Assays, Taqman® Gene Expression Master Mix, and the StepOnePlus™ Real-Time PCR System (Applied Biosystems). β-actin was used as the endogenous control primer alongside target primers in a multiplex format. Results were calculated using the ΔΔC_{T} method, and represented as fold expression relative to a 0.25% DMSO control unless otherwise stated.

9.4 ELISA Secreted Protein Analysis

Cell supernatants were harvested, centrifuged to remove floating cells and cellular debris, and were stored at -20°C until needed. Non-tissue culture treated 96-well plates purchased from BD Biosciences (San Jose, CA) were used in conjunction with the BD OptEIA Human IL-6 ELISA Set and Reagent set B (BD Biosciences) according to
manufacturer specifications. Plates were read using a 96-well plate reader at dual wavelengths of 450 nm and 570 nm. Samples were run alongside a kit-provided set of standards. The resultant OD readings from these standards, fit to a 4- or 5- parameter regression using the SoftMax software package (Molecular Devices), were used to interpret the concentrations of IL-6 present in each sample. Results were given as averages of percent (%) inhibition of IL-6 ± SEM over three experiments.

9.5 MTS Assay

Specific cell quantities were seeded in 96 well plates to allow for proper growth at each of the desired time points: 2.5x10^4 cells/well for 24 hours, 2.0x10^4 cells/well for 48 hours, 1.0x10^4 cells/well for 72 hours, and 5.0x10^3 cells/well for 96 hours. Each plate contained a set of “blank” wells containing media only, testing the culture media for background signal. Treatments were added and cells were incubated at 5.0% CO_2 and 37°C. The CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Kit (Promega)-provided MTS solution was added directly to the media of each well according to manufacturer protocol, at a confluence of at least 70% after the desired incubation period (McCall et al., 2007). The cells were then incubated for another 2 – 4 hours. Each plate was read on a plate reader at 490 nm for absorption. Results were given as percent (%) viable cells ± SEM.
9.6 Bromodeoxyuridine (BrdU) Incorporation Proliferation Assay

The Bromodeoxyuridine (BrdU) cell proliferation kit (Millipore) was used according to the provided protocol for this assay and as previously reported (Schwartz et al., 2009). Cells were seeded into 96 well plates at quantities of 25,000 cells/well, 20,000 cells/well, 10,000 cells/well, and 5,000 cells/well for 24 hour, 48 hour, 72 hour and 96 hour incubation respectively. Treatments were added to each well at a volume of 200 µL, along with 20 µL of BrdU solution. Fresh treatment solution and BrdU solution was re-administered every 24 hours. At the end of incubation, supernatants were removed and cells were blocked to the surface of the 96 well plate with a fixing solution provided with the kit. The amount of BrdU incorporation was measured via absorbance at a dual wavelength of 450/550 nm using a spectromax 96 well plate reader. Results were reported as percent (%) proliferation ± SEM for n=3 experiments.

9.7 Flow Cytometry Assay (FACS)

Flow cytometry was performed following a protocol initially published by the lab of Monica Burdick (Burdick M.M., 2006). Cells were seeded and treated in 10 cm tissue-culture treated dishes (BD Biosciences). Cells were harvested by the use of enzyme free dissociation solution (Thermo Fisher) incubation. Cells were suspended in culture media and counted using a Hauser Bright Line Hemocytometer (Thermo Fisher). The cells were centrifuged, the supernatant was removed, and the pellet was re-suspended in 1X HBSS+ with 1 wt% BSA (Lonza) to wash remaining media from the cells. Cells were then re-suspended in 1x10^7 cells/mL in HBSS+ with 1 wt% BSA. 20-25 µL (2.0x10^5
– 2.5x10^5 cells) of cell solution was aliquoted to micro centrifuge tubes or wells of a 96 well plate for each sample. The samples were centrifuged into a pellet and the supernatant was removed for disposal. Primary antibody or isotype control solution at a dilution of 1:50 was added to each applicable well and incubated on ice for 30 minutes. This was followed by several wash and centrifugation steps in HBSS^+ with 1 wt% BSA. Secondary antibody diluted in HBSS^+ with 1 wt% BSA to a concentration of 10 µg/mL was then added and the samples were incubated on ice for 30 min. Subsequent washes in HBSS^+ with 1 wt% BSA followed by tertiary staining were performed if necessary. All fluorophore conjugated stains were performed in the dark. Finally, cells were washed further and re-suspended in 4% paraformaldahyde. Samples were subsequently analyzed using the FACSARia II custom order flow cytometer apparatus (BD Biosciences).

For IL-6Rα expression, a three step staining protocol was used to enhance the signal. A mouse anti-IL-6Rα IgG monoclonal antibody (mAb) (M5) (BD Biosciences) was used for primary detection, followed by a secondary anti-mouse goat biotinylated polyclonal antibody (pAb) purchased from Santa Cruz (Santa Cruz, Ca). A tertiary staining utilizing a phycoerythrin (PE) conjugated strepavidin (SA) molecule (BD Biosciences) was used for fluorescent detection.

Gp130 staining was initiated using a mouse anti-human gp130 IgG mAb (BD Biosciences) for primary detection, followed by a FITC conjugated rabbit anti-mouse IgG (BD Biosciences) secondary antibody for fluorescent detection. A mouse IgG κ (BD Biosciences) was used for all isotype controls.
9.5 Nuclear Protein Western Blot Analysis

Cells were grown on 10 cm tissue culture treated dishes (BD Biosciences, Maryland) to allow for an adequate quantity of extracted protein. Cells were harvested by the use of trypsin-versene (Thermo Fisher) incubation. Cells were then suspended in media and spun at 1.2 x g for 5 min. The supernatant was removed and the remaining cell pellets were re-suspended in 1 mL of 1X DPBS (Lonza) and centrifuged at 500 x g for 5 minutes. Cytoplasmic and nuclear protein was isolated and extracted using the NE-PER nuclear and cytoplasmic isolation kit (Pierce) as previously reported (Schwartz, 2009). All procedures were performed as described in the manufacturer's protocols. The nuclear proteins were quantified using the Micro BCA protein assay kit (Pierce) and the Nanodrop 2000 Micro-volume UV-Vis Spectrophotometer (Thermo Scientific) (Schwartz et al., 2009). Samples were run alongside the Odyssey dual color molecular marker (Licor) on a Nupage 4-12% Bis-Tris gel (Invitrogen, NP0322), according to the manufacturer’s protocol. Completed blots were transferred to a Nitrocellulose membrane (NCM) purchased from Invitrogen (now Life Technologies, Carlsbad, CA) according to the manufacturer’s protocol. Transferred membranes were blocked in Odyssey Blocking Buffer (Licor) for 1 hour at room temperature on a rotating table. Blocking was followed by an overnight incubation at 4°C in a primary mAb solution at a dilution of 1:1000 in blocking buffer while gently oscillating. The membrane was incubated at room temperature in a secondary mAb solution at a dilution of 1:20,000. Following incubation, the NCM was read on the Licor Odyssey infrared imaging system. Subsequent staining for molecules of different molecular weights can be done on the same membrane following a
5-10 min incubation in Odyssey Stripping Buffer (5x) (Licor) at a dilution of 1:5 in deionized water.

Primary antibodies, mouse anti-human phosphorylated STAT3 at the tyrosine 705 residue and mouse anti-human β-actin control were purchased from Cell Signaling (Danvers, MA). Antibodies for total STAT3 (TSTAT3) were purchased from BD Biosciences, and antibodies for anti-human phosphorylated STAT3 at the serine 727 residue were purchased from Invitrogen. Secondary rabbit anti-mouse IgG conjugated with a 800 nm fluorescence dye along with the dual color molecular weight marker were purchased from Licor.

9.9 Statistics

All experiments were run in at least triplicate for each sample of cells or cell supernatants. Data sets are represented by mean ± SEM for each sample. Statistical relevance was analyzed using either a T-test, one-way, or two-way ANOVA test via the Statistica software package. All ANOVA analysis was followed by a Tukey T-test. All significant relevance was determined and reported by a 95% confidence interval or higher.
Chapter 10: Experimental Results

10.1 MDA-MB-231 cells express high levels of interleukin 6 (IL-6) and Toll-like receptor 3 (TLR3)

Previous studies performed by the McCall lab have shown that cancer cells constitutively expressing TLR3, like pancreatic and melanoma cancers, show decreased TLR3 signaling, along with growth and proliferation when treated with C10 (Schwartz et al., 2009). Figure 3 shows the results of the QRT-PCR for MDA-MB-231 breast cancer cell basal expression of TLR3 with respect to PANC-1 pancreatic adenocarcinoma cells. MDA-MB-231 cells were shown to express ~17 fold greater TLR3 expression than PANC-1 cells. The high basal expression of TLR3 in MDA-MB-231 cells initially suggests that C10 and COB-141 may elicit similar results in MDA-MB-231 as previously reported for other TLR3-expressing cancer cell lines.
Figure 3. MDA-MB-231 exhibit elevated expression of TLR3. MDA-MB-231 cells and PANC-1 cells were analyzed for basal mRNA expression of TLR-3 via QRT-PCR. MDA-MB-231 were shown to have an elevated expression of TLR3 when compared to TLR3 expression in PANC-1 cells. Graph is representative of n=3; **p≤0.01 as determined by t-test compared to PANC-1 cells.

MDA-MB-231 TNBC cells were also tested for basal expression of IL-6 using QRT-PCR. Previous research published by the McCall lab has shown that C10 was effective at decreasing the expression of IL-6 in TLR3-expressing pancreatic and melanoma cancers (Schwartz et al., 2009). PANC-1 pancreatic adenocarcinoma cells and MCF-7 ER(+) breast cancer cells were also tested for comparison. Figure 4 shows the results of the QRT-PCR analysis for basal expression of IL-6 with respect to that of MCF-7 cells. PANC-1 cells were shown to have twice the IL-6 expression of MCF-7 cells. Strikingly, the basal expression of IL-6 mRNA in MDA-MB-231 cells, was greater than 2,000 fold the expression of MCF-7 cells.
Figure 4. MDA-MB-231 cells exhibit high basal expression of IL-6. MDA-MB-231 cells were analyzed for basal mRNA expression via QRT-PCR. The cells exhibited a high expression when compared to PANC-1 pancreatic cancer cells and MCF-7 ER+ breast cancer cells. Graph is representative of n=3; *p≤0.05 as determined by one-way ANOVA with a t-test (Tukey) compared to MCF-7 cells.

10.2 C10 and COB-141 decrease IL-6 expression in MDA-MB-231 cells

In order to accurately determine the possible in vitro effects of C10 and COB-141 on MDA-MB-231 cells, treatments of each were added at a predetermined concentration prior to QRT-PCR for transcribed IL-6 mRNA. Each experiment was run in triplicate with untreated and 0.25% DMSO carrier control groups run in parallel with C10 and COB-141 groups. The concentrations of C10 and COB-141 were 500 µM and 40 µM respectively. As seen in Figure 5, both C10 and COB-141 exhibited a profound effect on the quantity of transcribed mRNA for IL-6 in MDA-MB-231 cells. C10 showed the ability to decrease IL-6 transcription by 80% at 500 µM. Similarly, COB-141 had a 75% decrease in IL-6 transcription at 40 µM, a tenfold lower concentration from C10.
**Figure 5. MDA-MB-231 production of IL-6 is decreased by treatment of C10 and COB-141.** MDA-MB-231 cells were treated with initial concentrations of 500 µM C10 and 40 µM COB-141 in order to test the effect of treatment on IL-6 transcription. IL-6 transcription was reduced by as much as 80% with 500 µM C10 and 75% with 40 µM COB-141. Graph is representative of n=3; *p≤0.05 as determined by one-way ANOVA with a t-test (Tukey) compared to 0.25% DMSO control.

**10.3 IL-6 half maximal inhibition concentrations (IC$_{50}$) of C10 in MDA-MB-231 cells**

Because C10 was effective at significantly decreasing the basal expression of IL-6 in MDA-MB-231 cells at the initial concentrations of 500 µM, a dose response measuring the attenuation of IL-6 over a range of concentrations was determined. The quantity of mRNA transcribed for IL-6 in MDA-MB-231 cells after 24 hours of C10 treatment at concentrations of 500, 250, 100, 80, 40, and 20 µM was tested via QRT-PCR. The supernatant of each sample was also tested for secreted IL-6 protein via ELISA. Figure 6 reveals that mRNA transcribed for IL-6 increases in MDA-MB-231 cells as C10 concentrations decrease. A regression fit reveals that in regards to mRNA transcribed for IL-6, the IC$_{50}$ for C10 is 157 µM. Figure 7 shows the dose dependent decrease in
translated protein found in the supernatant of cells cultured in the aforementioned C10 concentrations. While the decrease is still very much apparent, the calculated IC<sub>50</sub> for secreted protein is 238 µM, an increase in the recorded IC<sub>50</sub> value for transcribed mRNA. The disparity in the IC<sub>50</sub> values for transcribed mRNA and translated protein after 24 hours of incubation could be in part due to the already transcribed mRNA within the cells prior to treatment.

![Graph](image)

**Figure 6.** IC<sub>50</sub> of IL-6 transcription in MDA-MB-231 cells from treatment with C10. MDA-MB-231 cells were treated with a range of C10 concentrations to determine the dose dependent effect on IL-6 mRNA expression, ultimately calculating an IC<sub>50</sub> value. For IL-6 transcription inhibition, the calculated IC<sub>50</sub> based on a polynomial regression is 157µM. Data are averages of n=3 ±SEM.
Figure 7. IC$_{50}$ of IL-6 translation in MDA-MB-231 cells from treatment with C10. MDA-MB-231 cells were treated with a range of C10 concentrations to determine the dose dependent effect on IL-6 translation, ultimately calculating an IC$_{50}$ value. For IL-6 translation inhibition, the calculated IC$_{50}$ based on a polynomial regression is 238 µM. Data are averages of n=3 ±SEM.

10.4 IL-6 half maximal inhibition concentrations (IC$_{50}$) of COB-141 in MDA-MB-231 cells

As with C10, COB-141 significantly decreased the level of IL-6 transcription in MDA-MB-231 cells after a 24 hour incubation at the initial concentration of 40 µM. Therefore, COB-141 was likewise tested for transcription of IL-6 and translation of IL-6 over a range of concentrations: 250, 100, 80, 40, 20, and 10 µM. The shifted range of COB-141 was due in part to the affectivity of the compound at 40 µM, a much lower concentration compared to 500 µM of C10, as well as the lack of solubility of COB-141 at concentrations around 500µM in 0.25% DMSO (visual observation; data not shown). Figure 8 reveals the dose dependent response of COB-141 on IL-6 transcription tested via
QRT-PCR. Based on the regression fit, the calculated IC\textsubscript{50} for IL-6 transcription in MDA-MB-231 cells of COB-141 is 8.4 µM. The distribution of the data points reveals what appears as two distinct phases of transcription that were affected by COB-141. In order to achieve the best fit, two curves were implemented. The lower curve was used to calculate the IC\textsubscript{50} value for COB-141. Figure 9 reveals the dose dependent response of COB-141 on the quantity of IL-6 secreted from MDA-MB-231 via ELISA. Based on a regression fit, the calculated IC\textsubscript{50} of COB-141 with regards to IL-6 translation is 43 µM.

**Figure 8. IC\textsubscript{50} of IL-6 transcription in MDA-MB-231 cells after treatment with COB-141.** MDA-MB-231 cells were treated with a range of COB-141 concentrations to determine the dose dependent effect on IL-6 transcription, ultimately calculating an IC\textsubscript{50} value, based on the lower curve. For IL-6 transcription inhibition, the calculated IC\textsubscript{50} based on a polynomial regression is 8.4 µM. Data are averages of n=3 ±SEM.
Figure 9. IC$_{50}$ of IL-6 translation in MDA-MB-231 cells from treatment with COB-141. MDA-MB-231 cells were treated with a range of COB-141 concentrations to determine the dose dependent effect on IL-6 translation, ultimately calculating an IC$_{50}$ value. For IL-6 translation inhibition, the calculated IC$_{50}$ based on a polynomial regression is 43 µM. Data are averages of n=3 ±SEM.

10.5 Interleukin-6 does not affect phosphorylation of STAT3

In order to investigate whether or not IL-6 is capable of affecting MDA-MB-231 cells directly, experiments were conducted with the introduction of exogenous rhIL-6 or removal of endogenous IL-6 from the growth environment of the cells.

10.5.1 Treatment of MDA-MB-231 cells with exogenous IL-6 does not affect STAT3 phosphorylation

Previous research in the McCall lab has shown that PANC-1 pancreatic cancer cells treated with exogenous IL-6 exhibit an increase in phosphorylation of the tyrosine 705 residue of the STAT3 nuclear transcription factor. Similarly, the same research
showed that phosphorylation of the serine 727 residue of STAT3 increases in melanoma cancer cells treated with exogenous IL-6 (Schwartz et al., 2009). To further test the presence of IL-6 signaling in MDA-MB-231 cells, a similar experiment was conducted with MDA-MB-231 cells. The phosphorylation of STAT3 at both the tyrosine 705 and serine 727 residues was analyzed via western blot analysis of nuclear protein harvested from both MDA-MB-231 cells and PANC-1 cells treated with 20 ng/ml rhIL-6. Figure 10A reveals that PANC-1 cells show an increase in phospho-STAT3$^{Y705}$, while MDA-MB-231 cells show no change in expression with exogenous IL-6 treatment. Similarly, total STAT3 staining reveals no change in overall STAT3 expression, supporting the hypothesis that IL-6 is not causing a signaling event in MDA-MB-231 cells. Figure 10B shows the results for phosphorylation of STAT3 serine 727 following treatment with exogenous IL-6. Treatment with exogenous IL-6 did not elicit increases in phosphorylation of STAT3 serine 727 in either PANC-1 or MDA-MB-231 cells.
Figure 10. Phosphorylation of STAT3 at the tyrosine 705 and serine 727 residues in MDA-MB-231 cells is not affected by rhIL-6 exogenous treatment. Untreated MDA-MB-231 and PANC-1 cells were tested alongside rhIL-6 treatments of each cell for a change in STAT3 phosphorylation. A) rhIL-6 treatment was shown to have no effect on MDA-MB-231 cells at the tyrosine 705 residue of STAT3, while PANC-1 cells exhibit an increase in phosphorylation. B) MDA-MB-231 cells express small amounts of the phosphorylated serine 727 residue of STAT3, with no change in signal after rhIL-6 treatment. Data are representative of n=3.

10.5.2 Sequestration of IL-6 does not change the phosphorylation state of STAT3 in MDA-MB231

In order to further test whether or not IL-6 produced by MDA-MB-231 cells induces an autocrine signaling event, a polyclonal anti-IL-6 antibody was added to cells in culture, in an attempt to attenuate any basal phosphorylation that may be present in the cells due to an interleukin-6 signal cascade. Figure 11 shows that MDA-MB-231 cells with addition of the blocking antibody exhibit no change in p-STAT3$^{Y705}$ or the TSTAT3...
expression. This again shows that IL-6 produced by MDA-MB-231 cells does not signal in an autocrine fashion within the cells.

**Figure 11. Blocking of Interleukin-6 in culture does not alter STAT3 phosphorylation of MDA-MB-231 cells.** Untreated MDA-MB-231 and cells treated with an IL-6 blocking antibody were tested for changes in STAT3 phosphorylation at the tyrosine 705 residue. No changes were seen in any of the treatment cases. This was seconded by the lack of change in TSTAT3 top band, representing all phosphorylated residues of STAT3. Data are representative of n=3.
10.6 MDA-MB-231 cells lack expression of surface bound Interleukin 6 receptor α (IL-6Rα)

The data so far have shown that C10 and COB-141 effectively decrease IL-6 production in MDA-MB-231 cells. Because of the initial over expression of IL-6, it is important to see whether or not an intact IL-6 autocrine signaling pathway exists in the cells. MDA-MB-231 cells were tested alongside MCF-7 cells for IL-6Rα by QRT-PCR. Figure 12A shows that the relative expression of IL-6Rα in MDA-MB-231 compared to that of MCF-7 is considerably less. This decrease is so significant, that the results suggest that MDA-MB-231 may not express surface bound IL-6Rα. To further investigate this conjecture, MDA-MB-231 cells were ran in parallel with isolated human peripheral mononuclear cells (PBMCs) via flow cytometry for IL-6Rα cell surface expression. As a positive control, the monocyte cell population was isolated by gating of the PBMC population, and can be seen to express IL-6Rα, when compared to an isotype control (Figure 12B). In contrast, MDA-MB-231 cells do not express IL-6Rα when compared to the isotype control (Figure 12B). Red signal represents the isotype control, where the blue tinted signal represents cells treated with the mAb for IL-6Rα. These results, taken together with the QRT-PCR analysis (Figure 12A), provide compelling evidence that MDA-MB-231 cells do not express the membrane bound IL-6 receptor.
Figure 12. MDA-MB-231 cells lack a membrane bound IL-6 receptor α. A) Untreated MDA-MB-231 cells were tested alongside untreated MCF-7 cells, as a positive control, for presence of IL-6Rα mRNA expression. B) MDA-MB-231 cells and PBMCs were tested for membrane expression of IL-6Rα. PBMCs show a definite shift in fluorescence (blue) compared to isotype control (red), while MDA-MB-231 cells have no change, and thus no expression.
MDA-MB-231 cells express surface bound gp130 (a.k.a. IL-6ST/IL-6Rβ)

Despite the apparent lack of IL-6Rα on MDA-MB-231 cells, it is important to analyze the rest of the IL-6 signaling pathway. In order to determine the presence of gp130 (IL-6ST) on the surface of MDA-MB-231 cells, QRT-PCR was run with MCF-7 breast cancer cells as a positive control. The results show that both cell lines basally express gp130 (Figure 13A). However, the expression of gp130 in MDA-MB-231 cells is over four times higher than that of MCF-7 cells. The presence of gp130 was also tested via flow cytometry, using monocyte populations (gating of the PBMC population) as a positive control. Figure 13B shows that both monocytes and MDA-MB-231 cells test positive for gp130 surface receptor expression when compared to the respective isotype controls. Red signal represents the isotype control, where the blue tinted signal represents cells probed with the gp130 antibody.
Figure 13. MDA-MB-231 express gp130 signal transduction protein. A) Untreated MDA-MB-231 cells were tested alongside untreated MCF-7 cells as a positive control for presence of gp130 mRNA expression. MDA-MB-231 are shown to express more than 3 times the signal of MCF-7 cells. B) MDA-MB-231 cells and monocytes were tested for membrane expression of IL-6Rα. PBMCs show a definite shift in fluorescence (blue) compared to isotype control (red). Similarly, MDA-MB-231 cells also exhibit a shift, revealing positive expression.
10.8 Effect of C10 and COB-141 on growth/proliferation of MDA-MB-231 cells

The following data are preliminary, making it difficult to arrive at definitive conclusions in regards to the effects of the compounds on proliferation and viability in MDA-MB-231 TNBC cells. That being said, the data is presented below for archival purposes with limited statistical analysis. Statistical analysis was limited to data that exhibit a difference of greater than 30% compared to the DMSO control.

Previous research done by the McCall lab has shown that cancer cells, which basally express high levels of IL-6, treated with C10 exhibit a decrease in the growth and proliferation (McCall et al., 2007; Schwartz et al., 2009). Based on these findings, C10 and COB-141 have the potential to exhibit a change in the growth and proliferation of MDA-MB-231 cells. The effect on proliferation of MDA-MB-231 cells by both compounds was analyzed using a BrdU incorporation assay. Cells were treated with the maximal concentration used in the prior dose response experiments, and a second concentration representative of an average of the IC₅₀ found for transcription and translation of IL-6 for each compound. Figure 14 shows the effect of C10 on proliferation of MDA-MB-231 cells over 96 hours of continuous treatment. At a concentration of 500 µM, proliferation in MDA-MB-231 cells appeared to be decreased, in a time dependent manner. After 96 hours, the proliferation had decreased to less than 50% of the 0.25% DMSO control. The 250 µM concentration of C10 resulted in a 35% decrease at 96 hours.
Figure 14. C10 decreases cellular proliferation of MDA-MB-231 cells at higher concentrations. MDA-MB-231 cells were tested for a change in cellular proliferation via BrdU incorporation assay after time dependent dosage of C10. Treatment of 500 µM MDA-MB-231 cells are shown to have a decrease in proliferation in a time dependent manner and as much as a 50% reduction after 96 hours. At 250 µM, there is a 35% decrease in proliferation after 96 hours. The preliminary nature of this data made it difficult to draw any solid conclusions. Only data points that express visually significant changes of ≥30% compared to the DMSO control were analyzed for statistical relevance. Data is given as an average percent viable cells ±SEM of n=3; *p≤0.05 as determined by two-way ANOVA with a t-test (Tukey) compared to 0.25% DMSO treated MDA-MB-231 cells, #p≤0.05 as determined by one-way ANOVA with a t-test (Tukey) compared to 24 hour time points.

Treatment with COB-141 also lead to a decrease in cellular proliferation tested via the BrdU incorporation assay. Figure 15 shows a significant decrease in the proliferation of nearly 50% in MDA-MB-231 cells treated with 250 µM COB-141 at the 24 hour time point. Each consecutive time point shows a continual decrease in proliferation, ultimately showing a 90% decrease in proliferation after 96 hours of treatment with 250 µM of COB-141. At 25 µM, a concentration more relevant to the
experimental IC₅₀ value calculated for COB-141, the graph shows a steadily decreasing trend. However, because the 24 hour time point reveals an increase in proliferation from the controls, the inhibition of the proliferation does not drop below 100% until 72 hours of treatment. At 96 hours 25 µM of COB-141 reported nearly a 20% decrease in proliferation.

Figure 15. COB-141 decreases proliferation of MDA-MB-231 cells at higher concentrations. MDA-MB-231 cells were tested for a change in cellular proliferation via BrdU incorporation assay after time dependent dosage of COB-141. With treatment of 250 µM, MDA-MB-231 cells are shown to have a decrease in proliferation of approximately 50% after 24 hours, and nearly full inhibition at 96 hours. At a concentration of 25 µM, COB-141 treatments had little effect until 72 hours, and a total decrease of nearly 20% at 96 hours. The preliminary nature of this data made it difficult to draw any solid conclusions. Only data points that express visually significant changes of ≥30% compared to the DMSO control were analyzed for statistical relevance. Data is given as average percent viable cells ±SEM of n=3; *p≤0.05 as determined by two-way ANOVA with a t-test (Tukey) compared to 0.25% DMSO treated MDA-MB-231 cells, #p≤0.05 as determined by one-way ANOVA with a t-test (Tukey) compared to 24 hour time points.
10.9 *C10 and COB-141 show limited effects on the growth of MDA-MB-231 cells*

While a decrease in proliferation was observed in MDA-MB-231 cells after treatment with C10 and COB-141, a separate MTS assay was used to test the viability of cells treated with the compounds. Treatment with C10 at either concentration (Figure 16) resulted in no relative decrease in cellular viability.

![Figure 16](image_url)

**Figure 16. C10 causes no change in cellular viability of MDA-MB-231 cells.** MDA-MB-231 cells were tested for a change in cellular viability via MTS assay after time dependent dosage of C10. C10 at both 500 μM and 250 μM elicited no significant change in cellular proliferation of MDA-MB-231 cells. The preliminary nature of this data made it difficult to draw any solid conclusions. Only data points that express visually significant changes of ≥30%, compared to the DMSO control, were analyzed for statistical relevance. Data is represented as average percent viable cells ±SEM of n=3;
MDA-MB-231 cells treated with COB-141 tested with the MTS assay revealed no effect until after 72 hours of treatment (Figure 17), at which time a 50% decrease was seen at a concentration of 250 µM. An even further decrease in viability was observed at 96 hours. No effect was apparent at 25µM treatment of COB-141.

Figure 17. COB-141 at high dosage causes a decrease in MDA-MB-231 cell viability. MDA-MB-231 cells were tested for a change in growth and viability after time dependent dosage of COB-141. At 250 µM no effect was seen until 72 hours of treatment, at which time the cells exhibited a 50% reduction in viable cells. At 25 µM no significant changes were apparent. The preliminary nature of this data made it difficult to draw any solid conclusions. Only data points that express visually significant changes of ≥30%, compared to the DMSO control, were analyzed for statistical relevance. Data is given as average percent viable cells ±SEM of n=3; *p≤0.05 as determined by two-way ANOVA with a t-test (Tukey) compared to 0.25% DMSO treated MDA-MB-231 cells.
Chapter 11: Discussion of Results

In initial research performed by the Kohn lab and the Interthyr Corporation, Phenylmethimazole (C10) was shown to decrease TLR3 signaling in autoimmune disorder affected cells and tissues, including the expression of IL-6 (Harii et al., 2005). Further Research pioneered by the McCall lab revealed that the decrease in TLR3 and IL-6 seen with treatment with C10 was also true in several cancer cell lines. Along with this finding, the cells also exhibited a decrease in growth mediated by the phosphorylation of STAT3 signaling through a decrease in IL-6 (McCall et al., 2007; Schwartz et al., 2009). Based on these initial findings, the present study was performed to determine if similar results could be seen in MDA-MB-231 triple negative breast cancer cells, when treated with C10 and COB-141. Through QRT-PCR and ELISA analysis, it was revealed that MDA-MB-231 cells exhibited a significant decrease in a normally high basal expression of IL-6 after 24 hour incubation in 500 µM C10 or 40 µM COB-141. Furthermore, IC$_{50}$ for inhibition of IL-6 generation was calculated to be relatively low, especially in the case of COB-141. These results agree with previous results, and accurately show that C10 and COB-141 are potent inhibitors of IL-6 production.

While the current data suggests that traditional IL-6 autocrine signaling is not intact in MDA-MB-231 cells in this study, the paracrine signaling events that would occur in vivo as a result of the over expression of IL-6 by MDA-MB-231 cells could be significant. Therefore, a decrease in IL-6 could theoretically disrupt these signaling events, altering the local tumor microenvironment of triple-negative breast cancer.
Ultimately, this may change the paracrine signaling that would affect the growth of the tumor.

Other studies performed on the presence and role of IL-6 signaling in MDA-MB-231 cells have reported that autocrine signaling does exist via a membrane bound IL-6Ra/gp130 complex signal transduction through a decrease in STAT3 phosphorylation after treatment with an IL-6 blocking antibody (Berishaj et al., 2007). The results from this study have revealed the opposite to be true. MDA-MB-231 cells were revealed to not express a membrane bound IL-6R via flow cytometry. The disparity in the results could be due to individual methodology, as well as differences between cellular populations. However, the results confirming the presence of gp130 on the cell surface suggest that IL-6 may be able to effectively transducer a signal in MDA-MB-231 cells given the presence of soluble IL-6Ra. Further research will need to be performed to clarify these results.

It was believed that treatment of MDA-MB-231 cells with C10 and COB-141 would lead to a decrease in cellular growth and proliferation, based on the previous experimentation on pancreatic and melanoma cancers. While the cells did exhibit a decrease in viability and proliferation, as revealed by the MTS and BrdU incorporation data, the overall reason is not truly revealed. The MTS assay is often used for a quantification of cell viability, since a strong signal reveals relative mitochondrial activity of the cells. However, the inability to accurately control the growth of tumor cells in 96 well plates, and thus the confluence that the assays are performed, may have confounded interpretation of the results. Research performed but not reported has shown the cell
counts performed on MDA-MB-231 cells grown in 12 well plates, with the same treatment and time frame, reveal a significant decrease in number of cells when treated with C10 and COB-141, especially after 72 and 96 hours. However, since the media is exchanged every 24 hours, it is difficult to interpret the true nature of the decrease in cell count. The compounds could be acting as either a cytotoxic or cytostatic effector on the cells. In terms of adversely effecting tumor cells, the compounds are successful at higher concentrations, although the mechanisms are not fully understood.

The results of the effect of C10 and COB-141 on IL-6 production in MDA-MB-231 cells, is but one study of a singular mechanism of the cell type. While the results were compelling, we did not provide solid data linking this to a decrease in proliferation and growth. This may suggest that additional cellular mechanisms are affected by C10 and COB-141 treatment, and further research would be needed to elucidate any further mechanisms that may be inhibited or altered during treatment with these compounds.
Chapter 12: Future Aims

12.1 *In vitro study of the paracrine effects of MDA-MB-231 cell-produced IL-6 on cells commonly found in the tissue microenvironment*

Because of the reported high basal expression of IL-6, and the inflammatory implications of high quantities of IL-6 in localized tissue microenvironments, it can be assumed that if the IL-6 produced by MDA-MB-231 cells is biologically active, paracrine signaling events would play a key role in the characterization of the surrounding cells. Primary cells, epithelial cells, adipocytes, endothelial cells (e.g. HUVECS, HAEC), and isolated human monocytes would be cultured in the presence of MDA-MB-231 derived IL-6, and screened for altered expression of cytokines and chemokines like IL-6, IL-1\(\alpha\), TNF-\(\alpha\), IFN\(\beta\), NOS species, VEGF, and MMP species through QRT-PCR and ELISA. The potential change in expression of surface bound receptors would also be analyzed by flow cytometry. The results from these experiments may reveal characteristics that lead MDA-MB-231 cells and triple-negative cancer cells to their invasive phenotype. In a further study based on this premise, experiments could be performed screening MDA-MB-231 cells for changes in protein expression after being cultured in conditioned media from the previous IL-6 treated cells. This would be a step in imitating the possible paracrine loops that could exist in the tumor microenvironment, revealing if the IL-6 over-production by MDA-MB-231 cells indirectly initiates signaling events in MDA-MB-231 cells.
12.2 In vivo study of the effects of C10 and COB-141 on the 4T1 triple negative mouse mammary epithelial cancer model

The 4T1 mouse mammary epithelial cancer cell model has been classified as a mammary model of triple negative breast cancer. Because of results found in these studies, and the implications that C10 and COB-141 are important in decreasing immunological proteins, like cytokines, chemokines, and PAMP receptors (e.g. TLR3), the compounds should be tested in an *in vivo* mouse model that has an intact immune system and robust immune response. 4T1 cells would be cultured and injected into the mammary fat pads of female BALB/c mice (Kaur et al., 2012). The tumor would be allowed to grow for 2 weeks, at which time C10 and COB-141 would be administered at relative doses based on prior *in vitro* testing. At the end of the study tumors would be resected and comparatively measured for changes in size based on a control non-treated group. This will also allow for immunohistochemical staining for proteins present in the tumor microenvironments, and any relative changes seen after treatment with C10 or COB141.
References


Anderson, K. C. (2009). Janus kinase inhibitor INCB20 has antiproliferative and
apoptotic effects on human myeloma cells in vitro and in vivo. Molecular Cancer
Therapeutics, 8(1), 26-35.

Calo, V., Migliavacca, M., Bazan, V., Macaluso, M., Buscemi, M., Gebbia, N., & Russo,
A. (2003). STAT proteins: From normal control of cellular events to

Castell, J. V., Gomezlechon, M. J., David, M., Hirano, T., Kishimoto, T., & Heinrich, P.
C. (1988). Recombinant human interleukin-6 (IL-6/BSF-2/HSF) regulates the
synthesis of acute phase proteins in human hepatocytes FEBS Lett, 232(2), 347-
350.

Cancer, 96(4), 591-599.

across high endothelial venules via an interleukin 6 trans-signaling mechanism.
Nature Immunology, 7(12), 1299-1308.

Chen, Q., Wang, W. C., Bruce, R., Li, H., Schleider, D. M., Mulbury, M. J., . . . Evans, S.
S. (2004). Central role of IL-6 receptor signal-transducing chain gp130 in
activation of L-selectin adhesion by fever-range thermal stress. Immunity, 20(1),
59-70.

Chin, K., DeVries, S., Fridlyand, J., Spellman, P. T., Roydsgupta, R., Kuo, W. L., . . .
cancer pathophysiology. Cancer Cell, 10(6), 529-541.

growth factor in human mammary carcinoma cell lines. Clinical Cancer
Research, 2(1), 215-221.

(2001). Autocrine production of interleukin 6 causes multidrug resistance in

Curnow, S. J., Scheel-Toellner, D., Jenkinson, W., Raza, K., Durrani, O. M., Faint, J. M.,
. . . Salmon, M. (2004). Inhibition of T cell apoptosis in the aqueous humor of
patients with uveitis by IL-6/soluble IL-6 receptor trans-signaling. Journal of
Immunology, 173(8), 5290-5297.


McCall, K. D., Harii, N., Lewis, C. J., Malgor, R., Kim, W. B., Saji, M., . . . Kohn, L. D. (2007). High basal levels of functional toll-like receptor 3 (TLR3) and noncanonical Wnt5a are expressed in papillary thyroid cancer and are coordinately decreased by phenylmethimazole together with cell proliferation and migration. *Endocrinology, 148*(9), 4226-4237.


